THE PARACASPASE MALT1 IS A CRITICAL MEDIATOR OF PAR1-DRIVEN NF-κB ACTIVATION AND TUMOR METASTASIS

by

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J. Randall McAuley, Ph.D.

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Protease Activated Receptor 1 (PAR1), a G-protein coupled receptor that is stimulated via thrombin-mediated proteolytic cleavage, is implicated in promoting metastasis in a variety of tumor types, including both carcinomas and sarcomas. The molecular mechanisms underlying PAR1-driven tumor metastasis remain largely unknown. Our laboratory previously discovered that PAR1 stimulation in endothelial cells leads to activation of the NF-κB transcription factor, and this is mediated by a protein complex comprised of the CARMA3 scaffolding protein, the Bcl10 adaptor protein, and the protease MALT1 (CBM). Given the strong association between NF-κB and tumor metastasis, we hypothesized that this CBM complex also mediates PAR1-driven, NF-κB-dependent tumor metastasis. In support of our hypothesis, we demonstrated that PAR1 stimulation in both osteosarcoma and in breast cancer cells results in NF-κB activation. siRNA-mediated MALT1 knockdown suppresses this NF-κB activation, suggesting that an intact CBM complex is required for PAR1-induced NF-κB activity in both tumor cell types. We identified several metastasis-associated genes that are significantly upregulated after PAR1 stimulation of osteosarcoma cells, and found that expression of the matrix remodeling protein MMP9, and the inflammatory cytokine IL-1β are both abrogated by MALT1 knockdown. We identified a similar, though distinct, PAR1-induced, MALT1-dependent gene expression profile in breast cancer cells. We next used CRISPR/Cas9 to knock out MALT1 in MCF7 breast cancer cells engineered to express PAR1 (MCF7-N55). In contrast to control MCF7 cells, which do not

express PAR1, MCF7-N55 cells are highly invasive in vitro and in vivo. We found that MALT1 knockout significantly blunts MCF7-N55 invasion and metastasis. Excitingly, we demonstrate that PAR1 stimulation induces MALT1 proteolytic activity in both osteosarcoma and breast cancer cells. Several small molecule MALT1 protease inhibitors have recently been described, and our study suggests that MALT1 could represent a promising new pharmaceutical target for the prevention/treatment of PAR1-driven tumor metastasis.

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PREFACE

If it takes a village to raise a child, I can only wonder at the multitudes it takes to raise that child into a physician-scientist. The work presented here is truly the product of scores of mentors, teachers, coworkers, fellow students, friends, and family over the course of the past ten years – the breadth of my scientific career to date. Absolutely none of this would be possible without the support, fellowship, and loving concern and care of innumerable people. I am certain that I will not sufficiently acknowledge every person whose contribution brought this work to realization; for these omissions I can only beg forgiveness.

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I cannot begin to thank Benjamin Ebert of Harvard Medical School and Brigham and Women's Hospital enough. From hiring me, a fresh college graduate, in 2008 when he was just starting his research group (and hadn't originally intended to hire another research technician), to his limitless support and generosity with his wisdom and insight, my time in Dr. Ebert's laboratory truly was an inflection point in my career: without his example and the experiences from his laboratory, I would not have considered pursuing an MD-PhD program.

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I am eternally grateful for the selfless support of my family. My family in Pittsburgh has been nothing but supportive of my endeavors since arriving here in 2010, and I am so grateful for their generosity. To Denis McAuley III, Mary McAuley, Bill Gugliotta, Carron McAuley, the members of the Pittsburgh Family Book Club, Becky Gloninger, John Gloninger, Gemma Gloninger, and Angela Gloninger, I am forever thankful. I truly am privileged to have such caring and loving family in Pittsburgh. The number of times that they have kindly hosted me during my busy medical school and graduate school schedule is beyond count, and there really is nothing like the knowledge that family is always there for you. Additionally, my family in Washington, DC, has been exceptionally supportive of me and my work: to Ellen and Rich Murray, Mimi Fleury, Anne Fleury, Kate and John Ryan, and all others in the DC area, I am so thankful and appreciative of your assistane and encouragement.

This work is dedicated in part to Anne McAuley and to the memory of Denis McAuley Jr. To say that I am lucky to get to live nearby while carrying out my graduate research is an understatement of the highest order. I am thrilled that she will be present for the defense of this dissertation.

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This work is dedicated to David McAuley, Laura McAuley, and Mia McAuley.

I owe you everything.

J. Randall McAuley

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To Celia

But with patience and faith

We remain unafraid

I'm home.

1.0 INTRODUCTION

1.1 DISSEMINATION OF CANCER: METASTASIS

The first known description of a cancer patient dates to the Edwin Smith papyrus of 1600 BC, over one thousand years before the time of Hippocrates, and itself thought to be a copy of a document from approximately 3000 BC[1]. While examples of disseminated cancer can be found throughout history[2], an understanding of cancer's propensity to spread throughout the body only began to emerge in the 19th century AD. First identified as "metastasis of milk," in the 17th and 18th centuries the term metastasis was applied to any affliction that appeared to transpose from one point of origin to another organ in the body (for a later-period example, see[3]). Only in 1829 did the French surgeon and gynecologist Joseph-Claude-Anthelme Récamier publish his *Research on the Treatment of Cancer* in which "metastasis" was used to refer to the dissemination and spread of cancer for the first time[4]. One of the pioneers of oncology, Récamier concludes his writing with a statement[5], the sentiment of which is no less true almost two hundred years after his work, and to which the author of this dissertation also aspires:

> *I will be happy if my work, imperfect as it is, may aid only one other observer, to discover the best way to cure the appalling disease which has been my particular concern for many years.*

1.1.1 General mechanisms of cancer metastasis

Over a century and a half of oncology research has shed an amount of light onto the process by which cancer spreads throughout the body. The picture of metastasis that has emerged is one of a highly complex and organized series of steps that requires precise genetic control of metastasizing cancer cells in order for distant colonization to be successful[6-12]. Further, it is becoming increasing clear that metastasis is not a cancer cell-intrinsic process, and the contributions of the origin tumor microenvironment as well as the pre-metastatic site niche are being recognized as equally important to the process of metastasis[13-15]. While the metastatic process can be divided into myriad specific steps (**Figure 1**), it is useful to think of the basic process in as simple a fashion as possible, composed of four elements which will be discussed below:

1. Primary tumor invasion and intravasation
2. Circulation
3. Distal extravasation
4. Colonization and proliferation

1.1.1.1 Primary tumor invasion and intravasation

Typically, it is thought that cancers are not normally invasive early in the course of the disease. Rather, after a period of relatively quiescent growth and proliferation, inflammatory cytokines, hypoxia, or numerous other insults induce a transformation from the initial epithelial character of the tumor cells to a more aggressive, mesenchymal phenotype, called the Epithelial-Mesenchymal Transition (EMT)[16-18]. EMT is necessary for early steps in metastasis, including

tumor cell motility and invasion[19]. In this step, tumor cells undergo cytoskeletal reorganization into a more elongated, spindle-shaped morphology[20]. Additionally, tumor cells undergoing EMT display a dramatic change in cell-surface adhesion protein expression. Cells suppress expression of E-cadherin (CDH1), an important factor in adherens junction formation with other cells expressing E-cadherin, and gain expression of N-cadherin (CDH2), an adhesion marker associated with increased motility in cancer cells[21,22].

In addition to the loss of adhesion proteins, the process of EMT also leads to upregulation of matrix remodeling enzymes such as Matrix MetalloProteinases (MMPs) and Urokinase (uPA, PLAU)[23,24]. These proteins are secreted by tumor cells and are known to play important roles in the promotion of cellular motility and tissue invasion (**Table 1**), as active matrix remodeling proteins catalyze the hydrolysis of protein components of the extracellular matrix such as collagen[25,26]. Degradation of the extracellular matrix allows for tumor cell mobilization and invasion into neighboring tissue[27].

Once tumor cells no longer adhere to neighboring cells and also have the capacity to degrade the extracellular matrix restricting their motility, the last step of early metastasis is intravasation: invasion through a vascular endothelium layer into the vessel lumen and into the blood circulation.

Table 1. Partial list of MMPs with known roles in cancer.

MMP	Cancer	References
MMP1	Breast, ovary	28-31
MMP2	Breast, fibrosarcoma	32,33
MMP3	Breast, pancreas	34-37
MMP7	Colorectal, bladder, esophageal, stomach, pancreas, prostate	38-42
MMP9	Breast, stomach, melanoma, leukemia	43-46
MMP10	Lung, cervix	47-49
MMP11	Breast, prostate, stomach	50,51
MMP12	Lung, squamous cell	52,53
MMP13	Breast, colorectal	54-57

1.1.1.2 Circulation

When in the circulation, tumor cells are thought to disseminate as either individual cells or as small groups of loosely associated cells[58-61]. Normally, non-hematological tissue cells initiate apoptosis, programmed cell death, when maintained in anchorage-independent suspension without contacts with neighboring cells or extracellular matrix (such as when in the circulation)[62]. This programmed cell death upon loss of contact with other cells or extracellular matrix is termed anoikis[63,64]. Anoikis resistance, that is, overcoming anoikis to enable anchorage-independent survival within the circulation, is a hallmark of metastatic cancer[65].

Tumor cells are able to evade anoikis through upregulation of survival associated factors such as cIAP2 and Bcl-xL, among other mechanisms[66-68].

1.1.1.3 Distal extravasation

After entering the circulation, tumor cells must extravasate – exit the vessel lumen through the endothelium into the target organ parenchyma – at a site away from the primary tumor. Circulating tumor cells appear to lodge in organs with extensive capillary beds or sinuses such as the lung, brain, bone, or liver; however, preferential metastasis of some tumor types to specific distant organs suggests that the targeting process is not solely mechanical in nature. Gene expression analysis of circulating tumor cells has demonstrated increased expression of tissue-specific adhesion proteins and homing markers such as CXCR4[69-72].

Emerging evidence suggests that distant communication between the primary tumor and metastatic target organ induces "pre-metastatic niche" formation in the future metastatic site[13,14]. Primary tumor secreted factors such as cytokines, cellular debris, and extracellular vesicles contribute to target organ microenvironmental changes that support metastatic colonization and growth[15,73]. Formation of a pre-metastatic niche is associated with vascular endothelium disruption, extracellular matrix remodeling, and inflammation: processes that enhance the ability of circulating tumor cells to successfully extravasate and grow in that site[14].

1.1.1.4 Colonization and proliferation

After circulating tumor cells have landed in distal sites and exited the circulation, the final step in metastasis is the tissue invasion, growth, and proliferation necessary to form a metastatic nodule. Importantly, there appears to be a selection gate separating initial micrometastatic growth from mature metastatic growth: for micrometastases to grow sufficiently into full-on metastatic

nodules, a number of growth-limiting hurdles must be overcome. Metastases that are to grow beyond a small size must initiate tumor angiogenesis to ensure a supply of oxygen and nutrients. Indeed, suppression of pro-angiogenic factors in tumor cells has been shown to decrease metastatic proliferation in sarcoma, breast cancer, and other tumor types[74-76]. In addition to the growth limitations imposed by angiogenesis requirements, the metastatic tumor microenvironment can inhibit or permit the proliferation of nascent metastases. Stromal cells and extracellular matrix can provide growth suppressing signals to dividing metastatic cells which must be overcome in order to successfully colonize the metastatic site[77-79]. The development of drugs that target the tumor microenvironment in an effort to make it even more anti-tumorigenic is an area of active clinical research interest[80,81].

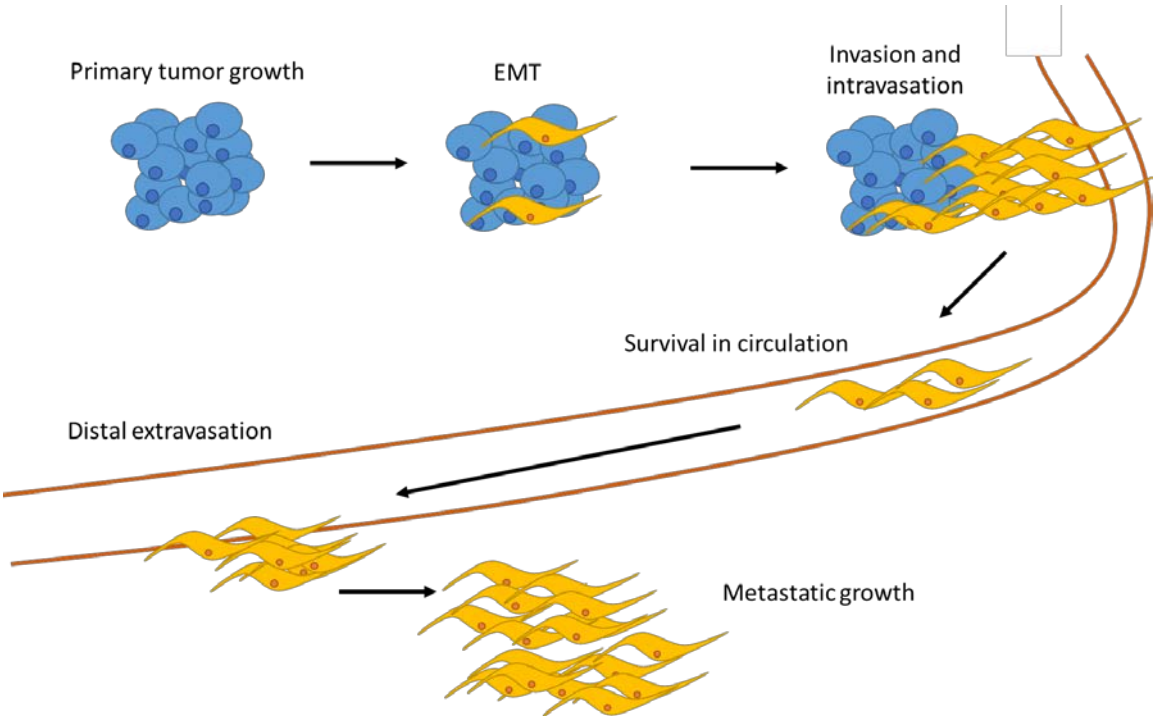

Figure 1 – Steps in metastasis. Primary tumor cells undergo EMT and invade into surrounding tissue. Invading tumor cells intravasate into the vessel and gain access to the circulation. Tumor cells survive in circulation by suppressing anoikis; distal extravasa

1.1.2 Prognosis and treatment of metastatic disease

The past decades have witnessed substantial gains in the survival of cancer patients with local or regional disease; however, effective treatment of metastatic cancer remains a major challenge. Formation of distant metastases confers poor prognosis for most types of malignancy, and, for many cancers, 5-year survival of patients with metastatic disease at presentation has not improved or has even decreased in the last 10 years (**Figure 2**)[80]. For breast cancer and osteosarcoma, development of distant metastases drastically worsens prognosis. Despite intensive study, the molecular mechanisms underlying tumor cell dissemination, as well as colonization of and growth in distant sites, are very poorly understood (reviewed in[9]). Elucidation of the cellular processes that drive metastasis could result in the discovery of new treatments that specifically target pathways promoting metastasis. Targeted therapeutic strategies preventing or treating metastases thus have tremendous potential to lead to significant gains in cancer patient survival.

The disseminated nature of metastatic disease favors systemic therapeutic modalities such as chemotherapy in combination with localized treatments such as surgical resection or radiation therapy. Standards of clinical care for both osteosarcoma and breast cancer will be discussed below.

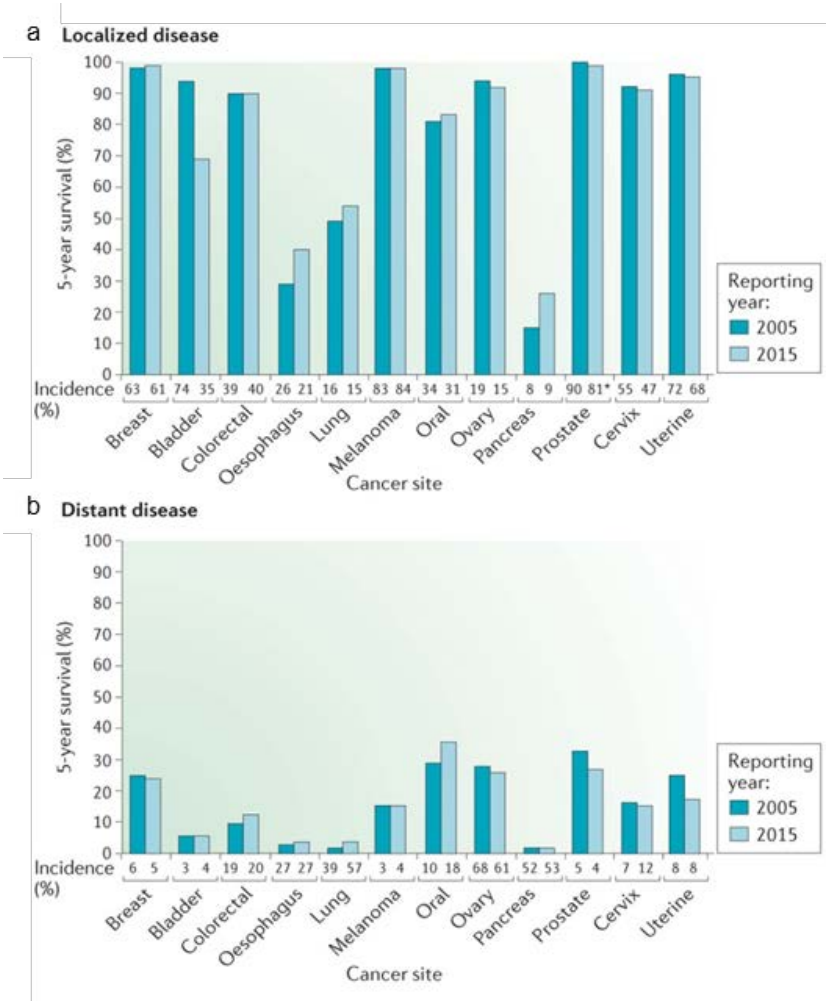

Figure 2 – Solid Tumor Patient Survival. 5-year patient survival of (a) localized disease versus (b) metastatic disease for various solid tumors. Adapted from[80].

