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ANGIOMOTIN IS A NOVEL CADHERIN-11 INTERACTING PROTEIN THAT MEDIATES MIGRATION IN PROSTATE CANCER CELLS

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ANGIOMOTIN IS A NOVEL CADHERIN-11 INTERACTING PROTEIN THAT MEDIATES MIGRATION IN PROSTATE CANCER CELLS

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ANGIOMOTIN IS A NOVEL CADHERIN-11 INTERACTING PROTEIN THAT MEDIATES MIGRATION IN PROSTATE CANCER CELLS

A

DISSERTATION

presented to the faculty of
The University of Texas
Health Science Center at Houston
Graduate School of Biomedical Sciences
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

by

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Houston, TX

August, 2014
DEDICATION

I dedicate this dissertation to my late father, Israel Ortiz Villalobos, and my mother, Salvadora Angelica Ortiz, who with sweat, heartbreak, and love pushed me to ask questions and gave me the strength to seek out the answers. Without their sacrifices, support, and faith,

I would not be here now.
ACKNOWLEDGEMENTS

This dissertation is a testament to the knowledge, passion, and support bestowed upon me from all the people involved in my academic and scientific endeavors.

First, I would like to thank my dissertation advisor Dr. Sue-Hwa Lin. Thank you, Dr. Lin for taking a chance on an English major and sharing the A-Z’s of surviving in academic research, and the advice on balancing work and life. I have learned a lot and will surely use most of it in the future. Moreover, I’m grateful for the opportunity to work in your lab and working with such kind and knowledgeable people. Thanks Yu-Chen Lee, Song-Chang Lin, Guo-Yu Yu, and Grace Liu for helping me learn and optimize new protocols to complete this work. And a special thanks to former lab member and dear friend Hyojin Cho, who was my soundboard and coffee-companion.

I'd like to say ‘thank you’ to my undergraduate research advisor Dr. Melanie Sohocki-Trapani for introducing me to the wonderful world of scientific research. I also thank the many friends I’ve made in GSBS and Houston who have contributed to the upkeep of my sanity by providing much needed non-science activities.

Lastly, I’d like to express my gratitude to my family. Marylin, my baby sister, whose sarcastic verbal abuse added much needed levity in times of doubt and stress. My darlings Amina Syed and Daniel Yoo who have many, many years of enduring my blunt behavior, I hope you are ready for my return to the Northeast. My Royal Holloway family who encouraged me to follow my dream in scientific research despite their constant suggestions I become a baker instead: Arpita Sinha, Douglas Maffei, Howard Wong, Helen Paul, and Anna Garefalaki.
Angiomotin is a novel cadherin-11 interacting protein that mediates migration in prostate cancer cells

Angelica Ortiz, B.A., M.A.

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Prostate cancer (PCa), the second leading cause of cancer-related deaths among men in the United States, has the proclivity to metastasize to bone resulting in sclerotic lesions. These cancer induced bone growths cause bone pain and fractures. Therefore, understanding the molecular mechanisms contributing to PCa bone metastasis is required in order to find better prognostic tools and suitable targets for metastasis treatment and/or prevention. Previous work in our laboratory showed increased expression of cadherin-11 (Cad11), a mesenchymal cadherin, during PCa progression. Furthermore, Cad11 expression endows PCa cells with increased migratory potential and metastasis to bone. Deletion of the Cad11 cytoplasmic region (cyto) resulted in loss of cell migration. How the Cad11 cytoplasmic domain mediates cell migration is unknown. We have identified angiomotin (Amot), a regulator of endothelial cell migration and epithelial cell polarity, as a Cad11 interacting protein. Deletion analysis showed that the last C-terminal 10 amino acids in Cad11 mediated Amot binding. Further, Cad11 preferentially interacts with the Amot-p80 isoform than the Amot-p130. Analysis of Amot mutants showed that Amot-p80 interacts with Cad11 through its middle domain, not the well characterized coiled-coil domains or PDZ motif. Deletion of the last ten Cad11 residues significantly reduced Cad11-mediated cell migration in C4-2B4, PC3-mm2, and HEK293T cells, compared to cells expressing wild-type Cad11. Together,
our studies identified and characterized Amot-p80 as a novel Cad11 binding protein that promotes Cad11-mediated PCa cell migration.
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ABBREVIATIONS

ΔAmot (deletion of Amot binding site)
Δβ-cat (deletion of β-catenin binding site)
ΔCBS (deletion of CBS domain)
ΔJMD (deletion of JMD domain)
AdenoCa (adenocarcinoma of the prostate)
Amot (Angiomotin)
Amot-L1 (Angiomotin Like-1)
Amot-L2 (Angiomotin Like-2)
Amp (Ampicillin)
ATP (Adenosine Triphosphate)
BAR (bar-amphyphisin-rvs domain)
β-cat (beta-catenin)
BPH (benign prostatic hyperplasia)
Ca^{2+} (Calcium)
Cad11 (Cadherin-11)
cDNA (complementary DNA)
cyto (cytoplasmic region)
CBS (catenin binding site)
Cdc42 (cell division control protein 42 homolog)
DAPI (4′, 6-diamidino-2-phenylindole)
DNA (deoxyribonucleic acid)
EC (Extracellular)
E-Cad (Epithelial-Cadherin)
E. coli (Escherichia coli)
EMT (Epithelial-Mesenchymal Transition)
FA (Focal Adhesion)
FACS (Fluorescence-Activated Cell Sorting)
FC (Focal contact)
GAP (GTPase activating protein)
GFP (green fluorescent protein)
GEF (Guanine nucleotide exchange factor)
GST (Glutathione-S-transferase)
GTP (Guanosine triphosphate)
GTPase (Guanosine triphosphate hydrolase)
HEK (Human Embryonic Kidney)
his\textsubscript{7} (7-histidine tag)
HSC (hematopoietic stem cell)
hr/hrs (hour/hours)
IB (Immunoblot)
IF (immunofluorescence)
IFil (intermediate filaments)
IHC (immunohistochemistry)
JMD (juxtamembrane domain)
Kan (Kanamycin)
K-Cad (Kidney cadherin)
mAb (monoclonal antibody)
mass spec (mass spectrometry)
MET (Mesenchymal-Epithelial Transition)
Mg\textsuperscript{2+} (magnesium)
min (minute)
MT (microtubules)
MTOC (Microtubule organizing center)
N-Cad (neuronal cadherin)
Neo (neomycin)
OB-Cad (osteoblast cadherin, i.e. Cad11)
ON (overnight)
pAb (polyclonal antibody)
p80 (The short Amot isoform)
p120 (p120-catenin)
p130 (The long Amot isoform)
PAGE (polyacrylamide gel)
PAP (prostatic acid phosphatase)
PatJ (Pals1 associated tight junction protein)
PBS (Phosphate Buffered Solution)
PCa (Prostate Cancer)
P-Cad (Placental Cadherin)
PCR (polychain reaction)
PDX (Patient Derived Xenograft)
PEI (polyethyleneimine)
rcf (relative centrifugal force)
PDZ (postsynaptic domain protein, Drosophila disc large tumor suppressor, and zonula occludens-1)
PSA (Prostate Specific Antigen)
Puro (puromycin)
Rac1 (Ras-related C3 botulinum toxin substrate 1)
RANKL (receptor associated of nuclear kappa-B ligand)
Rap1 (Ras-related protein 1)
R-Cad (Retinal Cadherin)
RhoA (Ras homolog gene family, member A)
RNA (ribonucleic acid)
rpm (rotations per minute)
RT (room temperature)
SCPCa (small cell prostate cancer)
SD (standard deviation)
SDS (sodium dodecyl sulfate)
shRNA (short hairpin RNA)
Syx (synectin-binding guanine exchange factor)
temp (Temperature)
Tris (Tris Buffered Solution)
WT (wild-type)
YAP (Yes-associated protein)
YFP (yellow fluorescent protein)
Chapter I: Background and Significance

Prostate Cancer (PCa)

PCa is the most commonly diagnosed cancer and second leading cause of cancer related death among American males\(^3\). PCa tumors are relatively slow-growing masses, therefore, it may be years before the prostate becomes large enough for detection thereby giving the cancerous cells time and opportunity to metastasize; unlike in patients with benign prostatic hyperplasia (BPH) whose prostates become so enlarged that pain is quickly felt for expedient diagnosis. In fact, upon diagnosis, most men with PCa present a Gleason sum of 6-7, with 2 representing normal prostate and 10 representing and extremely aggressive cancer\(^2\). Thus, there is a need for identification of biomarkers for early detection and accurate prognosis.