1.1.3 Metastasis in sarcoma: focus on osteosarcoma

Osteosarcoma is the most common primary bone tumor of children, representing over 50% of bone cancers of patients under the age of 20. With approximately 900 new cases diagnosed in the United States per year, it is the fifth most common tumor diagnosed in adolescents[82]. Osteosarcoma is a sarcoma – a tumor derived from cells of mesenchymal origin – characterized by the production of osteoid, the organic component of immature bone matrix. It primarily occurs in the long bones of the extremities, usually the tibia or femur[83]. The genetic etiology of osteosarcoma is not straightforward: no clear genetic lesions have been identified that drive osteosarcomagenesis, though the comparatively low number of cases per year has hindered search efforts[84]. Loss or inactivation of RB1 and TP53 has been demonstrated in instances, although 80% of osteosarcoma cases have no clearly driving genetic lesions[85]. The strongest risk factor for osteosarcoma is prior irradiation from earlier solid tumor therapy. Osteosarcoma is most frequent during the adolescent growth spurt in highly actively growing long bones, leading to the current hypothesis is that osteosarcoma occurs in rapidly dividing bone cells that are especially sensitive to oncogenic insults or mitotic errors[86,87].

Osteosarcoma is a clear example of a precipitous decline in prognosis associated with metastasis. In osteosarcoma, 5-year survival rates fall to 15 - 30% in patients with metastatic disease; whereas 5-year survival rates are 60 – 80% for patients with localized disease[88]. Prior to the advent of the modern osteosarcoma treatment protocol of adjuvant / neo-adjuvant chemotherapy and radical surgical resection, up to 90% of osteosarcoma patients went on to develop clinical metastases, usually to the lungs, even after surgery and localized control of their osteosarcoma. This observation led to the hypothesis, later shown to be correct, that most

osteosarcoma patients develop subclinical metastases extremely early on in the course of the disease[89,90].

Osteosarcoma survival has increased dramatically since the institution of adjuvant / neo-adjuvant chemotherapy regimens. The current standardized protocol is the MAP protocol, including both adjuvant (postoperative) and neo-adjuvant (preoperative) doxorubicin, cisplatin, and high-dose methotrexate[91-96]. With the MAP regimen, approximately 60 to 70% of patients that present without metastases will go on to be long-term survivors; whereas less than 25% of patients that present with clinical metastases will do so.

1.1.4 Metastasis in carcinoma: focus on breast cancer

Over one million cases of breast cancer are diagnosed around the world each year. In the United States, breast cancer is the most common cancer diagnosis and the second-highest cause of cancer death in women. Breast cancers generally arise from the ductal tissue of the breast, marking them as carcinomas – epithelium-derived neoplasias. In contrast to osteosarcoma, the genetic background of breast cancer points to clear hereditary factors as well as clear drivers of tumor growth[97]. The estrogen receptor family (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) have been shown to drive early breast cancer growth[98,99]. HER2 amplification defines a molecular subtype of breast cancer, the HER2-enriched subtype, which accounts for approximately 10% of breast cancer cases[100,101]. Broadly speaking, ER expression correlates with favorable prognosis; whereas HER2 enrichment and triple-negative (ER, PR, and HER2 negative) cancers have the poorest outcomes[102]. In addition to molecular drivers of early breast cancer, there is a strong hereditary component to breast cancer carcinogenesis. Although only 5 to 10% of breast cancer patients have a hereditary form

of the disease, women with deleterious mutations in the tumor suppressors BRCA1 or BRCA2 have exceedingly high cumulative risks of developing breast cancer by age 70: 65% and 45%, respectively[103-105].

Treatment of early breast cancer consists of surgical management (lumpectomy or mastectomy and regional lymph node dissection) with radiotherapy for patients at risk of recurrence. Radiation therapy can constitute of a wide variety of specific modalities, including external beam therapy and brachytherapy, and is generally indicated for patients with axillary lymph node involvement or poor surgical margins[106,107]. Detection of breast cancer metastases outside of regional lymph nodes defines stage IV cancer, currently considered incurable. While over 90% of patients with early-stage breast cancer survive past five years, patients with stage IV breast cancer have a five year survival rate of approximately 20%[108].

1.2 PROTEASE ACTIVATED RECEPTOR 1

PAR1 is a G-Protein-Coupled Receptor (GPCR) activated by thrombin, a serine protease central in the blood coagulation cascade. Thrombin acts at several points in the coagulation process, converting factors V, VIII, XI, and XIII into their active forms[109-112]. Thrombin also catalyzes the hydrolysis of fibrinogen into insoluble fibrin, the major matrix component of blood clots. Thrombin acts upon and activates PAR1 in a manner unique among cell-surface receptors: thrombin-mediated proteolysis of the PAR1 N-terminal extracellular domain uncovers a cryptic peptide ligand tethered to PAR1 itself[113]. After activation by thrombin, this peptide sequence is able to dock with the PAR1 extracellular receptor site and induce PAR1 intracellular signaling activation. Notably, this mechanism of action by thrombin is irreversible: PAR1 signaling

deactivation is accomplished through vesicular uptake and internalization of activated PAR1 molecules and subsequent proteasomal degradation and peptide recycling[114].

PAR1 is the best-characterized of a family of protease-activated receptors, encompassing PAR1, PAR2, PAR3, and PAR4[115]. Thrombin is known to act upon PAR1, 3, and 4. Notably, several other proteases have been shown to activate various members of the PAR family, including MMPs and trypsin, though the physiological role of such non-thrombin PAR activation is unknown. Further, PAR family members appear to be able to transactivate other cell surface receptors including other PARs and EGFR, though the extent and character of this signaling *in vivo* is currently debated[116-118].

1.2.1 PAR1 in cancer

PAR1, is implicated in promoting the progression and metastasis[119,120] of a wide variety of tumors including breast cancer, lung cancer[121-123], melanoma[124,125], prostate cancer[126-128], pancreatic cancer[129], and multiple subtypes of sarcoma[130,131]. Notably, this list of PAR1-driven tumor types includes both epithelial tumors, such as breast cancer, and mesenchymal tumors, such as osteosarcoma[131]. PAR1 is not expressed in normal breast epithelium, but is upregulated in invasive breast cancers, a finding that has led several groups to investigate a role for PAR1 in breast cancer metastasis. A recent study demonstrated that ectopic expression of PAR1 in non-metastatic MCF7 breast cancer cells causes dramatic changes in cell morphology and induces aggressive metastatic behavior both *in vitro* and *in vivo*[29,132]. These findings suggest PAR1-induced signaling can promote breast cancer metastasis and may represent a novel therapeutic target. In the case of osteosarcoma, an initial study showed that patients with pulmonary metastases have significantly higher levels of thrombin in the lungs as compared to patients

without metastasis[133]. Additionally, studies have demonstrated that thrombin promotes osteosarcoma cell migration *in vitro*[134,135], a necessary step in the process of metastasis. Further, thrombin stimulation of PAR1 in osteosarcoma cells induces the expression of extracellular matrix remodeling enzymes such as MMPs and the promotion of invasion[136]. Similarly, inhibition of thrombin signaling by low molecular weight heparin suppressed osteosarcoma proliferation[137], strongly suggesting a role for PAR1 signaling in the regulation of several steps in metastasis in osteosarcoma.

1.2.2 Coagulation-associated pathology associated with cancer

It has been observed that cancer patients are at increased risk of developing thrombi – a phenomenon known as the "hypercoagulable state of malignancy" – and a major cause of patient morbidity and mortality. Approximately 20% of cancer patients will experience venous thromboembolism[138]; venous thromboembolism in an otherwise healthy patient can be an indication of undetected malignancy. A number of hypotheses have been proposed to account for this clinical observation, from prothrombotic factors secreted by tumors to mechanical interactions between cancer cells and the extracellular matrix or stromal cells[139].

In osteosarcoma and breast cancer, coagulation-associated pathology appears correlated with progression and metastasis. Hypercoagulability has been noted in osteosarcoma patients[140], and increased expression of PAR1 has been discovered in primary osteosarcoma cells embedded in peri-tumor thrombus[137]. Further, osteosarcoma patients with venous thrombi experienced poor clinical outcomes relative to patients without thromboembolic events. Thrombin treatment of osteosarcoma[136] or breast cancer[141] cells *in vitro* induced invasion and motility. Suppression of thrombin signaling using low molecular weight heparin inhibited osteosarcoma cell growth

and invasion *in vitro*. Thrombin stimulation of both osteosarcoma and breast cancer cells also induced expression of pro-metastatic factors such as MMPs[136].

The mechanisms underlying this prothrombotic state are still under debate, as the etiology and incidence of the hypercoagulable state of malignancy appear to be heterogeneous. Most cancer cells express Tissue Factor (TF) on their surface, which is capable of converting prothrombin to thrombin through the activation of factors IX and X. Thrombin is a potent activator of platelets and can cause platelet aggregation and clot formation. It has been demonstrated that cancer cells can interact with platelets to form small emboli of loose platelet-tumor cell collections, and that tumor cell interactions with platelets prolong survival in the circulation[142]. Additionally, platelet-derived growth factors such as Vascular Endothelial Growth Factor (VEGF), LysoPhosphatidic Acid (LPA), Platelet-Derived Growth Factor (PDGF) are known to promote cancer growth and metastasis. Tumor-associated hypercoagulability is likely a byproduct of the beneficial interactions between tumor cells and activated platelets, at least in part.

1.3 THE CARMA3/BCL10/MALT1 SIGNALING COMPLEX

The CARMA – Bcl10 – MALT1 (CBM) signaling complex is an intracellular signaling complex that is found downstream of a wide variety of cell surface receptors. The three proteins that comprise the core complex itself are described below.

CARMA – so named as the family contains CARD (caspase activation and recruitment domain) and MAGUK (membrane-associated guanylate kinase-like) domains (**Figure 2**) – is the scaffold protein of the complex[143]. CARMA phosphorylation by PKC isoforms is required for

CBM complex oligomerization into long, signaling-active filaments comprised of many CBM heterotrimers[144,145]. Notably, there are three CARMA family members that appear to be functionally similar, albeit with differing expression patterns: CARMA1 (also commonly known as CARD11) is found in hematological and lymphoid cells, CARMA2 (CARD14) is expressed in the placenta, and CARMA3 (CARD10) expression is widely distributed in somatic cells[146]. CARMA phosphorylation allows for CBM complex formation and signaling activation.

Bcl10 is the smallest member of the complex and serves as a linker protein to bring phosphorylated CARMA and MALT1 together. Bcl10 and MALT1 form constitutive heterodimers in the cytosol[147]. The scope of Bcl10-specific signaling and interactions is increasing as Bcl10 has become an independent subject of research interest. Bcl10 phosphorylation appears to modulate NF-κB signaling downstream of the CBM complex[148], and Bcl10 cleavage by MALT1 is still not fully understood, though T cells expressing an uncleavable Bcl10 mutant demonstrated impaired adhesion[149].

MALT1 is broadly considered the downstream effector protein of the CBM complex, responsible for the propagation of downstream signaling via protein-protein interactions or direct proteolytic activity. It is discussed in detail below.

Signaling events upstream of the CBM complex were originally worked out in lymphocytes with the CARMA1-containing CBM and are presumed to be similar in non-lymphocytes with the CARMA2- and CARMA3-CBM: PKC activation downstream of cell surface receptor ligand binding phosphorylates CARMA. Different PKC isoforms have been found upstream of the CBM complex: PKCβ in B cells and PKCθ in T cells[150]. While signaling events upstream of the CBM have not been elucidated in non-CARMA1-containing cells, PKC agonists such as Phorbol Myristate Acetate (PMA) and ionomycin[151] have been shown to

activate CBM signaling in CARMA3-containing cells, suggesting that activation of the CARMA3-CBM complex is likely similar to that of the CARMA1-CBM coplex[152].

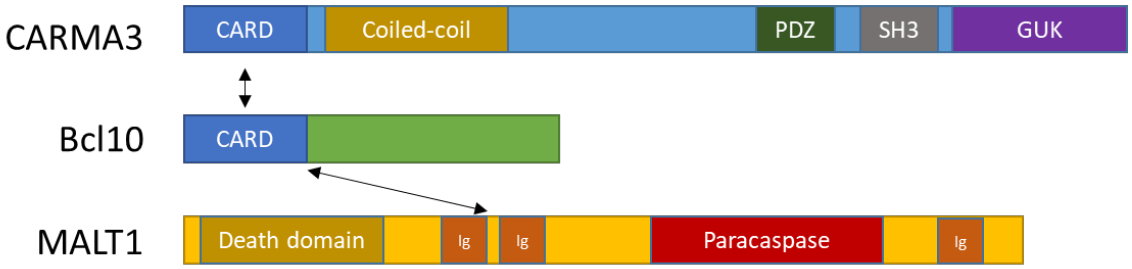

Figure 3 – Domain structure of CARMA3, Bcl10, and MALT1. Black arrows show regions of interaction between proteins. CARD: Caspase Activation and Recruitment Domain. SH3: Src-Homology 3 domain. GUK: Guanylate Kinase like domain. Ig: Immunoglobulin domain.

1.3.1 The CARMA1-CBM complex: a history rooted in lymphomagenesis

The CBM signalosome originally came into the research spotlight when it was noticed that a series of chromosomal translocations and abnormalities were associated with the development of B cell lymphoma of the Mucosa-Associated Lymphoid Tissue (MALT lymphoma). MALT lymphoma, first detailed in 1983[153], is the most common extra-nodal lymphoma, and most frequently develops in MALT associated with chronic inflammation such as in the stomach or lung. The t(11;18)(q21;q21) translocation was the first to be described in patients[154], although discovery of the gene product would occur ten years later: a fusion between Inhibitor of Apoptosis 2 (API2, also cIAP2) and MALT1 creating the API2-MALT1 oncoprotein[155-157]. MALT lymphoma with the API2-MALT1 translocation was found to be persist despite *H. pylori* antibacterial treatment[158], suggesting that API2-MALT1 conferred inflammation-independent

growth of the tumor. Other translocations were discovered that associated with the development of MALT lymphoma. The t(1;14)(p22;q32) translocation[159] was found to move the BCL10 gene such that its expression was constitutively driven by the immunoglobulin heavy chain enhancer promoter element (IGH-Bcl10)[160]. Similarly, the t(14;18)(q32;q21) translocation was discovered[161] to place the MALT1 gene under the control of the immunoglobulin heavy chain enhancer promoter element to make constitutively-expressed IGH-MALT1 in a fashion akin to the t(1;14)(p22;q32) translocation that creates IGH-Bcl10[162,163].

After members of the CBM complex were discovered to be targets of chromosomal translocation in MALT lymphoma, an inflammation-associated tumor, it rapidly became apparent that Bcl10 and MALT1 were intracellular mediators that activated the inflammatory transcription factor family NF-κB. Bcl10 was shown to be necessary for activation of NF-κB in mouse T and B cells[164], while T cell receptor (TCR) activation of NF-κB was shown to be MALT1 dependent[165].

CARMA proteins were added to the emerging model of the CBM complex when a novel Bcl10-interacting protein was found to activate NF-κB[166,167]. Other CARMA family members were rapidly identified[168,169], and it was subsequently shown that CARMA proteins interact with Bcl10 and MALT1 to activate NF-κB[170-172].

1.3.2 The CARMA3-CBM complex

Although most early studies of the CBM complex were performed in immune cells and examined the CARMA1-containing complex, initial reports suggested that other CARD-carrying MAGUK-domain proteins did exist[169]. Genetic studies of these other CARMA family members showed that knockout of CARMA3 induced an embryonic-lethal neural tube formation defect

similar to the one observed in CARMA1 knockout mouse embroys[173]. Furthermore, ectopic CARMA3 expression rescued NF-κB activation downstream of TCR stimulation in CARMA1-deficient T cells, strongly suggesting an amount of functional redundancy between CARMA3 and CARMA1[174]. While CARMA3 was found to interact with Bcl10 and MALT1, the context of such CARMA3-CBM signaling was not apparent.

A series of papers published in the *Proceedings of the National Academy of Sciences* in January of 2007 established the current model of the CARMA3-containing CBM complex downstream of G-protein coupled cell surface receptors. All at once, it was discovered that CARMA3-CBM complex activated NF-κB downstream of the LPA[175], angiotensin II[176], and endothelin receptors[177]. Subsequent studies identified even more cell surface receptors that activated NF-κB via the CARMA3-CBM complex, including the CXCL12/SDF-1 receptor CXCR4[176,178-180], the IL8 receptor CXCR2[181], and, most notably, PAR1[182].

1.3.3 MALT1: scaffolding signaling and proteolytic activity

MALT1, the effector protein of the CBM complex, possesses two distinct functions by which it carries out downstream signaling: protein-protein interactions and direct proteolysis by MALT1 itself.