In an effort to circumvent metastatic incidence and detect PCa at earlier stages, there have been studies focusing to identify markers from tissue, blood, and/or urine that can be used as diagnostic and prognostic factors. The most notable are the measurement of prostatic acid phosphatase (PAP) and prostate specific antigen (PSA). PAP is a glycoprotein predominately produced by the prostate. PAP in the serum was an indicator of metastasis, but was insufficiently sensitive to detect localized disease\(^{127}\). Focus was then lent to optimizing techniques to measure levels of PSA in serum. PSA is a glycoprotein enzyme secreted by the epithelial cells of the prostate gland. PSA is present in small concentrations
in the serum of healthy males, but is elevated in presence of PCa and other prostate disorders, as well as changes in medications and environment\textsuperscript{127}. Moreover, PSA levels could not distinguish between PCa stages nor was it a good indicator of metastatic lesions\textsuperscript{127}. Since the implementation of the PSA test, PCa mortality has decreased, however, there is insufficient evidence that this decrease is due to the accuracy of the PSA test, especially because of the false positives generated\textsuperscript{127}. As such, metastasis of the disease remains a concern in the diagnosis and treatment of PCa patients.

**Prostate Cancer Metastasis and the Bone Microenvironment**

Bone metastasis is the main cause of morbidity and mortality in patients suffering from advanced PCa\textsuperscript{104, 122}. After cancer cells disseminate from the primary site, they must enter the vasculature, survive the shearing forces in circulation, and exit circulation to colonize a distant site that provides an environment amenable to promote cancer cell survival and proliferation\textsuperscript{87}. Clinical observations and studies have shown that cancers of certain tissue origin have a pattern of metastasis to multiple sites\textsuperscript{87}. Interestingly, PCa predominantly metastasizes to bone with lesions appearing in the vertebra and ribs\textsuperscript{22, 104, 105, 108}. The PCa cells disseminate from the primary tumor to then enter the Batson’s venous plexus, a valveless network of veins that connects the deep pelvic veins to the internal vertebral venous plexus, thereby facilitating metastasis to bone\textsuperscript{78}. 
Once in the bone, invading cancer cells can either activate or inhibit the activity of bone stromal cells called osteoblasts and osteoclasts. Osteoblasts are responsible for the secretion of bone matrix and subsequent mineralization needed to form new bone. Osteoclasts lyse mineralized bone, in a process called resorption, in order to maintain shape but also in response to stimuli, such as loss of mechanical loading, low blood calcium, or changes in cytokine/hormone serum levels. The bone metastatic lesions resulting from tumor burden, which induce increased osteoclast activity, are called osteolytic, while those that result in increased osteoblast activity are called osteoblastic or sclerotic.

Under normal conditions, the processes of resorbing and forming bone are coupled and highly regulated to maintain skeletal integrity under changing physiological conditions. When cancer cells invade the bone microenvironment, this coupling is distorted resulting in increased osteolytic or osteoblastic activity. Multiple myeloma cells in the bone secrete factors resulting in osteolytic metastatic lesions. Breast cancer (BCa) metastatic lesions, though predominantly osteolytic in phenotype, have been observed to present a mixed osteoblastic and osteolytic phenotype.

Unlike other bone tropic cancers, PCa is uniquely and predominantly osteoblastic. Post-mortem analysis showed that approximately 60 to 80% of men with PCa have bone forming masses. Histological analyses found PCa cells surrounded by newly formed bone. Studies have shown that the osteoblastic masses found are a result of PCa cells secreting proteins that increase the proliferation and differentiation of osteoblasts resulting in the fast formation of new bone, such as BMP-4. This newly formed bone, also called woven bone, is not tightly regulated by normal bone resorption/absorption mechanisms resulting in haphazard organization of collagen fibers creating dense patches of sclerotic
lesions that are mechanically weak thereby leading to bone fractures and pain\footnote{57, 80}. The cause of the frail skeleton in patients with osteoblastic lesions may be the heterogeneity within the lesions that present both osteopenic and osteodense patches as determined by histomorphometric analysis of metastatic biopsy\footnote{105}. This new PCa cell/osteoblast coupling results in favorable conditions not only for osteoblasts, but for PCa cells as well.

Osteoblasts secrete factors that assist in PCa cell survival within the bone as well as promote PCa cell proliferation. Co-culture assays using primary mouse osteoblasts and PCa cells have shown osteoblasts secrete osteopontin to affect cell survival, and sialoprotein to increase PCa cell affinity to bone\footnote{29}. Osteoblasts also promote PCa growth indirectly. Formation of the new bone induces systemic repercussions because in making the new bone, nutrients are pulled in from the blood, which leads to hypocalcemia and other nutritional deficiencies\footnote{80, 98, 118}. The resulting hypocalcemia induces secretion of the parathyroid hormone leading to the secretion of receptor activator of nuclear factor kappa-B ligand (RANKL) in the bone marrow stromal cells and PCa cells to promote osteoclast differentiation and activation\footnote{57, 61, 85}. This osteoclast activation may be an attempt to balance the high bone forming rate. However, the resulting osteolysis results in the release of growth factors from bone that aid in PCa cell growth\footnote{94}. Thus, PCa invasion of the bone results in a vicious cycle between the PCa cells and the bone stromal cells. Therefore, it is important to identify proteins and signaling pathways contributing to PCa metastasis to bone and promote PCa growth in the bone that may be used to identify therapeutic targets to block or specifically treat these skeletal masses, as well as identify novel diagnostic/prognostic markers.
**Treatment and Prevention of Bone Metastasis**

Observing the role of osteoclast activation in promoting PCa bone metastasis, treatment strategies used for osteolytic cancers, such as multiple myeloma, were implemented for PCa patients. Treatments that target osteoclast activation include administration of bisphosphonates, such as zoledronic acid, and monoclonal antibodies, such as Denosumab, which have been successful in treating cancer patients with osteolytic metastatic lesions\textsuperscript{90, 146}. It was believed that suppressing the osteoclast activity in the PCa/bone microenvironment vicious cycle would yield similar effects in PCa patients.