Activated MALT1 forms important protein-protein interactions, termed "scaffolding signaling" by the field, with components of the NF-κB signaling machinery such as the TRAF6 ubiquitin ligase[183], Linear Ubiquitin Chain Assembly Complex (LUBAC), and kinase TAK1[184]. MALT1 interaction with these intermediates results in IKKγ polyubiquitination and subsequent IKK complex phosphorylation and activation[185,186]. This active IKK complex induces Inhibitor

of κB (IκB) phosphorylation and degradation, leading to translocation of NF-κB transcription factor dimers into the nuclear and NF-κB transcriptional activation[187].

In addition the scaffolding signaling capacity of MALT1, it has recently been discovered that the paracaspase domain[188] (**Figure 3**) of MALT1 is enzymatically active and that it acts to cleave a specific set of protein substrates[149,189,190]. Among other substrates, MALT1 cleaves and destroys the NF-κB family transcription factor subunit, RelB, and the deubiquitinases, CYLD and A20[188,190-192] (**Table 2)**. The effects of MALT1 protease activity on CBM signaling outputs are not completely understood, though the current hypothesis is that MALT1 proteolytic activity enhances scaffolding signaling. MALT1 substrates appear to include inhibitors of the NF-κB signaling pathway, and thus MALT1 proteolytic activity may serve to maximize MALT1 scaffolding signaling activation of NF-κB by cleaving and inactivating these inhibitory substrates.

Table 2. List of known MALT1 substrates and effects of MALT1 proteolysis.

Substrate	Role	Effect of MALT1 proteolysis	References
A20	Deubiquitinase	NF-κB activation enhancement	190
RelB	Non-canonical NF-κB transcription factor	NF-κB activation enhancement	191
MALT1	CBM complex protein	NF-κB activation enhancement	193
CYLD	Deubiquitinase	AP-1 transcription factor activation, endothelial cell permeability	192,194
Regnase-1	RNAse	mRNA stabilization, increased mRNA synthesis	195,196
Roquin-1/2	mRNA deadenylation promoter	mRNA stabilization	196
Bcl10	CBM complex protein	Reduced leukocyte adhesion	149
HOIL1	LUBAC subunit	NF-κB activation enhancement	197

1.3.4 The role of the CBM complex in solid tumors

Given the tight association between the CARMA1-CBM complex and lymphoma, it was surmised that the CARMA3-CBM complex might play a similarly oncogenic role in solid tumors. Lymphomagenic activating mutations and translocations of the CARMA1-CBM complex have been described in the literature and are discussed above. Somewhat surprisingly, no similarly oncogenic mutations or translocations of the CARMA3-CBM complex in solid tumors have been discovered to date. Despite this apparent lack of an observation, it is clear that

CARMA3-CBM signaling and activation of NF-κB is important to the development and progression of multiple solid tumors, including ovarian cancer, oral squamous cell carcinoma, lung cancer, and breast cancer.

Enrichment of LPA receptors, GPCRs known to act through the CARMA3-CBM complex, has been seen in malignant populations of ovarian cancer[198]. Additionally, suppression of CARMA3, Bcl10, or MALT1 inhibits *in vitro* invasion of ovarian cancer cells[199]. Outside of ovarian cancer, MALT1 silencing suppresses CXCR4 stimulation-induced invasion of oral squamous cell carcinoma cells[180]. Further, in lung cancer, CARMA3 expression correlates with poor prognosis, NF-κB activation, and EGFR expression[200]. In a subsequent finding, MALT1 was found to mediate EGFR stimulation-induced NF-κB activation in lung cancer, and suppression of MALT1 increased lung cancer anchorage-independent growth and invasion and reduced tumor burden in an EGFR-driven model of lung cancer in mice[201].

In breast cancer, the CARMA3-CBM complex has been implicated in mechanisms linked to tumor promotion and metastasis. A recent study demonstrated that CBM-complex mediated activation of NF-κB downstream of HER2 stimulation, and that MALT1 knockout delayed tumor formation in a mouse model of spontaneous breast cancer with HER2 overexpression[202]. Other studies have implicated the CARMA3-containing CBM complex in NF-κB activation and chemotherapeutic resistance in breast cancer cells[203].

1.4 THE INFLAMMATORY TRANSCRIPTION FACTOR NF-KB

While NF-κB is commonly spoken of as a single transcription factor, in actuality it is a complex formed from five separate Rel-homology domain-containing proteins that comprise the NF-κB family: RelA (p65), RelB, c-Rel, p50, and p52. NF-κB members assemble in a wide variety of hetero- and homodimers which are then able to bind to NF-κB DNA response elements to activate or repress transcription at those sites[204,205]. Of these family members, RelA, RelB, and c-Rel contain transactivation domains that are necessary for target gene transcription.

1.4.1 NF-κB signaling: canonical and non-canonical activation

Canonical – or "classical" – NF-κB activation is defined by IKK complex-mediated phosphorylation of IκB. Upstream signaling events result in IKKγ (NEMO) ubiquitination and formation of the active IKK complex, composed of IKKα, IKKβ, and IKKγ[206]. The active IKK complex phosphorylates IκB at two sites, leading to its ubiquitination by ubiquitin ligases and subsequent proteasomal degradation. Degradation of IκB releases previously-sequestered NF-κB transcription factor dimers, which are then free to translocate to the nucleus and bind to NF-κB response elements. Release of RelA/p50 heterodimers is most closely associated with the canonical NF-κB pathway[207].

In contrast, non-canonical – or "alternative" – NF-κB signaling results in the nuclear translocation and DNA-binding of RelB/p52 complexes. Receptor stimulation results in activation of NF-κB Inducing Kinase (NIK), which then phosphorylates and activates IKKα.

Activated IKKα homodimerizes and phosphorylates RelB-bound p100, triggering its proteolytic processing into p52. p100 acts similar to IκB in the classical NF-κB pathway, inhibiting RelB translocation to the nucleus and suppressing alternative NF-κB signaling; however, upon processing to p52, this inhibition is lost, and RelB/p52 heterodimers are capable of nuclear translocation and signaling activation[208].

1.4.2 NF-κB activation in cancer metastasis and progression

NF-κB activation promotes metastasis and cancer progression via several mechanisms (**Figure 4**) [209,210]. First, NF-κB stimulates cell proliferation and protects against apoptosis directly through upregulation of a variety of survival factors and tissue invasion effectors, such as cIAP and MMPs[211]. NF-κB promotes specific aspects of cell survival important to the metastatic process: for example, NF-κB promotes survival in the face of cytotoxic chemotherapeutic agents (ie chemotherapy resistance) and suppresses anoikis[67,212]. Second, NF-κB induction is associated with MMP expression and tissue invasion[27,213]. Third, NF-κB activation is tightly linked with the expression of Epithelial-Mesenchymal Transition (EMT) markers such as Twist and Snail[214,215]. Fourth, NF-κB signaling has been implicated in the expression of cellular adhesion proteins, necessary in the vascular extravasation step of metastasis[209].

In breast cancer patients, NF-κB activation in tumor samples correlates with poor prognosis and aggressiveness of disease[216,217]. In other studies, NF-κB transcriptional activation has been linked to increased cell motility, *in vitro* invasion, and increased proliferation[218]. NF-κB also plays a role in drug resistance of breast cancer cells: NF-κB activation was found to suppress apoptosis in response to chemotherapeutic treatment[219], while inhibition of NF-κB dramatically increased breast cancer sensitivity to chemotherapeutic agents *in vitro*[220].

Furthermore, an apparent population of breast cancer stem cells or tumor-initiating cells requires NF-κB activation in order to continue self-renewal of stem cell capacity[221].

In osteosarcoma, pharmacological NF-κB suppression has been shown to reduce tumor volume of osteosarcoma xenografts[222] as well as spontaneous metastasis of orthotopically injected tumor cells to the lungs in murine models[223]. Additionally, genetic suppression of NF-κB has been shown to markedly decrease pulmonary metastasis in mouse models and reduce tumor angiogenesis[224].

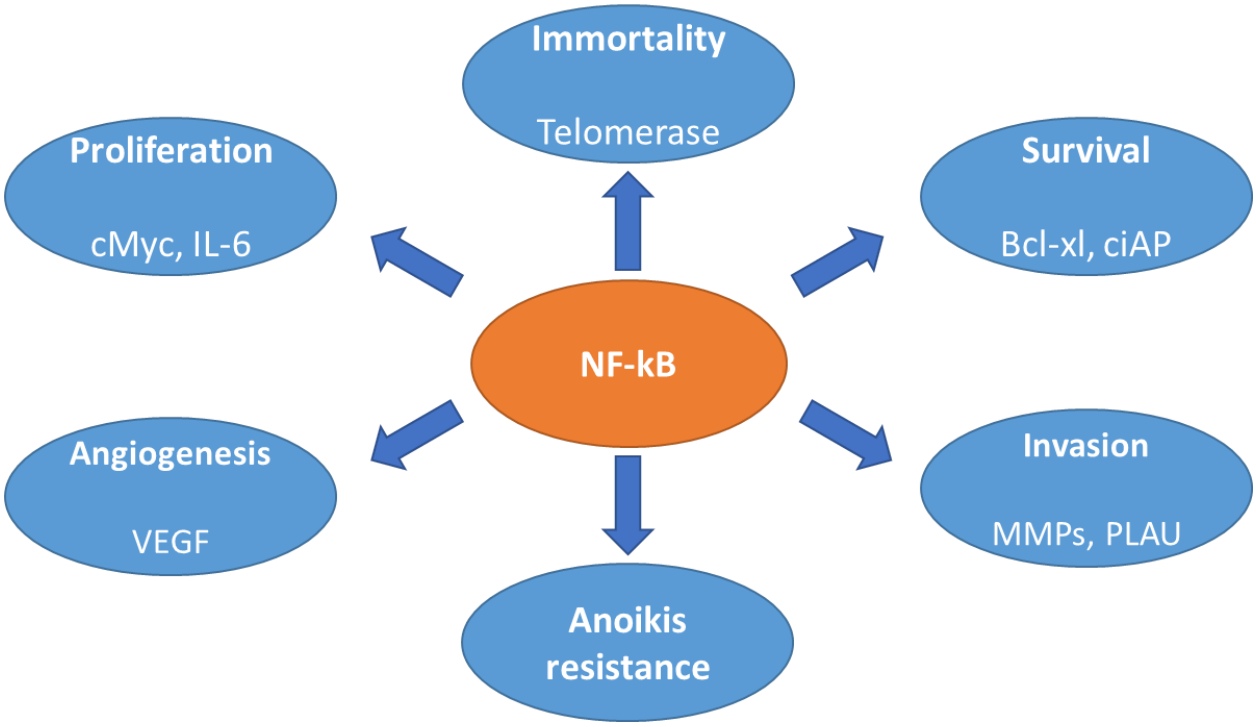

Figure 4 – Roles of NF-κB in cancer metastasis. The NF-κB transcription factor family is implicated in many steps of metastasis.

1.5 HYPOTHESIS

While PAR1 has been shown to be important in driving tumor metastasis in a wide variety of contexts in multiple studies, the precise molecular mechanisms underlying this effect are unclear. **We hypothesize that PAR1 induced CBM-mediated NF-κB activation promotes tumor invasion and metastasis, and in this study we specifically test this hypothesis using both breast cancer and osteosarcoma model systems (Figure 5).** Our analysis of the signaling events responsible for NF-κB transcriptional activation and promotion of metastasis in these cancers has great potential to lead to the identification of novel future therapeutic targets. For example, MALT1 proteolytic activity inhibitors are currently under development and may prove to be effective in preventing and/or treating PAR1-driven tumor metastasis.

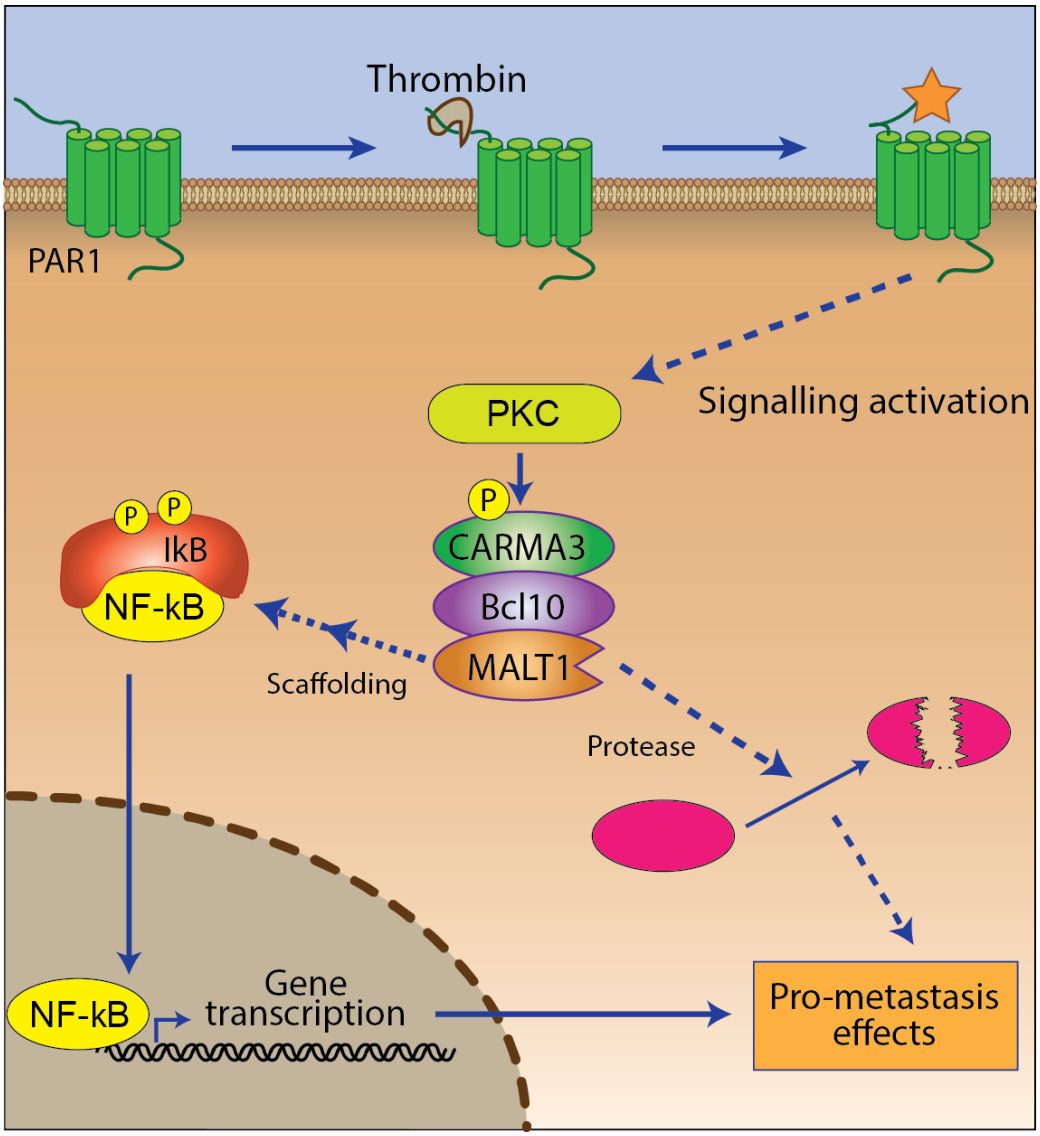

Figure 5 – Hypothesis. The CBM complex mediates PAR1 activation of NF-κB and subsequent pro-metastatic effects in solid tumors.

2.0 RESULTS

2.1 TUMOR PAR1 EXPRESSION PREDICTS CLINICAL OUTCOME IN OSTEOSARCOMA AND BREAST CANCER

2.1.1 Osteosarcoma

We explored available clinical datasets in order to assess whether tumor expression of PAR1 correlates with clinical outcome and presence of tumor metastases. We first studied osteosarcoma using a published clinical case series in which patient tumor sample gene expression profiles were examined[225]. The authors of this study harvested primary tumor samples and assessed gene expression using mRNA microarrays. Sorting cases according to PAR1 (F2R) gene expression revealed a significant survival advantage for patients with tumors that expressed low levels of PAR1 (**Figure 6a**). Median survival time of high-PAR1 cases was approximately 60 months; whereas median survival time for patients with low tumor PAR1 expression was approximately 120 months. Due to the low number of osteosarcoma cases available for study (n = 46), high PAR1 expression was defined as above-median tumor PAR1 expression, while low PAR1 expression was set as below-median tumor PAR1 expression.

We also studied whether patients that died during the study period showed any difference in tumor PAR1 expression relative to patients who survived. While patients that died during the

study appeared to have somewhat higher levels of tumor PAR1 expression over surviving patients (**Figure 6b**), the low number of osteosarcoma cases available did not allow for sufficient power to state that the result was significant at an alpha of 0.05 (Student t test).

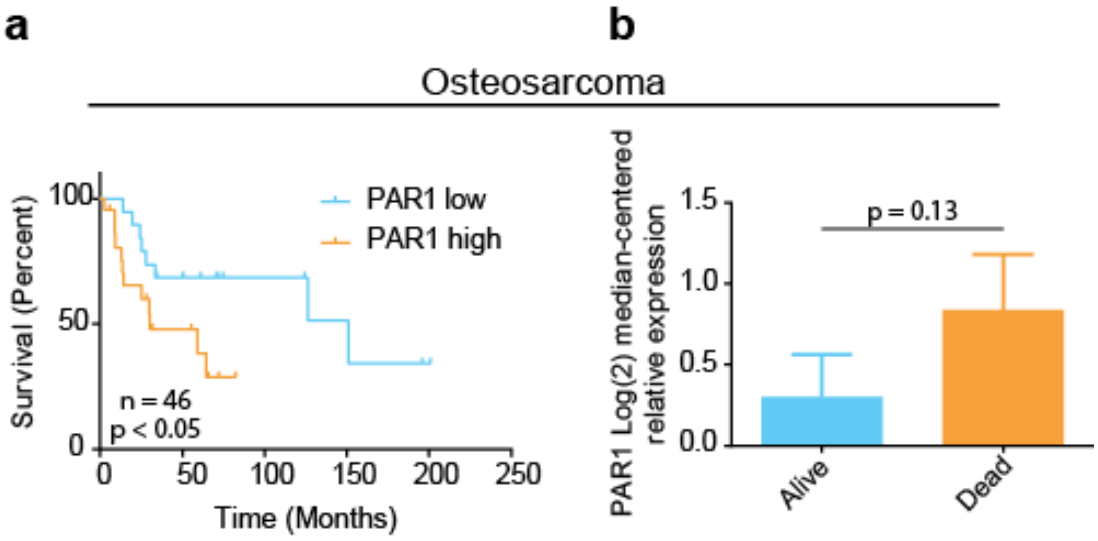

Figure 6 – PAR1 expression is associated with poor clinical outcome and increased tumor metastasis in osteosarcoma. (a) Kaplan-Meier survival plot of osteosarcoma patients sorted by high (above median) and low (below median) PAR1 expression in tumor samples. (b) Expression of PAR1 in osteosarcoma patient tumor samples by survival status; this result is not statistically significant, likely due to the low number of cases analyzed (n = 46). Data are from Kelly *et al.*[225].

2.1.2 Breast cancer

We also examined the relationship between tumor PAR1 expression and survival in breast cancer. Using data from The Cancer Genome Atlas (TCGA)[226] – a database containing mRNA microarray and next-generation exome sequencing expression data – we observed that PAR1 expression negatively correlates with survival in stage 3 breast cancer patients (**Figure 7a**). Patients with high tumor PAR1 expression show significantly greater rates of mortality and did not live as long as patients with low PAR1 (median survival of approximately 100 months vs. >300 months). Given the greater number of breast cancer clinical cases available for study (n = 723), high PAR1 was defined as the patients in the top quartile of tumor PAR1 expression; whereas low PAR1 was set as the patients in the bottom quartile.

Additionally, we found a strong positive relationship between tumor PAR1 expression and the number of metastasis-positive lymph nodes (**Figure 7b**). Patients with no lymph node metastases had significantly ($p < 0.05$) lower levels of primary tumor PAR1 expression compared with the primary tumor PAR1 expression of patients with the highest number of metastasis-positive lymph nodes. PAR1 expression increased steadily with the number of -positive lymph nodes.

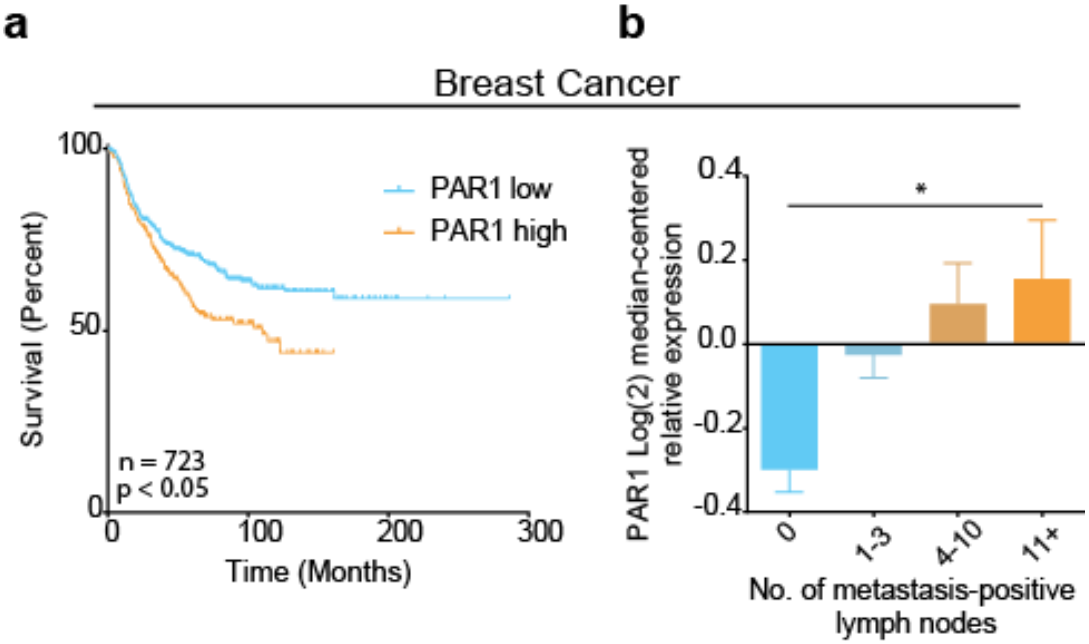

Figure 7 – PAR1 expression is associated with poor clinical outcome and increased tumor metastasis in breast cancer. (a) Kaplan-Meier survival plot of grade 3 breast cancer patients. Patients with high (top quartile) tumor PAR1 expression show decreased survival over time compared to patients with low (lowest quartile) tumor PAR1 expression. (b) PAR1 expression correlates with metastatic lymph node number in breast cancer patients. Data are from the TCGA[226]. *: $p < 0.05$

2.2 PAR1 STIMULATION INDUCES NF-KB IN OSTEOSARCOMA CELLS

2.2.1 Osteosarcoma cell line identification

In light of our observation that PAR1 tumor expression correlates with adverse clinical outcome and metastasis, we next wished to investigate the molecular mechanism by which PAR1 signaling promotes metastasis. Our laboratory previously discovered that PAR1 stimulation

triggers CBM complex-mediated canonical NF-κB activation in vascular endothelial cells[182]. We thus tested whether similar signaling events also take place downstream of PAR1 stimulation in tumor cells. We utilized data from the Cancer Cell Line Encyclopedia (CCLE)[227], a database with characterizations of approximately 1000 cancer cell lines, to examine PAR1 expression in human osteosarcoma cell lines (**Figure 8**), and noted that U2OS, a well-studied untransformed human osteosarcoma cell line, expresses a relatively high level of PAR1 relative to other osteosarcoma lines[136]. While other osteosarcoma cell lines showed slightly higher PAR1 expression, U2OS was the best-characterized line with elevated PAR1 expression. Additionally, we noted that all osteosarcoma cell lines demonstrated some degree of above-median PAR1 expression, where the median was set at the median expression of PAR1 in all cell lines in the CCLE.

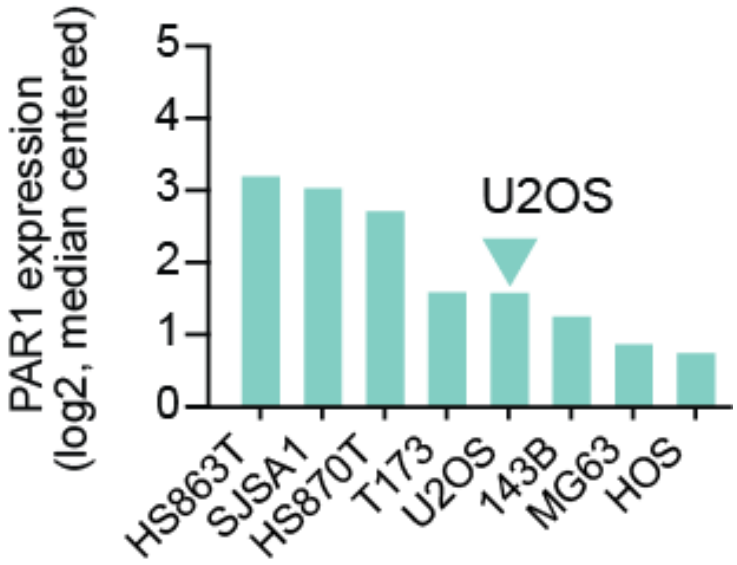

Figure 8 – PAR1 expression of osteosarcoma cell lines. U2OS osteosarcoma cells express PAR1. Log(2) median-centered expression of PAR1 mRNA in selected osteosarcoma cell lines is shown, where the median is median expression of PAR1 in all cancer cell lines studied in the CCLE[227].

2.2.2 U2OS cells activate NF-κB: pIκB, RelA nuclear localization, and luciferase assay

We then analyzed U2OS cells *in vitro* by stimulating with two distinct PAR1 agonists: 1) thrombin, the natural ligand which stimulates PAR1 as well as the related PAR3 and PAR4[228] via proteolytic cleavage, and 2) TRAP6, a synthetic peptide ligand (SFLLRN) which specifically mimics thrombin-induced PAR1 activation[229]. We found that stimulation of U2OS cells with either thrombin or TRAP6 induces robust phosphorylation of IκB, a proximal step in NF-κB activation (**Figure 9a** – see **section 1.4.1**).

We then examined the steps required for NF-κB activation downstream of IκB phosphorylation, and observed that thrombin stimulation of U2OS cells leads to translocation of the canonical NF-κB signaling-associated subunit RelA (p65) into the nucleus as shown by nuclear fractionation and immunoblot (**Figure 9b**). Additionally, thrombin stimulation of U2OS cells enhanced RelA DNA binding activity as detected by NF-κB DNA-binding ELISA (**Figure 9c**). Finally, we performed an NF-κB luciferase reporter assay to assay NF-κB-driven transcriptional activity downstream of PAR1 stimulation. U2OS cells stimulated with thrombin demonstrated increased luciferase expression relative to cells treated with a vehicle control (**Figure 9d**).

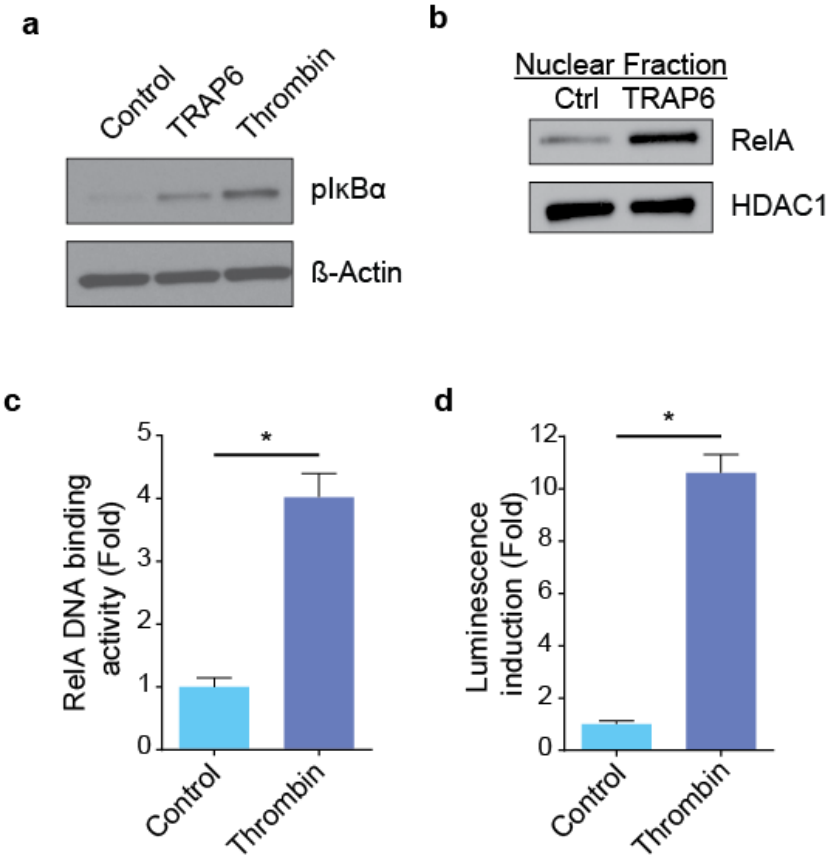

Figure 9 – U2OS osteosarcoma cells activate NF-κB in response to PAR1 agonist stimulation. (a) Stimulation of U2OS human osteosarcoma cells with the 25 μM TRAP6 or 2 U / mL thrombin for 20 minutes induces phosphorylation of IκB. (b) Nuclear fractionation of U2OS cells stimulated with 25 μM TRAP6 for 1 hour shows increased nuclear translocation of the NF-κB subunit RelA as compared to vehicle control. (c) NF-κB consensus-sequence DNA ELISA demonstrates that 2 U / mL thrombin stimulation of U2OS cells for 3 hours leads to increased RelA DNA binding. (d) U2OS cells transfected with an NF-κB reporter plasmid induce expression of luciferase when treated with 2 U / mL thrombin for 8 hours. Data are normalized to transfection efficiency using *Renila* co-transfection.

2.2.3 U2OS cells require MALT1 for PAR1-driven NF-κB activation

We next sought to characterize the specific mechanism by which PAR1 activates NF-κB in osteosarcoma cells, asking whether the CBM complex is required for intracellular signal transduction. We performed siRNA knockdown of CARMA3, Bcl10, and MALT1 and found that phosphorylation of IκB subsequent to PAR1 stimulation is suppressed in U2OS cells transfected with siRNA directed against any constituent of the CBM complex (**Figure 10a**). We then focused our subsequent efforts on the effector protein, MALT1, and found that siRNA suppression of MALT1 completely abrogates TRAP6 or thrombin-induced NF-κB luciferase reporter activity (**Figure 10b**).

Taken together, these results show that PAR1 stimulation does result in NF-κB activation, from phosphorylation of IκB to RelA nuclear localization and transcriptional activation, in U2OS human osteosarcoma cells. Further, this NF-κB activation appears to be dependent on the CBM complex, as loss of MALT1 suppresses both phosphorylation of IκB and NF-κB transcriptional activity.

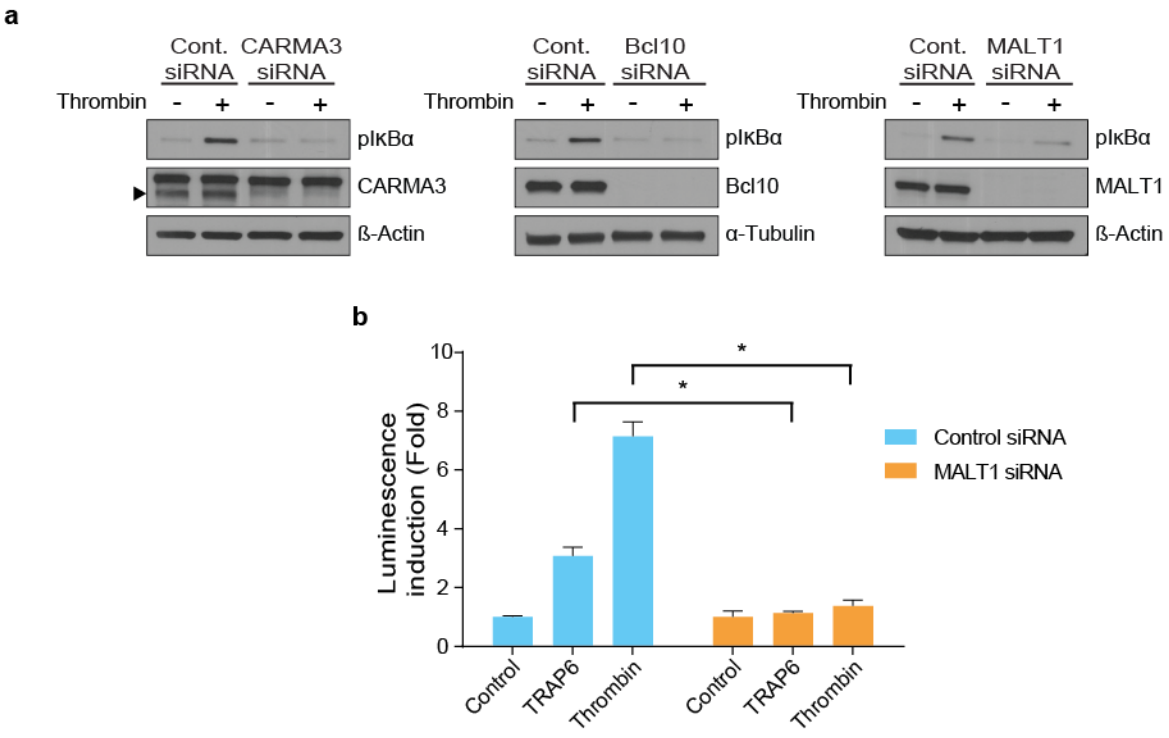

Figure 10 – PAR1 activation of NF-κB signaling requires the CBM complex in U2OS cells. (a) PAR1-induced phosphorylation of IκB in U2OS cells is lost when any member of the CARMA3-Bcl10-MALT1 (CBM) complex is suppressed using siRNA. Cells were stimulated with 2 U / mL of thrombin for 20 minutes. (b) siRNA-mediated knockdown of MALT1 suppresses PAR1-induced NF-κB luciferase reporter activity. Cells were stimulated with 2 U / mL thrombin for 8 hours. *: $p < 0.05$.