In multiple myeloma, treatment with zoledronic acid, a potent synthetic analog of pyrophosphate, is able to reduce osteolytic markers, skeletal related events, and improve overall survival\textsuperscript{79}. Though zoledronic acid treatment did show a reduction in bone pain for some PCa patients, they found greater efficacy when administered prior to the existence of bone pain\textsuperscript{106}. Moreover, there was little benefit in overall PCa patient survival\textsuperscript{106}. Histopathologic analyses of PCa patient bone lesions by Roudier et al found that treatment with bisphosphonates changed little of the overall bone/tumor architecture\textsuperscript{105}. To understand this underwhelming outcome for the use of zoledronic acid in treating PCa, Thudi et al injected canine prostate cancer cells into nude mice then treated with zoledronic acid\textsuperscript{122}. The cancer cells generated mixed osteoblastic and osteolytic lesions, and, while treatment with zoledronic acid did reduce osteolysis, it had no significant effect on osteoblastic activity\textsuperscript{122}. These studies suggest that perhaps this method of targeting PCa cell interaction with the bone microenvironment was not an efficacious strategy\textsuperscript{94}. 
In a different strategy to target osteoclast activity to reduce PCa tumor burden in the bone, the human monoclonal IgG2 antibody against RANKL, called Denosumab, was used. Denosumab is administered every six months with a dose of 60mg with the purpose of preventing bone loss and fractures. Some clinical trials showed improvement in bone mineral density in patients with nonmetastatic breast cancer and prostate cancer after hormone deprivation therapy. Though bone fractures are a concern for a patient’s overall quality of life, suppressing osteoclast activity in PCa using Denosumab results in severe hypocalcemia and vitamin D deficiency. Together this further shows that while suppressing osteoclast activity in osteolytic bone metastasis in BCa and multiple myeloma is effective, treatment of PCa would need to disturb either the PCa/osteoblast interaction or prevent PCa from exiting the prostate and entering the bone microenvironment.

Once PCa metastasis occurs, effective treatment strategies are limited and shift from curative to palliative, therefore designing strategies for prevention are needed to improve patient care. At the time of diagnosis, PCa patients have tumors that are hormone dependent so are subsequently subjected to androgen ablation treatment, by either surgical or pharmacological castration, in order to reduce testosterone levels in the blood circulation. Androgen ablation has been shown to decrease PCa cell proliferation and increase apoptosis. Moreover, this treatment resulted in remission for almost 70% of patients. However, this patient response does not last long and the relapsing tumor is able to thrive independent of androgen, thus called castration resistant. The resulting tumor relapse is found in the bone in more than 80% of cases. Though hormone deprivation therapy in treatment of PCa while still localized in the primary site seems to be highly effective, the risk of relapse is coupled by the emergence of a more aggressive cancer. Therefore, elucidating
the mechanisms by which PCa cells are able to disseminate from the primary site can help identify the proteins that mediate metastasis. In turn, further study of these proteins could be used as targets for developing novel therapies. Previous studies suggest proteins involved in epithelial-mesenchymal transition (EMT) may be a mechanism contributing to the progression of prostate cancer.\textsuperscript{55}

Epithelial-Mesenchymal Transition (EMT)

During development, epigenetic signals induce embryonic cells to undergo numerous rounds of EMT and mesenchymal-epithelial transition (MET) to form complex tissue architectures.\textsuperscript{120, 121} As cells undergo EMT, the cell polarity needed to maintain tissue architecture is lost and the once differentiated epithelial cells begin to have similar morphology to stromal cells.\textsuperscript{55, 121} As the cells lose their epithelial cell markers, they begin to upregulate several transcription factors, such as Snail, Slug, and Twist.\textsuperscript{88} In MET, mesenchymal cells express epithelial proteins to reestablish apical polarity and cell-cell contacts.\textsuperscript{55, 144} These transitions are not irreversible, therefore, even in adult tissue, epithelial cells maintain cellular plasticity governed by various cellular and molecular events.\textsuperscript{55, 120, 121, 144} And it is this plasticity that aids in cancer cell adaptation to a variety of physiological changes.

In differentiated tissue, EMT can be induced by mechanical injury, chronic inflammation, and molecular abnormalities resulting in upregulation of mesenchymal
molecules and downregulation of epithelial cell molecules\textsuperscript{120, 121}. These molecular variations are tightly regulated and result in changes to cellular phenotype and tissue architecture. However, under conditions of serious injury and excessive inflammation, deregulation of EMT can result in the formation of fibrosis or promote cancer progression. Once cancer cells begin expression of mesenchymal proteins, we observe changes not only in morphology, but also cellular function\textsuperscript{55, 83, 113, 120}. The upregulation of Snail during EMT, for example, results in conferring cancer cells with stem cell-like properties and increased metastatic potential\textsuperscript{130}. A hallmark of EMT during cancer progression is the loss of E-Cad and an upregulation of mesenchymal molecules, such as mesenchymal cadherins in a process called “cadherin switching”\textsuperscript{113, 133}. The expression of mesenchymal cadherins causes alterations in cellular phenotypes, such as loss of cell polarity, changes in cell morphology, and increased cell migration. Understanding how cadherin switching affects the cell can help in understanding the role of cadherin switching in EMT and PCa progression.

**Cadherins and Cadherin Switching**

The cadherin superfamily is a family of transmembrane glycoproteins involved in Ca\textsuperscript{2+}-dependent cell-cell interactions and the maintenance of tissue integrity and morphogenesis. The cadherin cell adhesion molecules are classified into three groups: classic cadherins, proto-cadherins, and atypical cadherins (Fig. 1)\textsuperscript{8}. Cadherins known as classic cadherins typically express 5 calcium-binding domains extracellular (EC) domains,
while Fat, Dachsous, and proto-cadherins can possess up to 34 calcium-binding domains (Fig. 1)\textsuperscript{8}. The variety of EC domains participate in regulating homophilic and heterophilic interactions, which mediates adhesion and cell sorting during organogenesis \textsuperscript{42}.

Classic cadherins are further classified as type I or type II. Type I cadherins possess the HAV tripeptide motif in the first EC domain and its members include epithelial-cadherin (E-cad), neuronal-cadherin (N-cad), placental-cadherin (P-cad) and retinal-cadherin (R-cad)\textsuperscript{42}. Type II cadherins lack the HAV motif and its members include kidney-cadherin (K-cad) and osteoblast-cadherin (OB-cadherin), also known as Cad1\textsuperscript{42}. There are further variations in the EC domains which ensure homophilic cadherin interaction and facilitate interaction between cadherin EC domains and other proteins. For example, the N-Cad EC4 domain is able to bind the fibroblast growth factor receptor in order to activate downstream signaling\textsuperscript{42}.

The ‘classic’ cadherins express a highly conserved classical cytoplasmic region with a juxtamembrane domain (JMD) domain and catenin binding site (CBS), which bind p120-catenin (p120) and β-catenin (β-cat) respectively. The function of p120 is to stabilize the cadherin expression and localization at the plasma membrane\textsuperscript{11, 89}. Loss of p120 interaction to the cadherin molecule would result in endocytosis of the cadherin and loss of cell adhesion\textsuperscript{50}. The role of β-cat begins as the cadherin molecule is being translated at the endoplasmic reticulum\textsuperscript{28}. There β-cat binding to the CBS aids in the stability of the cadherin molecule for targeting to the plasma membrane\textsuperscript{28}. Once at the plasma membrane, β-cat then functions to connect the cadherin molecules to the actin cytoskeleton through interaction with α-catenin\textsuperscript{32, 117}. Though the classic cadherin cytoplasmic domains are conserved and bind
p120 and β-cat, there is still variety within these domains that allow for protein interactions specific to the cadherin. These unique interactions are particular to the cells that express the cadherin\textsuperscript{8, 42}, which contribute to their unique functions in a variety of cellular and physiological contexts in response to adhesion.