2.3 BREAST CANCER CELLS ACTIVATE NF-KB IN RESPONSE TO PAR1 STIMULATION *IN VITRO*

2.3.1 Breast cancer cell line identification

We then turned to analysis of PAR1 signaling in breast cancer. In examining CCLE PAR1 expression data, we found that MDA-MB-231, a highly metastatic human breast cancer cell line thought to represent an aggressive subtype of breast cancer, exhibits relatively high endogenous PAR1 expression (**Figure 11**).

In addition to the MDA-MB-231 human breast cancer cell line, we noted that the human breast cancer line MCF7 appeared to have low PAR1 expression (**Figure 11**). MCF7 is a well-studied untransformed non-metastatic human breast cancer cell thought to represent an early-stage, less aggressive subtype of the disease. For comparison with the parental MCF7 cell line, we obtained the MCF7-N55 cell line. MCF7-N55 is an engineered derivative of MCF7 in which PAR1 has been stably expressed[29]. Notably, ectopic PAR1 expression in MCF7 cells causes significant morphological and phenotypic alterations: MCF7-N55 cells take on an elongated, mesenchymal shape relative to the parental MCF7 line[132]. Additionally, MCF7-N55 cells readily invade through extracellular matrix *in vitro* and colonize mouse lungs *in vivo* in a tail vein injection assay of metastasis; whereas MCF7 cells remain quiescent *in vitro* and do not metastasize *in vivo*[29].

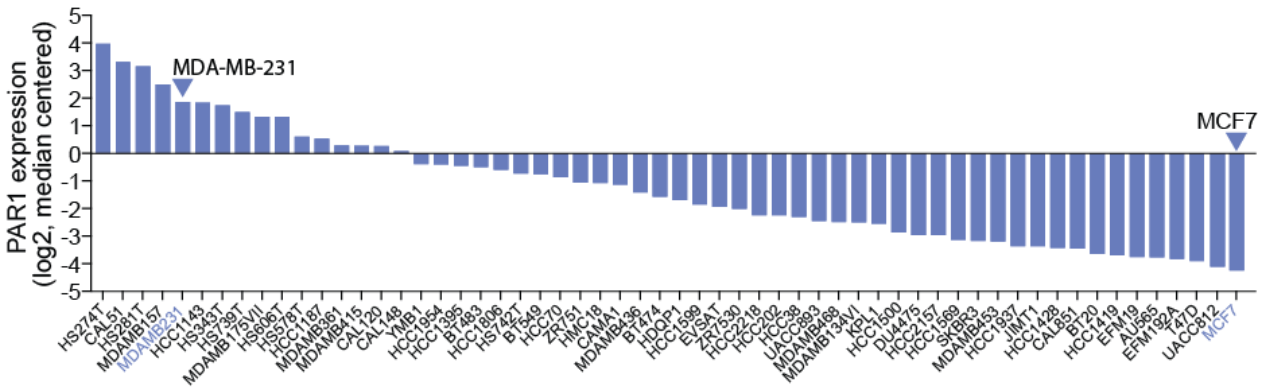

Figure 11 – PAR1 expression of breast cancer cell lines. MDA-MB-231 cells express comparatively high PAR1; whereas PAR1 levels are low in MCF7 cells. Log(2) median-centered expression of PAR1 mRNA in selected breast cancer cell lines is shown, where the median is median expression of PAR1 in all cancer cell lines studied in the CCLE[227].

2.3.2 Breast cancer cells activate NF-κB when stimulated with the PAR1 agonist TRAP6

Stimulation of MDA-MB-231 cells with either TRAP6 or thrombin induces phosphorylation of IκB, indicating activation of canonical NF-κB signaling (**Figure 12a**). Additionally, TRAP6 stimulation of parental MCF7 cells does not result in phosphorylation of IκB, an expected finding given the lack of PAR1 expression in these cells (**Figure 12b**). Stimulation of MCF7 cells with TNFa elicited phosphorylation of IκB, indicating that NF-κB induction machinery was intact in MCF7. In contrast, MCF7-N55 cells demonstrate robust phosphorylation of IκB when stimulated with TRAP6, as expected given the stable expression of PAR1 in these cells.

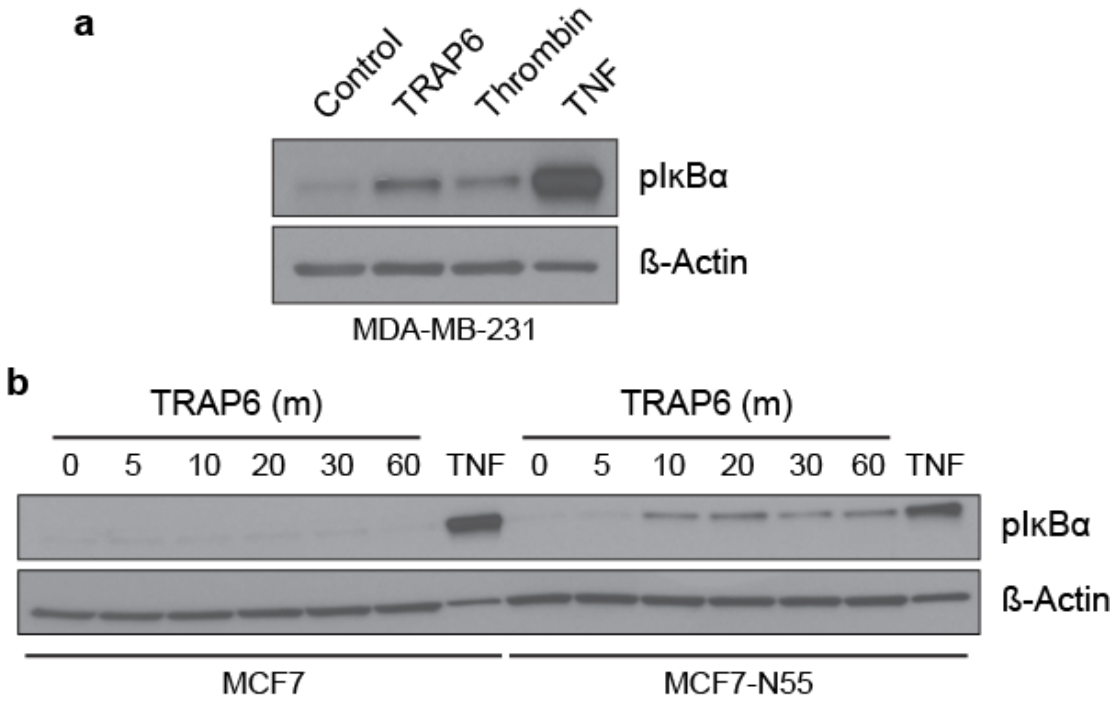

Figure 12 – Breast cancer cells activate NF-κB in response to PAR1 agonist stimulation. (a) Stimulation of MDA-MB-231 cells with the 50 μM TRAP6 or 2 U / mL thrombin for 15 minutes induces phosphorylation of IκB. (b) PAR1 stimulation with 50 μM TRAP6 fails to induce phosphorylation of IκB in MCF7 human breast cancer cells; whereas TRAP6 treatment induces robust phosphorylation of IκB in MCF7-PAR1 cells.

2.3.3 MALT1 is required for PAR1-driven NF-κB activation in breast cancer

MALT1 knockdown results in loss of IκB phosphorylation in MDA-MB-231 cells stimulated with TRAP6 (**Figure 13a**). Further, we observed a similar suppression of IκB phosphorylation after TRAP6 treatment in MCF7-N55 transfected with siRNA directed against MALT1 (**Figure 13b**).

Taken together, these results indicate that TRAP6 stimulation of the PAR1-expressing breast cancer cells MDA-MB-231 and MCF7-N55 induces MALT1-dependent NF-κB activation, similar to the results we observed in osteosarcoma cells.

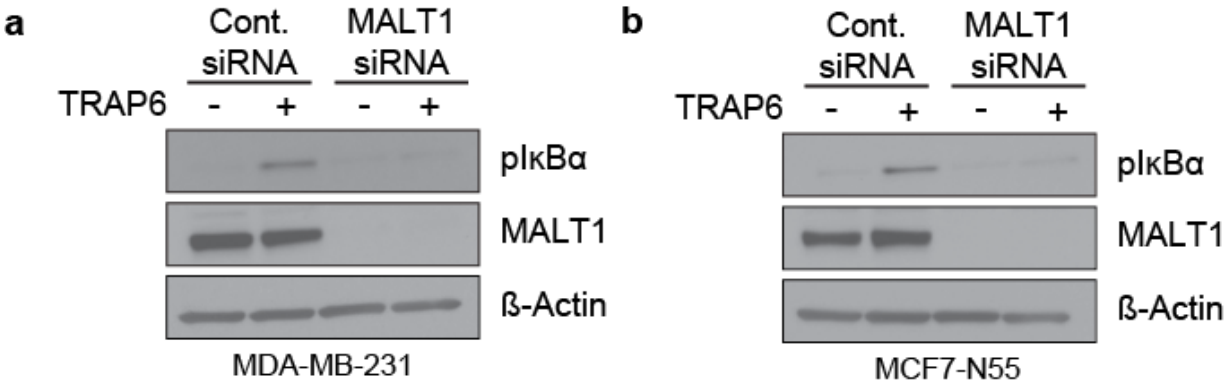

Figure 13 – Breast cancer cells require MALT1 in order to activate PAR1 – NF-κB signaling. (a) PAR1-induced phosphorylation of IκB in MDA-MB-231 cells is lost when MALT1 is suppressed using siRNA. Cells were stimulated with 50 μM TRAP6 for 20 minutes. (b) PAR1-induced phosphorylation of IκB in MCF7-PAR1 cells is lost when MALT1 is suppressed using siRNA. Cells were stimulated with 50 μM TRAP6 for 20 minutes.

2.4 PAR1 ACTIVATION OF NF-KB DRIVES PRO-METASTATIC GENE EXPRESSION IN OSTEOSARCOMA

2.4.1 RT-PCR microarray

We next asked whether PAR1 stimulation of NF-κB in cancer cells caused gene expression reprogramming known to be associated with metastasis. First, we employed a custom RT-PCR microarray to assess changes in the expression levels of genes known to be NF-κB targets and to promote metastasis (**Figure 14**). Data in Figure 14 are shown as a heat map of relative quantification Z-scores across three independent biological replicates: significant upregulation of expression is colored red, while blue denotes significant downregulation. We detected several significant gene expression changes in U2OS cells after stimulation with thrombin. Several

metastasis-associated matrix remodeling proteins such as MMP9, 10, and 12 were significantly upregulated in thrombin-treated U2OS cells. We also observed a significant increase in expression of the inflammatory cytokine IL1B.

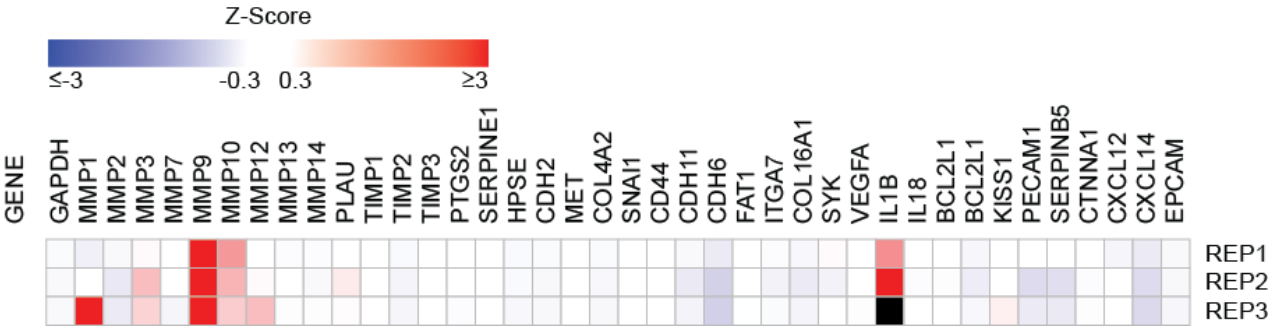

Figure 14 – Invasion-associated RT-PCR microarray results. Heat map of Z-scores for invasion-associated RT-PCR microarray. U2OS human osteosarcoma cells were stimulated with 2 U / mL thrombin for 6 hours. Three biological replicates of the array were performed. Black: no value returned from replicate (technical error).

2.4.2 Microarray hits: MMP9 and IL1B

We then examined whether MALT1 was required for the PAR1-stimulation associated upregulation of genes detected in the metastasis-associated RT-PCR microarray in U2OS osteosarcoma cells. We noted that MALT1 loss suppresses thrombin-dependent induction of inflammatory cytokine IL1B (**Figure 15a**). Additionally, MALT1 is also required for PAR1-driven upregulation of the matrix metalloproteases MMP9 and MMP12 (**Figures 15b and 15c**). siRNA was used to knock down MALT1; a representative demonstration of MALT1 knockdown detected by RT-PCR is shown (**Figure 15d**).

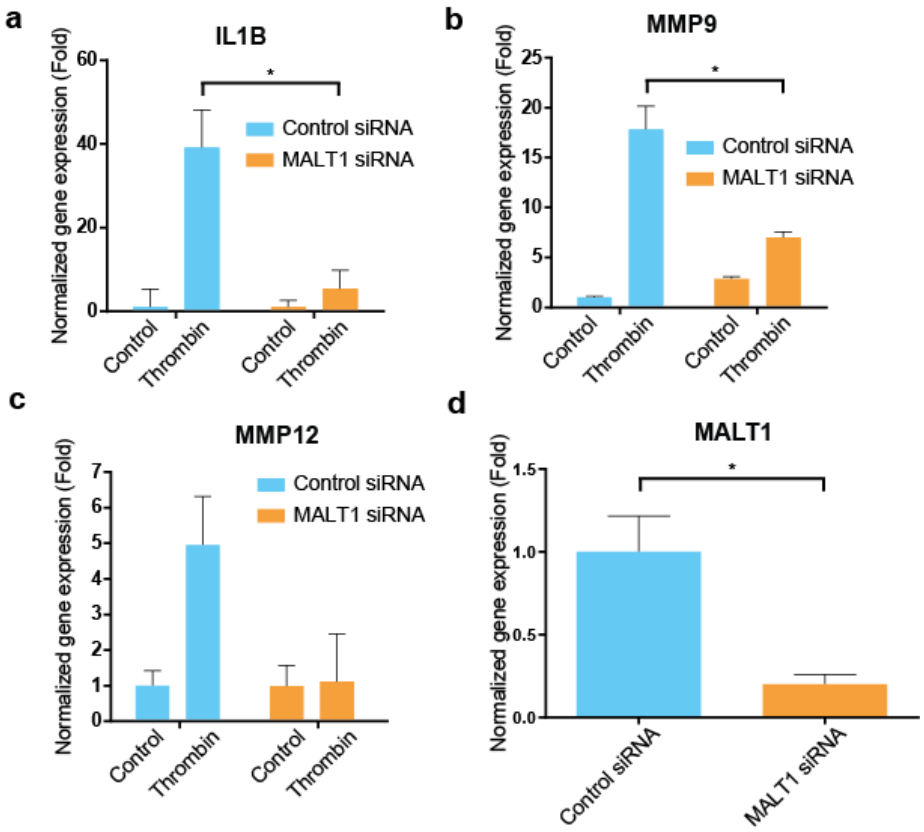

Figure 15 – Upregulation of invasion-associated genes requires MALT1 in osteosarcoma cells. (a) IL1B, (b) MMP9, and (c) MMP12 mRNA expression is induced by stimulation of U2OS cells with 2 U / mL thrombin for 6 hours. (d) siRNA-directed MALT1 knockdown suppresses PAR1-dependent gene induction.

2.5 PAR1 DRIVES PRO-METASTATIC GENE EXPRESSION IN BREAST CANCER

Having identified upregulation of metastasis-associated genes downstream of PAR1 signaling activation in osteosarcoma, we next looked at gene expression reprogramming in breast cancer. We observed that stimulation of PAR1 in both MDA-MB-231 breast cancer cells and MCF7-N55 breast cancer cells results in gene expression changes that are similar to, though distinct from, those observed in osteosarcoma cells. In common with U2OS osteosarcoma cells, MDA-MB-231 and MCF7-N55 breast cancer cells demonstrate upregulation of IL1B in response to

TRAP6 stimulation (**Figure 16a and 16b, top left**). Further, PAR1 stimulation in MDA-MB-231 and MCF7-N55 cells leads to upregulation of the inflammatory cytokine IL-8 (**Figure 16a and 16b, bottom left**) and of SERPINE1 (also referred to as PAI-1, **Figure 16 a and 16b, bottom right**), both of which are highly implicated in breast cancer metastasis. Additionally, the parental MCF7 breast cancer line did not show expression of IL1B or IL8 when treated with TRAP6, as expected given the low PAR1 expression in this cell line (data not shown).

Unlike in U2OS cells, we did not observe MMP9 expression when either MDA-MB-231 or MCF7-N55 breast cancer cells were stimulated with a PAR1 agonist (**Figure 16 and 16b, top right**).