As cadherin molecules rely on homophilic adhesion for cell-cell interaction, cadherin switching results in mesenchymal cadherin expressing cells disseminating from the surrounding cells similar to the cell sorting observed in the organogenesis during development\textsuperscript{42}. This process may contribute to mesenchymal cadherin expressing cancer cells escaping from the primary site and homing to a distant site populated by cells expressing the same mesenchymal cadherin molecule. A common observation in several cancers types is the loss of E-Cad expression and upregulation of N-Cad\textsuperscript{88, 133}. During normal development the E-Cad to N-Cad cadherin switch is an EMT progression needed normal embryonic development\textsuperscript{42, 120}, but N-Cad expression in cancer cells results in increased cell migration and invasion\textsuperscript{88}. Though PCa cells may express N-Cad to induce cell migration, PCa cells have a propensity for bone metastasis. Therefore, a cadherin that would enable interaction between PCa cells and bone stromal cells, such as Cad11, may give insight into the molecular mechanisms that govern PCa cell migration and survival in bone.
Figure 1. The Cadherin Superfamily. The figure shows the varying structures of the molecules that comprise the cadherin superfamily (blue) and their common cytoplasmic interacting proteins. Approximately 80 members of the cadherin superfamily have been identified and classified as either classic cadherins, protocadherins, or atypical cadherins. Classic cadherins have been identified as Cad^{12}-dependent cell adhesion molecules. Cell adhesion is regulated by catenins bound to the cytoplasmic region.
Cadherin-11 (Cad11)

The mechanisms mediating PCa preferentially metastasizing to bone are not fully understood. Interactions of PCa cells with stromal cells present in the bone through direct cell-cell contact and/or soluble factors secreted by PCa cells or cells in the bone microenvironment could be contributing to this phenotype. Hematopoietic stem cells (HSCs), for example, are able to mobilize and home to their HSC niche utilizing cell-cell adhesion molecules for selective retention to the niche. It is, therefore, likely that PCa cells mimic this mechanism via cell adhesion molecules mutually expressed by PCa cells and cells within the bone microenvironment. A gene array analysis comparing acinar and ductal PCa patient samples, which are bone tropic and non-bone tropic respectively, identified Cad11, also known as OB-cadherin, as a cell adhesion molecule potentially involved in mediating/enhancing bone metastasis.

Cad11 is expressed preferentially in osteoblasts and synoviocytes\textsuperscript{60, 92} and is associated with osteoblast differentiation\textsuperscript{58}. Though Cad11 knockdown in mice did not result in embryonic lethality, there was a reduction in bone density in the calvaria and long bone metaphyses\textsuperscript{56}, suggesting that Cad11 functions in osteoblast maturation\textsuperscript{56}. Clinical PCa specimens showed no Cad11 expression in normal prostate epithelial cells, but demonstrated comparatively increasing levels of Cad11 expression in lymph node and bone metastasis samples\textsuperscript{30}. Moreover, our laboratory found that Cad11 was highly expressed in the bone metastasis derived PCa cell line PC3-mm2\textsuperscript{30}. When injected intracardially into mice, the PC3-mm2 cells were able to metastasize to the femur showing that PCa cells expressing Cad11 have a preference for colonizing bone\textsuperscript{30}. Furthermore, knockdown of Cad11 in PC3-
mm2 using shRNA was able to significantly stifle the bone tropic phenotype\textsuperscript{30}. To understand the functions that Cad11 mediates to facilitate metastasis to bone, we expressed Cad11 in L-cells and C4-2B4 cells, and found that Cad11 expression confers cells migratory potential, increased cell-cell adhesion, and the ability to interact with osteoblasts \textsuperscript{47, 64}. These observations suggest that Cad11 may play a role in various key steps throughout PCa metastasis and PCa colonization of the bone. Therefore, identification of the proteins that specifically interact with Cad11 can be used to elucidate the mechanisms which contribute to these unique cellular functions and how those mechanisms are regulated in PCa cells. Moreover, these novel and unique Cad11 interacting proteins may be used as prognostic or diagnostic markers, as well as targets for novel therapies.

**Angiomotin (Amot)**

Amot is a member of the Motin Family, which consists of Amot, Amot-like 1 (Amot-L1), and Amot-like2 (Amot-L2)\textsuperscript{20}. Amot is expressed as two isoforms via alternate splicing: p80 and p130\textsuperscript{20, 33, 35}. Though the members of the Motin Family possess similar protein interacting domains, such as the PDZ motif and two coiled-coil (C-C) domains, they have varying functions\textsuperscript{20}.

The p80 isoform, for example, promotes endothelial cell migration while the p130 stabilizes endothelial cell shape during tubule formation\textsuperscript{33, 35, 132}. Studies have shown that using the PDZ motif at the C-terminal, p80 is able to induce endothelial cell motility via the
PatJ/Syx signaling complex, which directs RhoA activity to the leading edge of the migrating cell\textsuperscript{35}. The p80 isoform is able to interact with Merlin, a key component of the tumor suppressing Hippo Pathway\textsuperscript{145} using the first C-C domain, which is rich in amphipathic helices to promote binding to Merlin’s C-C domain via hydrophobic interactions. The Amot-Merlin interaction increases Rac1-mediated migration and proliferation by releasing Rich1 from the apical complex\textsuperscript{145}. Recent studies have shown that the p130 Amot isoform also participates in the Hippo Pathway but much further downstream by promoting YAP cytoplasmic retention to enable its phosphorylation by Lats 1/2\textsuperscript{5, 65, 147} (Fig 2).

The p130 Amot isoform, as well as Amot-L1 and Amot-L2, have motifs in their N-terminals that facilitate YAP binding in the cytoplasm to inhibit YAP nuclear translocation and transcription activity\textsuperscript{147}. However, there are circumstances in which YAP transcription, in conjunction with TEAD, is necessary to induce cellular senescence\textsuperscript{101, 138} and tissue regeneration in cardiac and pancreas tissue\textsuperscript{41}; therefore, p130 expression and function need to be regulated. The extended N-terminal expressed in p130 contains motifs that promote Amot phosphorylation and subsequent degradation in the cell\textsuperscript{129}. Another way p130 function can be suppressed is by forming a heterodimer with the p80 isoform\textsuperscript{34}. The p80 Amot isoform utilizes its C-C domains not only to interact with other proteins, but also to form homodimers and heterodimers with p130\textsuperscript{34}. As expression of p80 suppresses p130 function in neovascularization, it is thought this regulation may occur through the formation of a p80-p130 heterodimer\textsuperscript{34}. Together, these studies suggest an important role for Amot in development and possibly during cancer progression.
Cancer metastasis occurs when tumor cells lose contact inhibition, such as that provided by p130 through the Hippo Pathway, and exhibit migratory potential, such as that observed by p80 expression through the PatJ/Syx/RhoA complex. In breast cancer, upregulation of Amot transcription correlated with increased angiogenesis and poor patient survival\textsuperscript{51}. Recently, studies have shown that mouse aortic endothelial cells with increased Amot expression resulted in more aggressive and invasive tumors\textsuperscript{66}. In epithelial BCa cells, expression of the p80 Amot isoform was shown to mediate increased cell proliferation\textsuperscript{100}. Though the functional role of the Amot isoforms in PCa cells and PCa stromal cells has not
yet been studied, the Oncomine database as able to identify two independent gene array analyses of PCa patient samples looking at molecular alterations due to hormone ablation and comparing primary to metastatic lesions that showed upregulation of Amot (Fig. 3). In a comparison of 20 PCa patient samples, which include 10 hormone naïve and 10 hormone refractory samples, Best et al measured changes in 12,624 genes and found Amot in the top 19% of overexpressed genes. In a study looking at molecular differences between primary and metastatic lesions in 31 PCa patient samples, which included 10 primary and 21 hormone-refractory metastatic lesions, Chandran et al measured changes in 14,738 genes and also found Amot in the top 19% of overexpressed genes. However, neither study distinguished between the mRNA levels of the individual isoforms, a distinction that would allow us to understand the role of the Amot isoforms in the context of PCa.