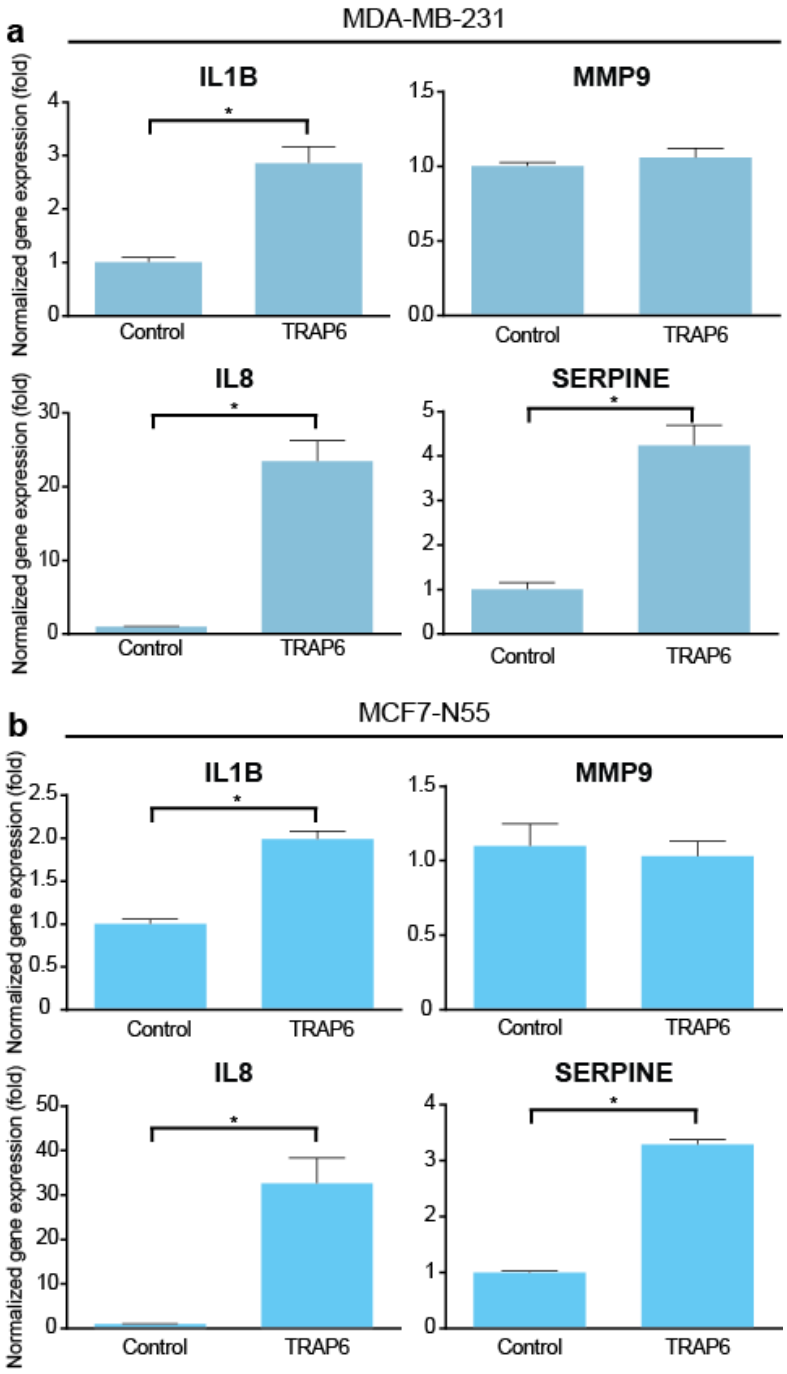

Figure 16 – Breast cancer cells upregulate invasion-associated genes in response to PAR1 stimulation. (a) IL1β, IL8, and SERPINE1 mRNA, though not MMP9 mRNA, is upregulated in MDA-MB-231 human breast cancer cells stimulated with 50 μM TRAP6 for 6 hours. (b) Similarly, in MCF7-PAR1 cells stimulated with 50 μM TRAP6 for 6 hours, IL1β, IL8, and SERPINE1mRNA is upregulated. TRAP6 treatment did not affect MMP9 mRNA expression.

2.6 MALT1 KNOCKOUT SUPPRESSES PRO-METASTATIC GENE EXPRESSION IN BREAST CANCER

2.6.1 CRISPR: generation of the N55-ΔMALT1 knockout pool

In order to investigate the requirement for MALT1 in PAR1-induced gene expression changes, we used CRISPR[230-234] to generate stable MCF7-N55 clones deficient in MALT1 (for methodological details, see **section 4.8**). MALT1 knockout clones were identified by immunoblot; three MALT1 knockout clones were selected (4-2, 5-3, 6-5) and evenly pooled for use in experiments to avoid single-clone dependent observations. This knockout pool is referred to as **N55-ΔMALT1** below. For use as a control, three clones of MCF7-N55 cells that underwent the CRISPR protocol but did not demonstrate detectable MALT1 knockout (as seen on immunoblot) were selected (5-2, 9-2, 12-1). These clones were evenly pooled for experiments and are referred to as **N55-Ctrl** below. All clones resulting from the CRISPR protocol were maintained separately in culture: N55-Ctrl and N55-ΔMALT1 pools were freshly prepared for each experiment.

2.6.2 Validation of MALT1 knockout

We regularly confirmed MALT1 knockout status in CRISPR clones by immunoblot and found that MALT1 knockout status remains stable for over six months following clonal selection (**Figure 17, top panel**). Control CRISPR clones also demonstrated stable MALT1 expression over six months past single-clone selection. .

Consistent with loss of MALT1, pooled N55-ΔMALT1 cells did not show phosphorylation of IκB when stimulated with TRAP6; whereas the N55-Ctrl pool demonstrated robust induction of pIκB when treated with TRAP6 (**Figure 17, bottom panel**).

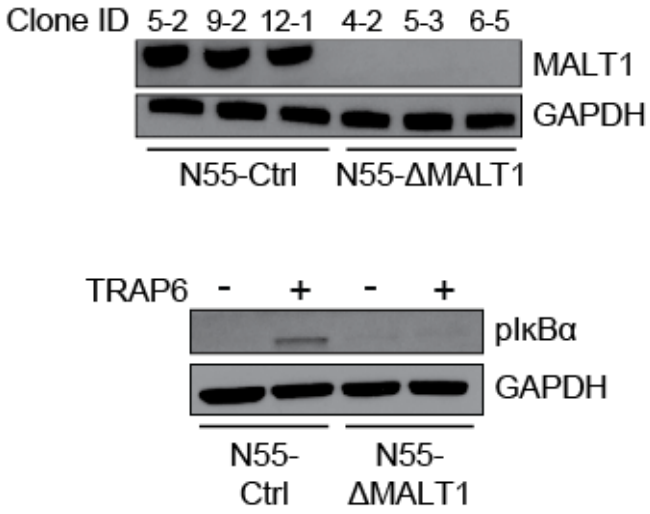

Figure 17 – Generation and validation of MALT1 CRISPR knockout cells. Confirmation that N55-ΔMALT1 cells are deficient in MALT1 (top panel), and fail to respond to TRAP6-mediated stimulation of PAR1 by inducing pIκB (bottom panel; cells treated with 50 μM TRAP6 for 20 minutes).

2.6.3 MALT1 knockout suppresses IL1B and IL8 induction downstream of PAR1 in breast cancer

The pooled N55-Ctrl cells behave similarly to the parental MCF7-N55 cells when stimulated with TRAP6 and assayed for gene expression reprogramming changes. TRAP6 stimulation of N55-Ctrl cells induces expression of IL1B; however, N55-ΔMALT1 cells do not demonstrate IL1B induction under the same conditions (**Figure 18, top left**), suggesting that MALT1 is required for IL1B upregulation after PAR1 stimulation in a fashion similar to that observed in U2OS osteosarcoma cells. Additionally, TRAP6 stimulation of N55-Ctrl cells induces IL8 expression, again consistent with MDA-MB-231 cells, and MALT1 is required for this effect (**Figure 18, bottom left**).

Additionally, we found that MMP9 is not upregulated in N55-Ctrl or in N55-ΔMALT1 cells in response to TRAP6 stimulation (**Figure 18, top right**). This is consistent with our observations in MDA-MB-231 breast cancer cells, giving us high confidence that MMP9 is not in fact expressed downstream of PAR1-associated NF-κB activation in breast cancer. Intriguingly, we noted that PAR1-induced SERPINE1 expression does not require MALT1 (**Figure 18, bottom right**). Stimulation of both N55-Ctrl cells and N55-ΔMALT1 with TRAP6 induced similar amounts of SERPINE1 expression, indicating that SERPINE1 expression is likely PAR1-depdent but MALT1-independent in breast cancer.

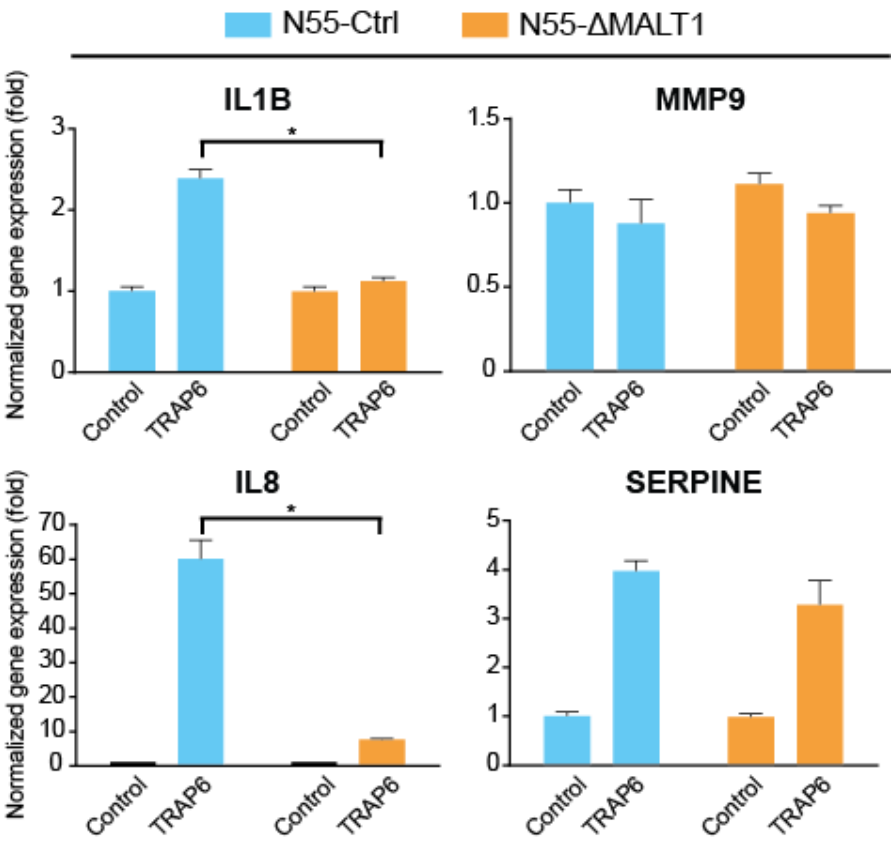

Figure 18 – MALT1 is required for IL1B and IL8 expression downstream of PAR1 in breast cancer cells. PAR1-dependent IL1β and IL8 mRNA upregulation is abrogated in N55-ΔMALT1 cells as compared to N55-Ctrl cells. MMP9 is not upregulated in response to PAR1 stimulation in N55-Ctrl or N55-ΔMALT1 cells. PAR1-dependent upregulation of SERPINE1 is not affected by MALT1 knockdown. Cells were stimulated with 50 μM TRAP6 for 6 hours.

2.7 MALT1 KNOCKOUT ATTENUATES *IN VITRO* INVASION AND *IN VIVO* METASTASIS

2.7.1 Transwell assay of invasion

After identification of a number of a pro-metastatic genes downstream of PAR1/CBM signaling, we sought to determine whether stimulation of this signaling axis had functional, phenotypic effects relevant to metastasis on human cancer cells. We first asked whether MALT1 is required for PAR1-dependent invasion *in vitro*. It has been previously shown that PAR1 expression specifically induces aggressive *in vitro* invasion and migration in the MCF7/MCF7-N55 system[29,132]. In a transwell chamber assay of *in vitro* invasion, we found a similar result in that PAR1-high MCF7-N55 cells invade through a collagen matrix at a significantly higher rate than the parental PAR1-low MCF7 cells (**Figure 19a**).

We then asked whether MALT1 was required for this PAR1-specific induction of *in vitro* invasion. We compared invasion of the N55-Ctrl pool to the N55-ΔMALT1 pool in the transwell invasion assay. We found that N55-Ctrl cells invade similarly to the MCF7-N55 cells from which they were derived; however, *in vitro* invasion of N55-ΔMALT1 cells is substantially blunted relative to the N55-Ctrl pool. Taken together these results suggest that MALT1 is required for the invasive cellular phenotype specifically induced by PAR1 expression (**Figure 18b**).

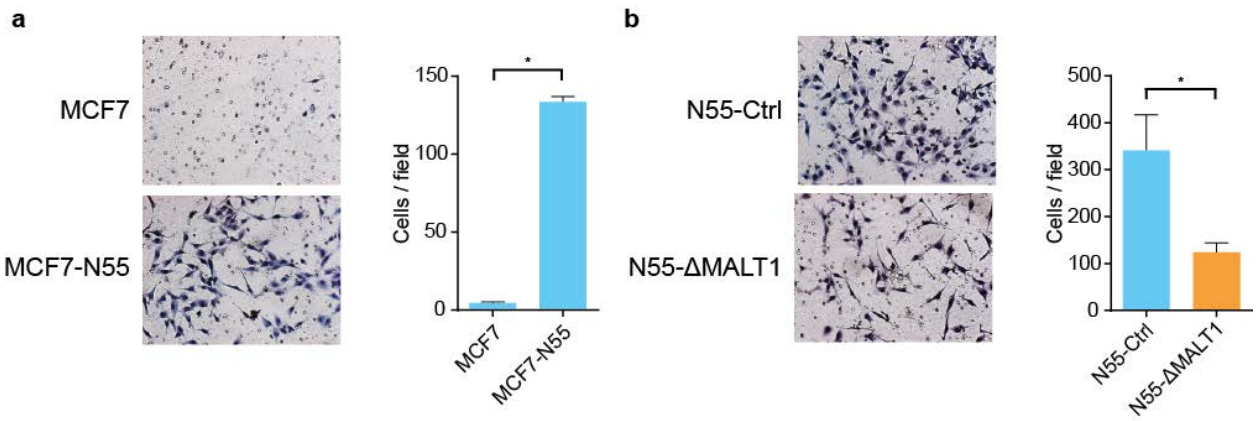

Figure 19 – CRISPR knockout of MALT1 in MCF7-N55 cells attenuates invasion *in vitro*. (a) MCF7-PAR1 (PAR1-high) cells demonstrate significantly inceased invasion in a Boyden chamber transwell assay as compared to parental MCF7 (PAR1-low) cells. (b) Pooled N55-ΔMALT1 cells demonstrate significant loss of invasiveness as compared to pooled N55-Ctrl cells.

2.7.2 Mouse model of breast cancer metastasis

We then proceeded to compare the formation of lung metastasis using N55-Ctrl cells or N55-ΔMALT1 cells in a tail-vein injection model. See **Appendix A** for details of ongoing experiments.

2.8 PAR1 STIMULATION ACTIVATES MALT1 PROTEOLYTIC ACTIVITY IN SOLID TUMORS

2.8.1 RelB: a target of the MALT1 protease

We investigated whether PAR1 stimulation in cancer cells induces MALT1 proteolytic activity, specifically by testing for cleavage of the NF-κB family member RelB, a known substrate of MALT1[189,191]. We found that stimulation of U2OS osteosarcoma cells with thrombin induces accumulation over time of a RelB cleavage fragment detectable by immunoblot (**Figure 20a**).

We then looked to breast cancer, examining whether PAR1 stimulation-associated MALT1 cleavage of RelB could be detected in MDA-MB-231 cells. In MDA-MB-231 cells, we found that TRAP6 stimulation induces generation of a RelB cleavage fragment (**Figure 20b**). As expected, no RelB cleavage is observed upon TRAP6 treatment of parental MCF7 cells, which do not express appreciable levels of PAR1. In contrast, TRAP6 -induced RelB cleavage is readily detected in MCF7-N55 cells (**Figure 21b**).

All of the RelB cleavage experiments performed above were carried out in the presence of the proteasome inhibitor MG132 in order to increase RelB cleavage fragment detection. RelB cleavage fragments were rapidly cleared from the cell by proteasomal degradation without MG132, consistent with published reports in lymphoma cell lines[191].

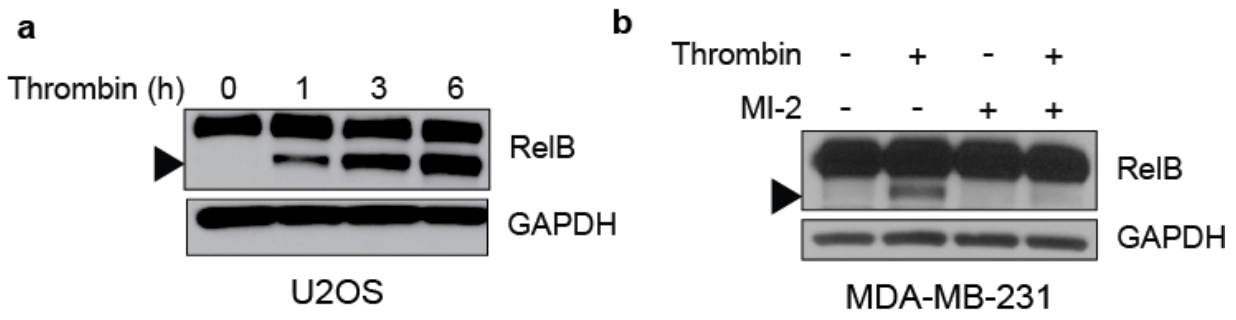

Figure 20 – PAR1 stimulation activates MALT1 proteolytic activity in solid tumors. (a) U2OS human osteosarcoma cells were stimulated with 25 μM TRAP6 for the indicated times. A RelB proteolytic cleavage fragment of approximately 40 kD can be seen accumulating (arrowhead). (b) RelB proteolytic cleavage also occurs in MDA-MB-231 breast cancer cells stimulated with thrombin, and treatment with the MALT1 inhibitor, MI-2, suppresses RelB cleavage. Cells were pretreated with 2 μM MI-2 or DMSO vehicle before treatment with 2 U / mL thrombin for 3 hours. All experiments above were performed in the presence of the proteasome inhibitor MG132 to prevent proteasomal degradation of the RelB cleavage fragment.