Figure 3. Results of Oncomine database inquiry for changes in Amot mRNA levels in independent PCa gene array analyses. (A) In 2005, Best et al found a slight increase in Amot mRNA levels comparing hormone naïve versus hormone refractory PCa samples. (B) In 2007, Chandran et al found that Amot mRNA levels were higher in hormone refractory metastatic lesions compared to primary tumor.
Cell Migration

Migratory potential is a necessary process in the development and maintenance of complex, multicellular organisms. During embryonic development, wound healing, and immune responses, cells are directed to specific locations\textsuperscript{49}. Induction of cellular migration is prompted by a variety of chemical and mechanical signals and different cells react to different signals using different mechanisms\textsuperscript{49}. Prokaryotic cells use flagella or cilia to generate motion. Eukaryotic cell migration is vastly more complicated involving changes in cellular shape driven by changes to the proteins that comprise the cytoskeleton resulting in crawling or blebbing movements\textsuperscript{49}. These processes are tightly regulated and errors in these signals lead to serious consequences, such as physical deformations in fetal development and cancer progression.

For the most part, cells migrate individually but under certain physiological conditions a cluster of cells can migrate together in what is called collective cell migration (Fig. 4)\textsuperscript{103,119}. The blebbing and crawling cell migration phenotypes for single cell migration require a substrate, such as extracellular matrix (ECM), to activate integrins, which are cell adhesion molecules that use the ECM to form focal adhesion points in order restructure the actin cytoskeleton\textsuperscript{128}. However, integrin mediated migration can be used to move across the ECM in concert with cadherin-mediated cell-cell contacts to facilitate collective migration (Fig. 4)\textsuperscript{103,119}. 
During collective migration, both epithelial and mesenchymal cells utilize cell adhesion molecules, such as classic cadherins, to promote coordinated movement. The cell-cell interactions can consist of tightly or loosely associated groups of cells. These groups can move by forming cellular sheets, small groups, or cellular strands. Though epithelial tissue is generally a constrained environment that holds cells in a fixed position by promoting apical polarity, there are conditions, as observed during embryogenesis, that involve cell movements within a tissue sheet. This tissue rearrangement is due to cadherin-mediated cell-cell adhesion complexes. As cadherin interaction is homophilic, neighboring cells need to express the same cadherins to engage in coordinated migration. Coordinated migration of a cell population has been observed during embryogenesis in the neural crest formation with N-Cad expressing cells, as well as wound healing and cancer metastasis. The cell-cell contacts formed by classic cadherins allow the cells to exert pulling forces to generate motion of the cell body forward.

Cell-cell interactions mediated by classic cadherins are formed in three steps: initiation, expansion, and stabilization. During initiation, cells move randomly throughout the local environment using protrusions called lamellipodia to explore until they encounter a
favorable cell-cell interaction at random\textsuperscript{119}. In the expansion and stabilization steps, the cell membranes meet and cadherins expressed on the cell surface form homophilic interactions inducing a transient peak of Rac1 and RhoA activity to promote changes in the cytoskeleton\textsuperscript{119}.

![Figure 5. Collective Cell Migration.](image)

Collective cell migration mediated by classic cadherin cell-cell contacts can result in cell migrating as a sheet, as a small cluster of cells, or as a strand of cells.\textsuperscript{119}

The eukaryotic cell cytoskeleton is comprised of actin, intermediate filaments (IFils) and microtubules (MTs). Actin and MTs, unlike IFils, are polar structures that require ATP or GTP hydrolysis to provide at the plus and minus ends to create unsymmetrical polymerization and depolymerization dynamics resulting in treadmilling of the cytoskeleton to propel the cell forward (Fig. 6)\textsuperscript{49}. Changes to the actin in the cytoskeleton at specific sites in the cell are induced by Rho proteins, which are in turn regulated by guanine nucleotide exchange factors (GEFs) or GTPase activating proteins (GAPs)\textsuperscript{43, 115, 136}. Though humans express approximately 20 Rho GTPases, the most studied are RhoA, Rac1, and Cdc42. Rho GTPases form protein complexes to directly or indirectly affect the assembly or disassembly of filamentous actin (F-actin) (Fig. 6)\textsuperscript{35, 43, 115}. Studies in fibroblast cells showed Rho activation resulted in the assembly of contractile actin-myosin filaments and associated focal adhesion complexes, while Rac1 generated actin filament clusters to generate lamellipodia.
and Cdc42 created actin-rich protrusions called filopodia (Fig. 6)\textsuperscript{43}. Presence of actin stress fibers is a characteristic of migrating mesenchymal cells to mediate myosin II-based contractility via associated focal adhesions\textsuperscript{125}, which may be a mechanism acquired by cancer cells during cancer-mediated EMT. Therefore, studying proteins that promote RhoA, Cdc42, and Rac1 activity and localization of activity, such as Amot, can be candidates for understanding the mechanisms mediating cancer cell migration.

\textbf{Figure 6. Rho GTPases Reorganize Cytoskeleton.} This schematic shows how the Rho GTPases RhoA, Cdc42, and Rac1 work in concert at focal adhesion points and the leading edge to reorganize MTOC/ MT and actin contraction to mediate cell migration directionality. (\textbf{MTOC}= MT organizing center; \textbf{FA}=focal adhesion; \textbf{FC}=Focal contact)\textsuperscript{16}. 
Summary of the problem and hypothesis

PCa is the second leading cause of cancer related deaths among males living within the United States. Organ confined PCa tumor can be treated by surgical removal and is sensitive to androgen deprivation therapy. Unfortunately, once PCa metastasizes to bone, androgen deprivation only marginally improves overall survival and as the disease becomes castration resistant, it is no longer dependent on AR signaling for promoting PCa growth. Though slow growing, PCa cells secrete various factors once within the bone microenvironment that increase osteoblast proliferation and activity to generate woven bone resulting in pain and fractures. This new equilibrium between the PCa cells and osteoblasts also allows for increased PCa growth. Treatments used for other bone tropic cancers have limited success in admonishing this equilibrium. Therefore, we must elucidate the mechanisms that contribute to PCa metastasis in order to identify novel therapeutic targets for prevention and/or treatment.

Cancer associated EMT offers a variety of signaling pathways that could contribute to the progression of PCa disease. Loss of E-Cad expression and upregulation of Cad11 in PCa, BCa, and renal cell carcinoma has been shown to increase cancer cell motility to promote metastasis to bone. Therefore, the identification of proteins that interact with Cad11 in PCa cells to induce metastasis can be used as prognostic/diagnostic markers or even possible targets in order to design novel pharmacological treatments.

The aim of this Ph.D. dissertation is to identify and characterize novel interacting protein for the mesenchymal cadherin Cad11 that elicit changes contributing to PCa
progression and metastasis. One of the unique properties of mesenchymal cadherins is to increase cell migration in order to promote dissemination from the primary site to a metastatic niche, while the epithelial cadherin (E-cadherin) has opposite effects\textsuperscript{27, 30, 97, 113}. Deletion of the Cad11 cyto domains abrogated PCa cell migration and invasion\textsuperscript{47} suggesting intracellular signaling via one or both the Cad11 cyto domains plays a role in Cad11-mediated migration. Using a protein subtraction strategy, Amot was identified as a Cad11 specific interacting protein. Previous studies showed the p80 Amot isoform mediates endothelial cell migration by binding to the PatJ/Syx signaling complex to concentrate RhoA activity to the leading edge of migrating endothelial cells during neovascularization\textsuperscript{35, 123}. It is possible that expression of p80 may play a similar role in PCa cell migration. Though previous studies have shown the contribution of Amot in development and in the proliferation of Schwann cells\textsuperscript{145} and BCa\textsuperscript{100}, this is the first endeavor to understanding the functional role of Amot in PCa cell migration. We hypothesized that Cad11 interacts with Amot in order to increase PCa cell migration.

In order to address and validate this hypothesis, I needed to confirm Amot as a unique and specific Cad11 interacting protein. Then, I identified the domains or motifs that mediate the Cad11/Amot interaction. Using human PCa cells, I showed the endogenous expression of Amot and that the Cad11/Amot interaction is an endogenous phenomenon within the context of PCa. Finally, I studied how the Amot/Cad11 interaction may affect Cad11-mediated functions, such as cell aggregation and migration. Together this work identified Amot as a novel Cad11 interacting protein whose function is to promote Cad11-mediated PCa cell migration.
Chapter II: Materials and Methods

Cell Lines

The L-cells cell line purchased from ATCC are fibroblast cells of mouse origin. L-cells are cultured, transformed fibroblasts from mouse subcutaneous connective tissue that express no cadherins and adhere poorly to themselves or to other cultured cells.

The HEK293 derivatives HEK293T and Phoenix cell lines were purchased from ATCC. HEK293T cells are cultured human embryonic kidney cells transformed with SV40 T-antigen to increase transfection efficiency. Phoenix cells express Moloney Murine Leukemia virus packaging proteins to support the production of ecotropic and amphotropic retroviruses.

The HEK293 derivative 293FT was purchased from Life Technologies (Carlsbad, Ca). 293FT cells are modified to generate high-yield lentiviral titer.

The PC3-mm2 cell line was a gift from Dr. Isaiah J Fiddler, and is a highly metastatic derivative of PC3 cells.

The C4-2B4 a cell line derived from LnCap cells that metastasized to lumbar spine region were a gift from Dr. Sikes (University of Delaware, Delaware). C4-2B4/Cad11 cell line was previously generated as described in Huang et al.
**Patient Derived Xenografts (PDX)**

The xenograft samples MDA-PCa 133-4, 180-30, 183, 118b, 144-13, 146-10, and 155-2 are sublines generated using primary castration-resistant prostate carcinomas acquired during palliative surgical resections at The University of Texas MD Anderson Cancer Center to implant into immune compromised mice\textsuperscript{124}. The MDA-PCa 144-13, 146-10, and 155-2 were derived from patient prostate and have been characterized as small cell prostate carcinoma (SCPCa)\textsuperscript{9,124}. The MDA-PCa 118b, 133-4, and 183 sublines are derived from patient bone metastatic lesions and have previously been characterized as adenocarcinomas (AdenoCa)\textsuperscript{36,63,124}. The MDA-PCa 180-30 xenograft was derived from the cystoprostatectomy of a Gleason 9 (4+5) patient who relapsed despite receiving maximum androgen blockade and docetaxel-carboplatin combination therapy\textsuperscript{114}. cDNA from these patient derived xenografts were obtained from the laboratory of Dr. Sankar Maity for qPCR using SYBR green. p80 and p130 specific primers were used to look at the differential expression of the AMOT isoforms in the different xenografts (Table 1).

**Plasmids**

**Bacterial Expression**

The vector pGEX4T-1 was used to generate GST-tagged fusion proteins expressed in *Escherichia coli* (*E. coli*). The vectors pET28a and pET28b were used to generate His
tagged fusion proteins expressed in *E. coli*. The Amot-p80 cDNA in the pCR4-TOPO vector was purchased from Open Biosystems (Pittsburgh, PA).

**Mammalian Expression**

The vector p3xFlag-CMV10 was purchased from Sigma-Aldrich and used to generate 3x-Flag-tagged fusion proteins. The v180-Flag (p80-Amot-3xFlag), Amot mutant 1 (pΔBAR-Flag), Amot mutant 3 (pBAR-flag), Amot mutant 5 (pΔEYLI) and pEYFP (Amot-YFP) vector set were gifts from Dr. Clark Wells (Indiana University, Indiana). A different set of Amot deletion mutants, Amot mutant 4 (pCMV-Flag-p80-CC), Amot mutant 2 (pCMV-Flag-p80-DCC) and the p130/Amot (p130-Amot -3xflag) were gifts from Dr. Joseph L. Kissil (The Wistar Institute, Pennsylvania). The retroviral vectors pBMN-I-GFP and pBMN-I-Neo were used to generate stable cell lines were previously described in Huang *et al*[^47]. The lentiviral vectors for shRNA pLKO and pGIPZ were purchased from Thermo Scientific (Waltham, MA).

**Construction of retroviral vectors**

The bicistronic retroviral vectors pBMN-Amot-his7-GFP and pBMN-Amot-his7-Neo were constructed by inserting Amot-his7 cDNA into pBMN-I-GFP or pBMN-I-Neo through
BamHI and NotI sites. The bicistronic retroviral vector pBMN-Cad11ΔAmot-GFP was constructed by PCR using pBMN-Cad11WT vector as the template using the primers Cad11-EC-forward and CBS-10-reverse (Table 1). The resulting PCR product was subcloned and its sequence verified by DNA sequencing. The Cad11-ΔAmot cDNA was isolated from pCR2.1 and subcloned into the pBMN-I-GFP or pBMN-I-Neo vectors. The Cad11-Δβcat insert was constructed using the Stratagene Site-Directed Mutagenesis Kit and the primers (Δβcat-Forward and Δβcat-Reverse) listed in supplemental Table S1, using Cad11-WT as the template. The resulting product was verified by DNA sequencing.
Table 1. List of Oligos. Above are the names and sequences of the oligos and oligo pairs used to complete this study. The last column describes the use of the oligo or oligo pair.
**Generation of GST-Amot or Amot-his\(_7\) proteins**

CDNA for GST-Amot or Amot-his\(_7\) fusion protein was generated by PCR using pCR4-TOPO-Amot as template using primers Amot-F1 and Amot-R1 (Table 1). The PCR product was ligated into the pCR2.1-TOPO vector and the DNA sequence was confirmed by using a series of forward Amot oligos, Amot F2 to F4 (Table 1). The Amot insert was removed from pCR2.1-p80-Amot via digestion with BamHI and NotI endonuclease enzymes. The resulting insert was then subcloned into the pGEX4T1 or pET28b vectors to express Amot as a GST or his\(_7\) fusion proteins, respectively, in *E. coli*. GST-Amot or Amot-his\(_7\) protein was purified using Thermo Scientific glutathione-agarose (Waltham, MA) or Qiagen Ni-NTA agarose (Vinlo, Limburg), respectively.

**Antibodies**

The polyclonal antibody (pAb) goat anti-Amot (L-16) against a C-terminal peptide was purchased from Santa Cruz (Santa Cruz, CA). Anti-Flag antibody was purchased from Sigma-Aldrich. Monoclonal antibody (mAb) against the C-terminus of Cad11 (5B2H5) was purchased from Invitrogen (Invitrogen, Carlsbad, CA). Generation of mAb 2C7, an antibody against the extracellular domain of Cad11, was described in Lee *et al* \(^{64}\).

Polyclonal and monoclonal anti-p80-Amot antibodies were generated as follows. Purified GST-Amot protein was used to immunize rabbits for generating polyclonal anti-
Amot antibody and mice for generating both polyclonal antibodies (mouse sera) and monoclonal antibodies. Rabbit anti-Amot antibody was affinity purified as follows. Freshly purified Amot-his$_7$ protein was allowed to dry on a strip of nitrocellulose membrane, which was then incubated with the rabbit final bleed overnight at 4°C. The nitrocellulose strip was washed to remove nonspecific protein present in the rabbit bleed and the Amot antibodies were eluted using Gentle Elute elution buffer (Thermo Fisher).