2.8.2 RelB degradation in osteosarcoma and breast cancer is MALT1-specific

In order to determine whether the detected cleavage of RelB was in fact MALT1 specific, we utilized two specific inhibitors of the MALT1 protease: ZVRPR-fmk, a covalently-bonding irreversible peptide inhibitor that binds to the MALT1 active site, or MI-2, an irreversible suicide substrate small molecule inhibitor of MALT1 proteolytic activity[149,235]. Thrombin stimulation-induced cleavage of RelB in U2OS cells was suppressed in a concentration-dependent manner by both ZVRPR-fmk and MI-2 (**Figure 21a**), strongly suggesting that PAR1 stimulation is activating MALT1 proteolysis of RelB in osteosarcoma.

Additionally, MI-2 treatment of MDA-MB-231 (**Figure 20b**) and MCF7-N55 (**Figure 21b**) cells stimulated with TRAP6 suppressed generation of a RelB cleavage fragment, suggesting that RelB cleavage in breast cancer cells is also MALT1-specific. Further, the N55-

Ctrl pool of CRISPR clones with intact MALT1 demonstrated generation of a RelB cleavage fragment when stimulated with TRAP6; however, the MALT1-deficient N55-ΔMALT1 pool lost RelB cleavage, indicating that MALT1 is required for RelB cleavage in breast cancer cells.

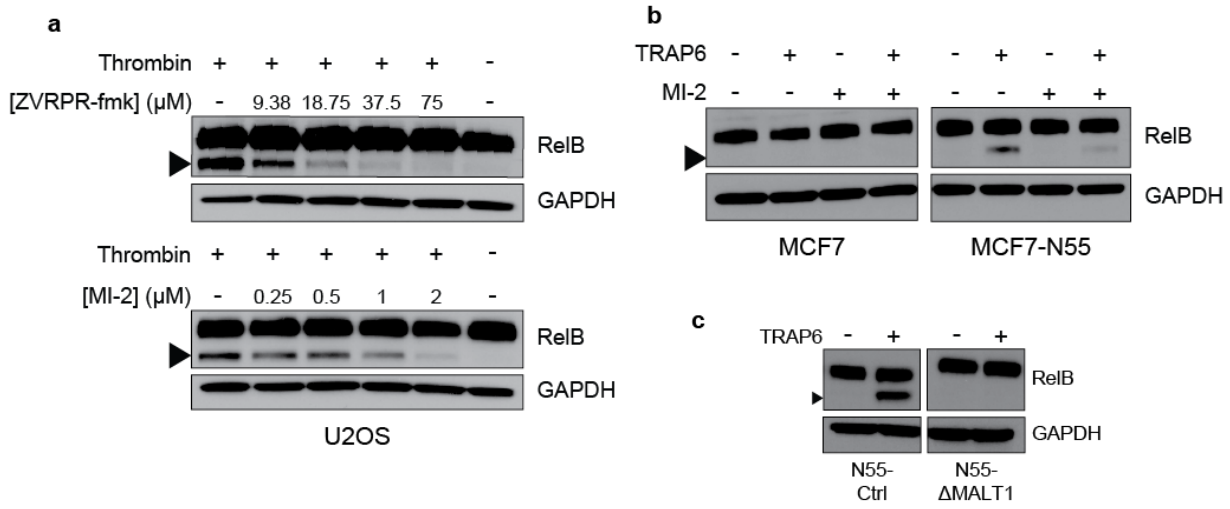

Figure 21 – Cleavage of RelB in osteosarcoma and breast cancer is MALT1-specific. (a) The MALT1-protease inhibitors ZVRPR-fmk and MI-2 both suppress the formation of the RelB cleavage fragment in a dosage-dependent manner in U2OS osteosarcoma cells. Cells were pretreated with the indicated concentrations of inhibitor for 1 hour before stimulation with 2 U / mL thrombin for 3 hours. (b) MALT1 dependent RelB cleavage occurs after PAR1 stimulation in MCF7-PAR1 cells, but not in control parental MCF7 cells. Cells were pretreated without or with 2 μM MI-2 before treatment with 50 μM TRAP6 for 3 hours. (c) CRISPR knockout of MALT1 suppresses RelB cleavage fragment formation after TRAP6 stimulation. N55-Ctrl and N55-ΔMALT1 cells were treated with 50 μM TRAP6 for 3 hours with MG132. All experiments above were performed in the presence of the proteasome inhibitor MG132 to prevent proteasomal degradation of the RelB cleavage fragment.

We asked whether MALT1 proteolytic activity inhibition had any effect on gene expression, so we next treated U2OS cells with the MALT1 protease activity inhibitors ZVRPR-fmk or MI-2. We found that PAR1-dependent induction of both IL1B (**Figure 22a**) and MMP9 (**Figure 22b**) are blocked by both MALT1 protease inhibitors.

Taken together, our results indicate that in both osteosarcoma and breast cancer cells, MALT1 proteolytic activity is induced by PAR1 stimulation, providing the first evidence of MALT1 proteolytic activity in non-hematopoietic tumors and further confirming PAR1-induced MALT1 activation in these malignant cells.

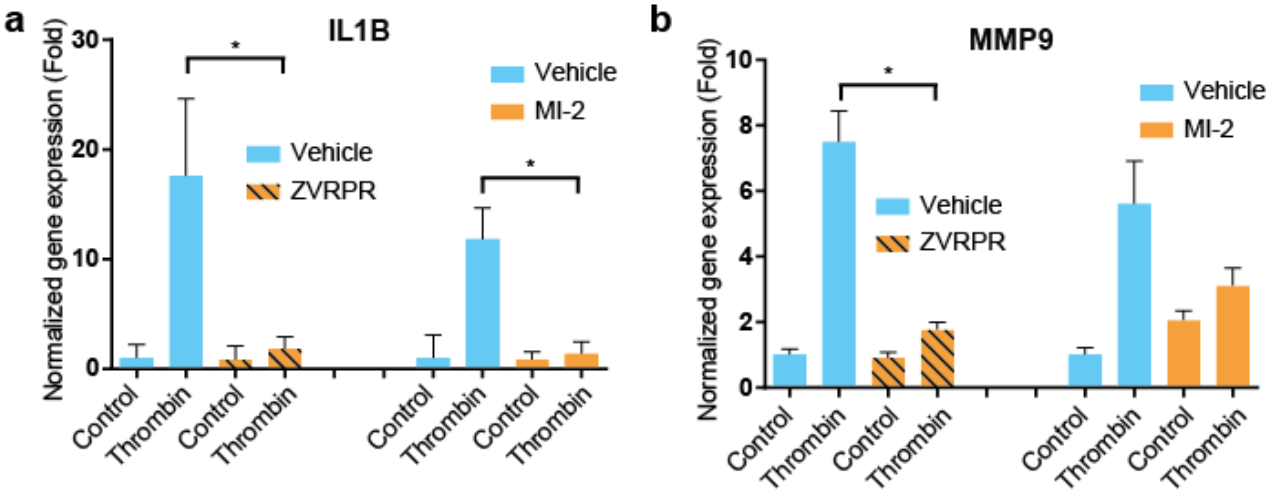

Figure 22 – MALT1 proteolytic activity is required for PAR1 stimulation-induced expression of IL1B and MMP9. MALT1 protease inhibitors ZVRPR and MI-2 suppress PAR1-dependent upregulation of (a) MMP9 and (b) IL1β. Cells were pretreated with 25 μM ZVRPR-fmk or 2 μM MI-2 for one hour before stimulation with 2 U / mL thrombin for 6 hours.

3.0 DISCUSSION

In this work, we show that the CBM complex is a critical mediator of PAR1-induced signaling to NF-κB. Our bioinformatics findings demonstrate that patients with high tumor PAR1 expression have worse clinical outcomes (**Figures 6a and 7a**). Further, we note that the CBM complex is required for PAR1 activation of NF-κB in both endogenous (MDA-MB-231, U2OS) and ectopic (MCF7-N55) systems of PAR1 expression, giving us confidence that our findings are representative of PAR1 signaling in cancer more broadly and not due solely to cell line and/or tumor type-specific factors. We demonstrate a requirement for MALT1 in PAR1-driven expression of genes tightly associated with metastasis: IL1β and MMP9 in osteosarcoma and IL1β and IL8 in breast cancer. Further, we show that MALT1 knockout attenuates *in vitro* invasion driven by PAR1 expression.

3.1 PAR1, CBM, AND NF-KB SIGNALING IN OSTEOSARCOMA AND BREAST CANCER METASTASIS

Our results show that PAR1 signaling activates NF-κB and promotes invasion through the CBM complex in tumor cells; however, this pro-metastatic behavior is not due to constitutively activating oncogenic mutations at any point in the signaling pathway, rather, it is due to endogenous PAR1 signaling that inappropriately activates inflammation. Taken together, these

results point to possible mechanisms by which the CBM complex mediates pro-metastatic signaling in cancer.

NF-κB activation, such as the activation we describe downstream of PAR1 / CBM signaling, promotes metastasis in myriad ways[209]. First, NF-κB stimulates cell proliferation and protects against apoptosis directly through upregulation of a variety of survival factors and tissue invasion effectors, such as cIAP and MMPs[211]. Second, NF-κB induction is associated with MMP expression and tissue invasion[213]. Third, NF-κB activation is tightly linked with the expression of EMT markers such as Twist and Snail[214,215]. Fourth, NF-κB signaling has been implicated in the expression of cellular adhesion proteins, necessary in the vascular extravasation step of metastasis. Given that pharmacological NF-κB suppression can attenuate osteosarcoma and breast cancer proliferation and invasion, it is likely that NF-κB plays an important role in the promotion of metastasis in these two cancers.

With our observation that PAR1-CBM signaling leads to activation of NF-κB in osteosarcoma and breast cancer, suppression of CBM signaling should block any contribution of PAR1 to NF-κB-mediated metastasis in these tumors. It is important to note that our results should not be interpreted beyond the extent of the PAR1 – CBM – NF-κB signaling axis. There are numerous pathways that can result in PAR1-independent and CBM-independent NF-κB activation, or indeed NF-κB-independent metastasis. We observed TNFa stimulation induction of pIκB, indicating NF-κB activation, independent of the CBM complex in both breast cancer and osteosarcoma. However, our results showing that PAR1 expression predicts poor outcome for osteosarcoma and breast cancer patients (**Figures 6a and 7a**), as well as previous reports indicating that PAR1 expression correlates with severity of disease, give us confidence that, while the PAR1 may not be the only cell surface receptor that can lead to NF-κB activation and

promotion of metastasis in these cancers, it is nevertheless an important contributor to tumor metastasis.

3.2 ADDING TO THE MODEL OF THE CBM COMPLEX IN SOLID TUMORS

An emerging model of the CARMA3-containing CBM complex as a putative "central hub" of GPCR activation of NF-κB promises exciting developments for future clinical and basic research. Several GPCRs, most notably AGTR1[236], CXCR4[180], LPA receptors[199], and now PAR1 have been demonstrated to activate NF-κB and promote solid tumor progression and metastasis via the CBM complex, and it seems likely that other GPCRs which engage the CBM complex in promoting NF-κB dependent tumor metastasis will be identified in the future. Several enticing questions remain unanswered, such as to what extent the CBM complex is capable of integrating intracellular signaling from multiple upstream GPCRs and whether multi-GPCR activation of CBM signaling has an additive effect on NF-κB activation and metastasis promotion. The position of the CBM complex as a common step in the intracellular signaling pathway that leads from cell surface receptors to NF-κB activation in cancer makes it an attractive therapeutic target: pharmacologically knocking out CBM signaling could, in theory, suppress pro-metastatic signals from multiple GPCRs.

3.3 MALT1 PROTEOLYTIC ACTIVITY: A POTENTIAL THERAPEUTIC TARGET

3.3.1 Clinical significance of this work

Our results suggest that MALT1 proteolytic activity inhibition could attenuate inappropriate PAR1-driven activation of NF-κB in solid tumors in order to prevent or treat metastatic disease. MALT1 protease activity inhibitors are currently in preclinical development. Thus far, three categories of MALT1 protease inhibitors are in active research use: modified peptides, small molecules, and phenothiazines. Modified peptides such as ZVRPR-fmk[149], and small molecules like MI-2[235], are irreversible inhibitors that covalently bond with MALT1. Most intriguingly from a preclinical standpoint, several phenothiazines, an FDA-approved class of antipsychotics, have recently been shown to reversibly inhibit MALT1 proteolytic capability at low concentrations[237]. Excitingly, several MALT1 inhibitors, including MI-2[235] and the phenothiazine derivatives mepazine[238,239] and thioridizine[237], have successfully been used to treat mice in models of disease with little observed toxicity.

Our evidence suggests that upregulation of metastasis-associated genes such as MMP9 and IL1B downstream of PAR1 stimulation in osteosarcoma are suppressed by treatment with these MALT1 inhibitors (**Figure 22**). Through downregulation of these important pro-metastatic targets, MALT1 proteolytic activity inhibition may decrease metastatic behavior in osteosarcoma, though this has yet to be demonstrated.

The majority of osteosarcoma patients do not present with clinically identifiable metastatic disease, and development of metastases during treatment has catastrophic prognostic consequences. It is possible that the addition of a MALT1 proteolytic activity inhibitor to the

current MAP protocol of adjuvant / neo-adjuvant chemotherapy in combination with surgery may serve to prevent the development of metastases in these at-risk patients.

3.3.2 Future applications of MALT1 inhibition in solid tumor treatment

Successful treatment of solid tumors in which CBM signaling is involved will require multiple modalities and strategies. It is doubtful that MALT1 inhibition alone will be enough to suppress cancer growth and progression sufficiently such that it could be used as a single-agent treatment for solid tumors. Similar to the current standard of osteosarcoma and breast cancer care in which multiple chemotherapeutics are used to treat an individual patient's disease, MALT1 proteolytic activity inhibition could be employed in combination with other targeted therapies, such as NF-κB suppression, to more effectively inhibit cancer progression. Selective NF-κB inhibition has long been sought after for use as an anticancer therapeutic; however, difficulties in limiting patient immunosuppression have hampered drug development efforts[240]. As part of a multi-agent protocol along with a MALT1 proteolytic activity inhibitor, though, it is possible that milder NF-κB suppression could be used to effectively target pathways that promote the growth and metastasis of CBM-associated solid tumors.

3.4 POSSIBLE FUTURE DIRECTIONS

As with many research projects, a surfeit of fascinating and compelling questions are raised by our results. Possible future research directions arising from this project are discussed below.

Our observation that PAR1 – CBM – NF-κB signaling in cancer cells induces the expression of several inflammatory cytokines such as IL1B and IL8 suggests the possible existence of an autocrine or paracrine positive-feedback loop in which initial NF-κB signaling events drive transcription, translation, and secretion of further inflammatory mediators. These inflammatory mediators may go on to trigger even more NF-κB activation in tumor cells and in the stromal microenvironment, making the tumor cells and microenvironment more metastatic or permissive of metastasis. The nature and extent of such CBM-mediated tumor / microenvironmental interactions has yet to be described; however, elucidation CBM complex-mediated tumor-stroma interaction would be a novel finding in the field of CBM cancer research.

NF-κB is not the sole downstream output of either PAR1 or of the CBM complex itself. The CBM complex is known to trigger JNK signaling[192], itself a pro-metastatic signaling pathway: JNK activation has been shown to increase sensitivity to chemotherapeutics in osteosarcoma[241]. The role of CBM-mediated signaling pathways aside from NF-κB and their contributions to solid tumor metastasis is an enticing research questions that has not been directly addressed in the literature.

Our results indicate a possible role for MALT1 proteolytic activity inhibition in the suppression of pro-metastatic genes in solid tumors; however, substantially more work on the effects of MALT1 proteolytic activity inhibition in solid tumors remains. While we see evidence for MALT1-mediated cleavage of RelB, it is only one of a variety of known MALT1 substrates (**Table 2**). It is not known whether MALT1 proteolytic activity degrades other targets in solid tumors and what, if any, effects that proteolytic cleavage has on pro-metastatic behavior in osteosarcoma and breast cancer.

3.5 CONCLUSIONS

The work described herein shows a clear role for CBM complex-mediated NF-κB activation in the promotion of breast cancer and osteosarcoma metastasis. We show that PAR1-driven expression of several pro-metastatic genes requires MALT1, and that suppression of MALT1 impairs PAR1-associated invasion *in vitro*. In addition to our findings demonstrating MALT1-mediated NF-κB activation downstream of PAR1 stimulation in these solid tumors, we present the first evidence of MALT1 proteolytic activity observed in non-lymphoid cancer: an exciting development in the field of CBM complex signaling in solid tumors, as MALT1 proteolytic activity represents a promising future therapeutic target. It our profound hope that, through the elucidation and investigation of the mechanistic pathways that drive cancer metastasis, new therapies and novel strategies will be found to aid in the treatment of this debilitating facet of cancer.