Construction of the cadherin cyto GST proteins

The cytoplasmic domain (cyto-domain, amino acids 641-796) of human Cad11 was amplified by PCR using full-length human Cad11 as a template. A GST fusion protein expressing two copies of Cad11 cyto domain was constructed as follows. Two fragments of cyto-domain with different restriction enzyme sites were generated. The first fragment, which includes four glycine residues at the C-terminus, was amplified by using primers EcoRI-BamHI-cyto-F and EcoRI-cyto-R (Table 1). The second fragment was amplified by using primers EcoRI-BamHI-cyto-F and NotI-cyto-ter-R (Table 1). After the PCR reactions, the cyto-domain1 DNA fragments were subcloned into the pCR2.1-TOPO TA vector (Invitrogen, Carlsbad, CA). After confirming by DNA sequencing, the cyto-domain1 fragment was isolated from pCR2.1-cyto-domain1 plasmid by digesting with BamHI and EcoRI. The second fragment was isolated by digesting pCR2.1-cyto-domain2 plasmid with NotI and EcoRI. These two DNA fragments were ligated with the pGEX-4T-1 plasmid through BamHI and NotI restriction sites to generate plasmid pGEX-Cad11-cyto-2X. The
pGEX-E-Cad-cyto-2X, pGEX-Cad11ΔJMD-cyto-2X and pGEX-Cad11ΔCBS-cyto-2X containing two copies of E-Cad cytoplasmic domain and mutant Cad11 cyto domains respectively, were constructed similarly. We then used the cyto-2X constructs to create Cad11-cyto-WT, Cad11-cyto-ΔJMD, and Cad11-cyto-ΔCBS GST fusion proteins expressing single copies of wild-type or mutant Cad11 cyto-domains as described in Huang et al. The serial deletion mutants within CBS domain were generated using pGEX-Cad11-cyto construct as a template and using the reverse primers listed in Table 1, which delete every ten amino acids from the C-terminus. p3xFlag-Cad11-cyto-2X was generated by restriction digestion of pGEX-Cad11-cyto-2X and subcloned into p3xFLAG-CMV (Sigma-Aldrich, St. Louis, MO) through HindIII/NotI sites (New England Biolabs, Inc. Ipswich, MA). These constructs were generated by Dr. Mehmet Asim Bilen.

**Isolation of proteins associated with Cad11 cyto domain**

GST-cyto constructs were expressed in *E. coli* and the induced protein was purified through glutathione-agarose. C4-2B4 cells were scraped from plate in cold distilled water with protease inhibitors and homogenized with a Dounce homogenizer. The cleared lysate was mixed with GST-E-Cad-cyto-2X protein immobilized on glutathione-agarose beads on a rocker at room temperature for 2 hrs. The beads binding E-Cad cyto-2X protein were removed and the supernatant was mixed with GST-Cad11-cyto-2X protein immobilized on glutathione-agarose beads at 4 °C overnight. The proteins bound to GST-E-Cad-cyto-2X and GST-Cad11-cyto-2X were resolved on a 4–12% gradient NuPage gels (Novex, San Diego,
CA). The gel was stained with GelCode (Thermo Fisher) and the proteins associated with GST-Cad11-cyto-2X were identified by mass spectrometry.

**Direct protein interaction assays**

Purified Amot-his\textsubscript{7} protein was incubated with GST(-), GST-E-Cad cyto-2X, GST-Cad11-cyto-2X or GST-Cad11-ΔCBS-2X in TBS with 0.1% Triton X-100 overnight at 4°C with rocking. The flow-through was removed and the beads were washed three times using TBS with 0.1% Triton X-100. Proteins eluted from the beads were examined by western blot analysis using a mouse anti-Amot mAb.

**Transfection of mammalian cells**

HEK293T were transfected with mammalian expression vectors using polyethylenimine (PEI) as previously described\textsuperscript{31}. After 48 hrs, the transfected HEK293T cell lysates were used for GST pull-down assay.

**Immunoprecipitation Assays**

Cells were washed twice with ice-cold PBS with 50mM CaCl\textsubscript{2} and 50mM MgCl\textsubscript{2} (PBS/+CaCl\textsubscript{2}/+MgCl\textsubscript{2}). The cells were then lysed in buffer containing 50 mM Tris pH7.2,
150 mM sodium chloride (NaCl), 1 mM sodium orthovanadate (Na$_3$VO$_4$), 50 mM sodium fluoride (NaF), 25 mM β-glycerophosphate, protease inhibitors, and 1% Triton X-100 and processed as described above for the isolation of Cad11 interacting proteins. The cleared lysates were incubated with antibody for one hour. Then protein G- and A-agarose beads were added to each reaction tube and incubated for two hours at room temperature with rocking. The agarose beads containing the protein-antibody complexes were washed four times with ice-cold PBS+/CaCl$_2$+/MgCl$_2$. The protein-antibody complexes were analyzed on a SDS-PAGE for western blot analysis.

Viral Infection

Lentivirus

293FT cells were transiently transfected with pGIPZ vectors expressing shRNA with a scramble sequence or target sequence along with the viral envelope vector pCMV-VSV-G and the packaging vector pCMV-dR8.2 using PEI. After 48 hours of PEI transfection, the cell culture media with lentivirus titer is collected and filtered through a .45μM filter to remove 293FT cells. Polybrene was added to the filtered lentivirus titer to improve transduction to the target cells. Target cells were infected until at least 30% of cells express GFP from the pGIPZ vector. The cells are then selected using the antibiotic puromycin (puro).
Retrovirus

These retroviral vectors were transfected into Phoenix cells using PEI transfection. After 48 hours, the culture media were collected for infection of mammalian cells as described previously.  

Generation of C4-2B4 cells overexpressing Cad11 mutants

To express Cad11 wild type (WT), Cad11-ΔAmot, and Cad11-Δβ-cat in C4-2B4 cells, cells were transduced with recombinant retroviruses with GFP or Neo selection marker. GFP positive cells were selected by fluorescence activated cell sorting (FACS) and Neo positive cells were selected using the antibiotic G418, also known as neomycin (Neo).

Generation of PC3-mm2 cells with knockdown of endogenous Cad11 followed with expression of Cad11 mutants

PC3-mm2 cells were transduced with Cad11 shRNA in the lentiviral vector pLKO.1-puro (TRCN0000303363 NM_001797.2-3233s21c1, Sigma). The shRNA sequence is located at the 3’-untranslated region. shRNA transduced PC3-mm2 cells (PC3-shCad11) were selected by puro. PC3-mm2 cells transduced with a control pLKO.1-puro vector (PC3-sh-vec) were used as a control. PC3-shCad11 cells were then transduced with Cad11
mutants in bicistronic retroviral vectors and the transfectants were selected by FACS based on GFP.

**Fluorescence-Activated Cell Sorting (FACS)**

To sort cells expressing the pBMN-I-GFP constructs, cells from a subconfluent 10-cm plate were lifted using trypsin digestion. The cells were washed with RPMI/10% FBS to neutralize and remove the trypsin. Then the cells were sorted on a FACScan analyzer (Becton–Dickinson, California). Cells that were positive with green fluorescence were collected, washed with RPMI/10% FBS, and plated on 10-cm tissue culture plates.