4.0 MATERIALS AND METHODS

4.1 CLINICAL DATA ANALYSIS AND STATISTICS

Publicly available gene expression data were obtained from Kelly *et al.*[225] (osteosarcoma) and TCGA[226] (breast cancer). Statistical analyses were performed using the Graphpad Prism (v7.01) software package. Kaplan-Meier P values were calculated using the Mantel-Cox log-rank test. Other P values were calculated using the Student t test (two sided) or by analysis of one-way ANOVA, followed by Bonferroni posttest as appropriate.

4.2 CELL LINES AND CULTURE

U2OS human osteosarcoma cells and MDA-MB-231 human breast cancer cells were obtained from the ATCC (Manassas, VA). MCF7 and MCF7-N55 human breast cancer cells were a kind gift from L. Covic of Tufts University (Boston, MA). U2OS and MDA-MB-231 cells were cultured in DMEM with GlutaMAX (Gibco) supplemented with 10% FBS and 1% streptomycin / penicillin. MCF7 and derivatives were cultured in phenol red-free RPMI 1640 (Gibco) with 10% FBS and 1% streptomycin / penicillin. Cell lines were regularly tested for *Mycoplasma* contamination using the MycoAlert Mycoplasma detection assay (Lonza, Allendale, NJ).

4.3 REAGENTS

TRAP6 and ZVRPR-fmk were purchased from AnaSpec (Fremont, CA). Thrombin was obtained from Enzo Life Sciences (Farmingdale, NY). TNFa and MG132 were from Sigma-Aldrich (St. Louis, MO). MI-2 was procured from R&D Systems (Minneapolis, MN).

4.4 NUCLEAR FRACTIONATION AND ELISA

Nuclear isolates were generated using the Active Motif (Carlsbad, CA) Nuclear Extract Kit according to manufacturer's instructions. Nuclear fractions were subjected to Immunoblot analysis as below, or tested for NF-κB subunit activation using TransAM NF-κB family ELISA kit (Active Motif).

4.5 LUCIFERASE REPORTER ASSAY

NF-κB firefly luciferase and transfection control renilla luciferase plasmids were procured from Agilent Technologies (Santa Clara, CA). Plasmids were forward-transfected into cells using lipofectamine 3000 and PLUS reagent (Thermo Fisher Scientific). Cells were stimulated 24 hours post-transfection and luciferase induction was evaluated using a microplate luminometer.

4.6 IMMUNOBLOT ANALYSIS

Cells were placed in serum-free medium for 4h prior to stimulation. After stimulation (see individual experiments for details) immunoblot analysis was performed as described previously[176]. Lysate protein content was determined by bicinchoninic acid assay. Antibodies used in our studies are detailed in **Table 3** (CST: Cell Signalling Technologies, Danvers, MA; Santa Cruz Biotech, Santa Cruz, CA; GeneTex, Irvine, CA).

Table 3. List of antibodies used.

Antigen	Source	Catalogue Number
RelB	CST	4922
MALT1	CST	2494
CARMA3	GeneTex	111222
Bcl10	Santa Cruz Biotech	Sc-9560
pIκB	CST	9240
GAPDH	CST	5174
Actin	CST	4970
HDAC1	CST	5356
Tubulin	CST	5335
2^o mouse	CST	7070
2^o rabbit	CST	7074

4.7 RT-PCR AND INVASION PCR MICROARRAY

RNA isolation and cDNA generation was performed as described in[182]. Quantitative PCR was performed using TaqMan probes and reagents (Thermo Fisher Scientific). A custom 96-well RT-PCR array was procured from Thermo Fisher Scientific. The MORPHEUS matrix visualization and analysis tool was used to generate and process heat maps[242].

4.8 GENERATION OF N55 -ΔMALT1

Human MALT1 CRISPR targets were identified using the GeneArt CRISPR Seach and Design tool (Thermo Fisher Scientific). The GeneArt Precision gRNA Synthesis Kit (Thermo Fisher Scientific), GeneArt Platinum Cas9 Nuclease, and Lipofectamine CRISPRMAX Reagent were used according to manufacturer instructions. Following single-clone expansion, MALT1 knockout was verified by immunoblotting. The GeneArt Genomic Cleavage Detection Kit was utilized to detect CRISPR cleavage products.

4.9 SIRNA KNOCKDOWN

SMARTpool siRNA pools directed against MALT1, Bcl10, CARMA3, and scramble control were generated by GE Dharmacon (Lafayette, CO). Cells were reverse-transfected with siRNA using lipofectamine RNAiMAX (Thermo Fisher Scientific, Waltham, MA). Cells were allowed

to incubate for 72 hours after transfection before stimulation or assays. Knockdown efficiency was determined by immunoblot or RT-PCR.

Table 4. siRNA sequences used.

Target	siRNA Sequences
Control	UGGUUUACAUGUCGACUAA UGGUUUACAUGUUGUGUGA UGGUUUACAUGUUUUCUGA UGGUUUACAUGUUUUCCUA
CARMA3	CAGAUGGACUGUCGUUUUA GCGUGUGGCCUUUGGGAAA CMGAGAUCCUUCGACUGG GAACUCGGCUGUACUUCGC
Bcl10	GCCACCAGAUCUACAGUUA CGAACAACCUCUCCAGAUC GGGCAUCCACUGUCAUGUA AAUCAUAGCUGAGAGACAU
MALT1	GGGAGUAUAUGGGUUAUUA GCAGUGUUCUCUUAAGGUA GCAAAUCUGUGUUGAACCA GGUAAUCCAAGUAAUGUUA

4.10 *IN VITRO* INVASION ASSAY

Transwell chamber invasion assays were performed as described previously[243,244]. Transwell inserts were imaged using an EVOSfl digital inverted microscope (Thermo Fisher Scientific) and manually quantified by averaging the total cells in 5 fields. Each condition was performed in duplicate per experiment and each experiment was performed in triplicate.

4.11 MOUSE MODEL OF METASTASIS AND IMAGING

Mouse metastasis studies were performed as described previously[132]. $2*10^6$ of N55-Ctrl or N55 -ΔMALT1 cells were injected into the lateral tail vein of 8 week old female NCr athymic nu/nu mice (Taconic Farms, Hudson, NY). All animal experiments and procedures were performed in full compliance with the University of Pittsburgh Institutional Animal Care and Use Committee. A Siemens (Berlin, Germany) Inveon micro-CT at the Children's Hospital of Pittsburgh animal imaging core laboratory was used to perform thoracic-CT on mice anesthetized using isofluorane. Image interpretation was performed by a clinical radiologist from the UPMC Presbyterian Hospital Department of Radiology in a randomized and interpreter-blinded fashion.

APPENDIX A

ONGOING EXPERIMENTS

A.1 MOUSE MODEL OF BREAST CANCER METASTASIS

In order to examine the requirement of MALT1 in PAR1-driven cancer metastasis, we are utilizing the CRISPR-modified MCF7-N55 system. It has been previously shown that PAR1 expression in MCF7-N55 cells specifically drives metastasis *in vivo*. MCF7-N55 cells rapidly colonize the lungs of mice in a tail vein injection xenograft model of metastasis; whereas both parental MCF7 cells and MCF7-R310E cells do not. MCF7-R310E are MCF7 cells which have been engineered to express PAR1 with a single point mutation that renders intracellular signaling inoperative (ie thrombin proteolysis of the PAR1-R310E N-terminal ligand has no downstream signaling effects)[132].

To test for MALT1 requirement of PAR1-driven metastasis, we used the N55-Ctrl and N55-ΔMALT1 CRISPR clone pools described in **section 2.6** above. We injected $2*10^6$ cells of freshly made, evenly pooled N55-Ctrl cells or N55-ΔMALT1 cells into the lateral tail veins of 8 week old female athymic nude mice (**Figure 23**). We are monitoring tumor development in the mice with regular examination and weight tracking, along with micro-CT imaging to visualize

developing pulmonary metastases. CT image sets are being evaluated by a clinical radiologist from the UPMC department of Radiology.

Whereas the MCF7-N55 cells appeared to colonize nude mouse lungs over the course of 6 weeks in a published paper[132], our initial cohort of N55-Ctrl mice – the mice injected with cells that we expect to be closest in behavior to the rapidly metastatic MCF7-N55 cells – has demonstrated an irregular pattern of death from disease. Two N55-Ctrl-injected mice were required to be sacrificed due to large subcutaneous tumor growth: one mouse grew a 2 x 1.5 cm tumor dorsally near the hind limbs, while the other had a 1.5 cm x 1 cm cervical tumor. The lungs of both of these mice were grossly metastasis-free upon examination after resection. In addition to these two events, one mouse in the N55-Ctrl cohort did die of pulmonary metastatic disease (**Figure 24**). Two mice in the N55-Ctrl cohort, as well as all 5 mice in the N55-ΔMALT1 cohort, are apparently healthy and disease-free almost 20 weeks post-injection.

Given that MCF7-N55 cells metastasize and colonize nude mice lungs in 6 weeks or less, and given the substantial amount of time our initial cohort has continued post-injection, we thought to repeat this experiment using a larger cohort: 10 mice injected with N55-Ctrl and 10 injected with N55-ΔMALT1. After identification of metastatic disease using micro-CT, lungs of these mice will be resected, fixed, and stained using hematoxylin and eosin staining to count metastases. We expect that N55-ΔMALT1-injected mice will have lungs with fewer metastases relative to the N55-Ctrl-injected mice.

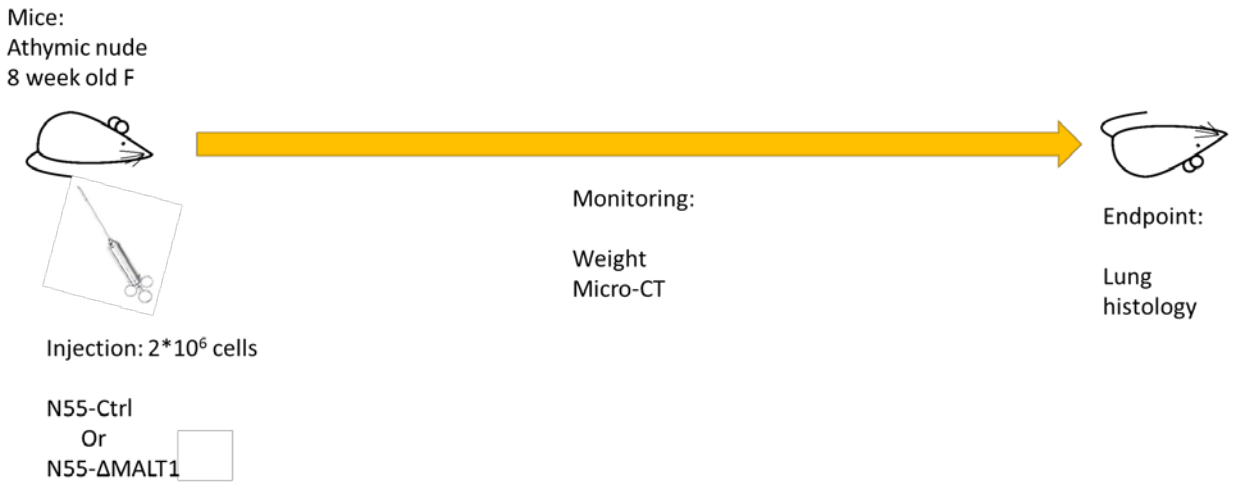

Figure 23 – Schematic overview of mouse experiment of breast cancer metastasis.

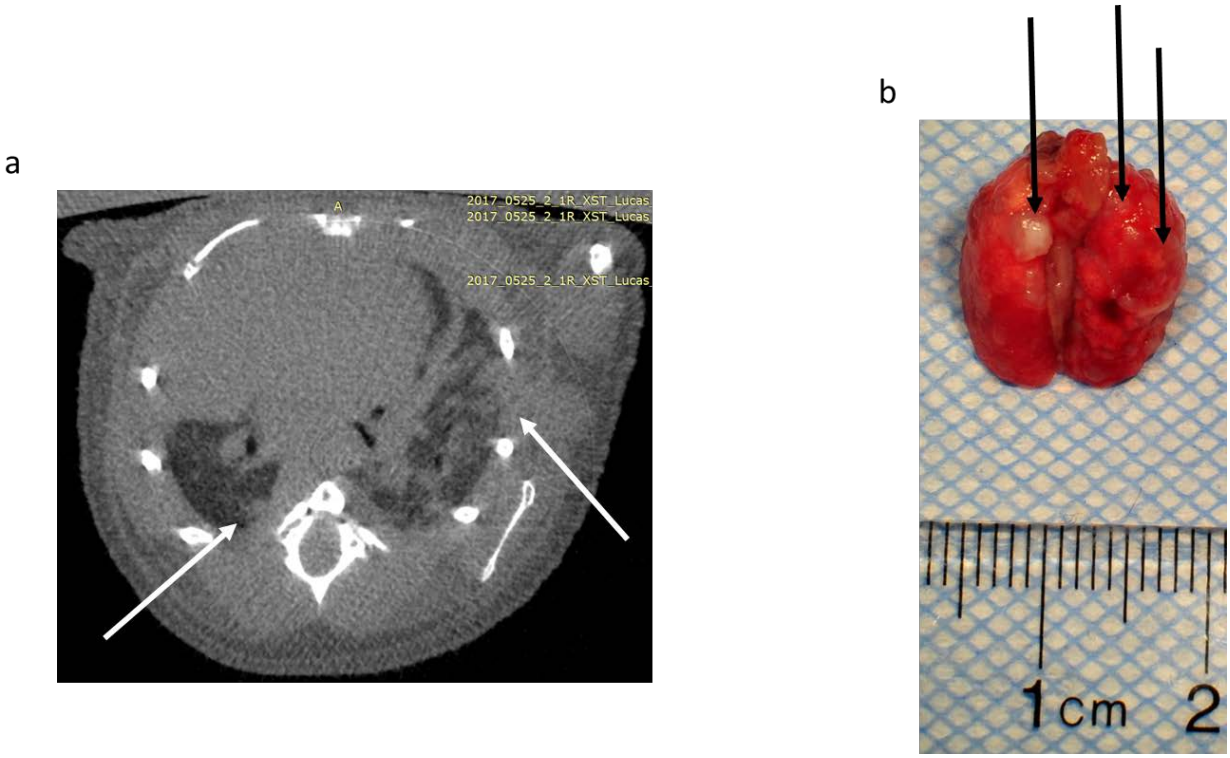

Figure 24 – Preliminary results from ongoing mouse experiment. (a) Micro-CT image of N55-Ctrl-injected mouse demonstrating identifiable pulmonary metastases (white arrows). (b) Photograph of lung from (a) immediately post-resection. Metastases are grossly visible (black arrows).

APPENDIX B

LIST OF ABBREVIATIONS AND NOMENCLATURE USED

Anoikis	Apoptosis triggered by loss of contacts to neighboring cells or extracellular matrix
Bcl10	Linker protein that binds to both CARMA and MALT1
Bcl-xL	Survival factor
BRCA1	Hereditary breast cancer marker
BRCA2	Hereditary breast cancer marker
CARD	Caspase activation and recruitment domain
CARMA1	(CARD11) Scaffolding protein that brings together the CBM, found in lymphoid cells
CARMA2	(CARD14) Scaffolding protein that brings together the CBM, found in placenta
CARMA3	(CARD10) Scaffolding protein that brings together the CBM, found in non-lymphoid cells
CBM	Signaling complex composed of CARMA proteins, Bcl10, and MALT1
CDH1	E-cadherin

CDH2	N-cadherin
cIAP2	Inhibitor of apoptosis 2, survival factor
CRISPR	Clustered regularly interspaced short palindromic repeats, gene modification and knockout technology
CXCR4	Chemokine receptor, linked to CBM activation of NF-κB
EGFR	Epidermal growth factor receptor 1
EMT	Epithelial-mesenchymal transition
ER	Estrogen receptor
ECM	Extracellular matrix, the non-cellular organic components of tissue
Extravasation	Invasion of tumor cells through the endothelial barrier to exit the circulation at a distant site
GPCR	G-protein coupled receptor
HER2	Epidermal growth factor receptor 2
IκB	Inhibitor of κB
IKK	Complex that phosphorylates IκB to activate canonical NF-κB signaling
Intravasation	Invasion of tumor cells through the endothelial barrier into the blood circulation
LPA	Lysophosphatidic acid
LUBAC	Linear ubiquitination assembly complex
MAGUK	Membrane associated guanylate kinase like domain
MALT1	Effector protein of the CBM complex through protein-protein interactions with and proteolytic degradation of downstream signaling mediators
Metastasis	The process by which cancer spreads from one organ to another

MMP	Matrix metalloproteinase
NF-κB	transcription factor family associated with inflammation
NIK	NF-κB inducing kinase, mediates non-canonical activation of NF-κB
P100	Precursor that is cleaved into p52
PAR1	Protease activated receptor 1
PDGF	Platelet derived growth factor
PKC	Protein kinase C, isoforms of which are responsible for CARMA phosphorylation
PR	Progesterone receptor
RB1	Retinoblastoma tumor suppressor
TAK1	Intracellular kinase important in IKK complex activation
TF	Tissue factor, cell surface mediator of the coagulation cascade
Thrombin	Serine protease central to the coagulation cascade
TP53	Tumor suppressor
TRAF6	Ubiquitin ligase important in IKK complex activation
VEGF	Vascular endothelial growth factor, important in angiogenesis

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