For analyzing Cad11 cell surface expression, cells from a subconfluent 10-cm plate were released from the plate using the non-enzymatic Cellstripper solution (Cellgro, Virginia). The cells were resuspended in DMEM/10% FBS and incubated with normal IgG or 2C7mAb as primary antibody for an hour. The cells were then incubated with the AlexaFluor 488 secondary antibody before sorting on a FACScan analyzer (Becton–Dickinson, California). Cells that were positive for 2C7 binding were positive for Cad11 membrane expression.
Immunofluorescence staining

Cells were plated on glass coverslips to 70% confluency. The adhered cells were washed with PBS before fixed with ice-cold methanol for 10 minutes at -20°C. The methanol was removed and the cells were allowed to dry. The cells were then re-hydrated with PBS and solubilized with 0.2% Triton in PBS. After the solubilization step, cells were washed three times with PBS before blocking in PBS with 5% normal donkey serum and 0.05% Triton for an hour at room temperature with rocking. The primary antibody was diluted in blocking solution and incubated on the cells at 4°C with rocking overnight. The cells were washed with PBS before incubating with secondary antibody. After secondary antibody incubation, the cells were washed and incubated with DAPI staining.

Migration Assay

PCa cells (1 × 10^5 cells) suspended in serum-free media were seeded in the upper chamber of FluoroBlok migration inserts\(^47\). The lower chamber contained media with 10% FBS. After incubation for 8 hours, the migrated cells in the bottom part of the insert were labeled with calcein AM. Values for migration were expressed as the average of migrated cells per microscope field (×100). Five microscopic fields per insert were counted. The 8 hour migration time was used to avoid the possibility of proliferation affecting the assay results.
Aggregation assays

L-cells (CCL1.3) expressing empty Neo vector, Cad11-WT, Cad11-ΔAmot, or Cad11-Δβ-cat were generated via retrovirus infection. Control L-vector cells express only Neo. The cells were released from culture plates using Cellstripper (Cellgro, Virginia) for 10 to 12 minutes at 37°C, suspended in DMEM with 10% FBS, mixed on a rocker, and counted using a hematocytometer. Cell clusters containing 3 or more cells were considered as aggregates as previously described in Lee et al. 64.

Statistical analyses

Student’s t test (two-tailed, paired) was used for statistical analyses. A P-value of less than 0.05 was considered statistically significant. Data are expressed as the means ± SD unless otherwise specified.
Chapter III: Identification of Amot as a novel Cadherin-11 interacting protein

Chapter III, Section I: Identification of Cadherin-11 interacting proteins

Rationale

A previous study found that expression of Cad11 in PCa cells increased cell migration \textit{in vitro} and metastasis to bone \textit{in vivo}\textsuperscript{30, 47}. Further \textit{in vitro} experiments showed that the aberrant expression of Cad11 in PCa cells facilitated interaction between PCa cells and osteoblasts\textsuperscript{47, 64}. We found that Cad11 enabled PCa cells to invade into a monolayer of osteoblast cells and Cad11 expression also increased the migration and invasion capacities of PCa cells\textsuperscript{47}. We also found that the juxtamembrane (JMD) and the \(\beta\)-catenin binding (CBS) subdomains of the Cad11 cytoplasmic tail are critical to Cad11-mediated cell migration and invasion\textsuperscript{47}. The results suggested that the cytoplasmic domain is involved in signal transduction to modulate Cad11 function. Though Cad11 binds common cadherin interacting proteins, such as p120-catenin (p120) and \(\beta\)-catenin (\(\beta\)-cat), like E-Cad, Cad11 has opposing functions to E-Cad and a different tissue expression pattern compared to other mesenchymal cadherins. We, therefore, suspected that Cad11 could interact with a unique subset of intracellular proteins to participate in Cad11-mediated functions, which may contribute to the progression of PCa and PCa metastasis to bone.
Results

Expression of mesenchymal cadherins during cancer progression has been noted to result in increased cell migration and invasion, while the expression of E-cad in cancer cells suppresses these effects. To identify the Cad11 interacting proteins that mediate PCa cell migration and/or invasion, a subtractive approach was used. The cytosolic fraction of the PCa cell C4-2B4 was incubated with agarose beads binding GST-E-Cad to deplete common cadherin-interacting proteins (Fig. 7A). The E-Cad depleted lysate was then incubated with GST-Cad11 beads to identify proteins that uniquely interact with the Cad11 cyto (Fig. 7A). Incubation with GST-E-Cad was able to remove the common ‘classic cadherin’ interacting proteins, such as p120 and β-cat (Fig. 7B). The beads were resolved on SDS PAGE and the area designated by the bold line was excised for mass spectrometry analysis (Fig. 7C).

Mass spectrometry analysis identified Amot as a novel Cad11 interacting protein (Table 2). The peptides sequenced by mass spectrometry correspond to Amot and not the other members of the Motin family, i.e. AmotL1 or AmotL2 (Fig. 8A). As previous studies showed Amot plays a role in cytoskeleton organization and endothelial cell migration, Amot was chosen for further study as a candidate for mediating Cad11 functions in PCa cells. To confirm Amot is indeed a unique Cad11 interacting protein, C4-2B4 cytosol was incubated with either GST-E-Cad or GST-Cad11. We found that in two separate experiments, Amot was able to bind to the GST-Cad11 fusion protein but not the GST-E-Cad fusion protein (Fig. 8B). To further show Amot is a specific Cad11 interacting protein and that this interaction requires no adaptor protein from the C4-2B4 cytosol, bacterially expressed Amot was incubated with GST, GST-E-Cad, or GST-Cad11. We found that Amot
binds directly and specifically to Cad11 (Fig. 8C). Together, these data suggest Amot may indeed be a Cad11-specific interacting protein.
Figure 7. Isolation of Cad11 specific interacting proteins. (A) Schematic of GST fusion proteins used to isolate cytoplasmic proteins that uniquely interact with Cad11. (B) Western blot analysis showing GST-E-Cad incubation removes common ‘classic cadherin’ interacting proteins. (C) Coomassie stain showing GST-Cad11 eluted proteins for mass spectrometry analysis.
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**Table 2. Mass Spectrometry Analysis Results.** Amot was identified as a Cad11 interacting protein. Through literature search, we found Amot shared a Cad11 function, i.e. cell migration. Thus, Amot was considered the most suitable candidate for further study.
Figure 8. Identification of Amot as a Cad11-specific interacting protein. (A) The peptides identified in the mass spectrometry analysis are highlighted in red. (B) In two separate experiments incubating C4-2B4 cytosol with GST-E-Cad or GST-Cad11 cyto proteins, we found Amot binds only to GST-Cad11. (C) Purified Amot protein was incubated with the GST fusion proteins to show that Amot binds specifically and directly to Cad11-cyto.
Chapter III, Section II: Identification of domains that mediate Angiomotin/Cadherin-11 interaction

Rationale

Classic cadherins express two major cytoplasmic domains that mediate interactions with intracellular proteins: JMD and CBS. We hypothesized that Amot would bind to one of these known Cad11 protein binding domains. Therefore, using GST-fusion proteins that individually delete these domains, we set out to identify the Amot binding domain on the Cad11 cyto region. Identification of the Amot binding domain would then lend insight to possible common cadherin-interacting proteins that may be regulating the Cad11/Amot interaction.

Identification of the Cad11 binding domain on Amot was also needed to understand the function of the Cad11/Amot interaction. Amot expresses domains that mediate protein-protein interactions for various functions. Both Amot isoforms contain C-C domains and a PDZ binding motif (YLI) in the C-terminus. The longer p130 isoform also contains an extended N-terminal domain with PPxY motifs, which mediates its distinct functions. Flag-tagged constructs deleting these known binding domains on Amot were used to identify the Cad11 binding domain needed to form the Amot/Cad11 interaction. Experiments done to confirm Amot as a Cad11 interacting protein showed stronger binding of the p80 Amot isoform in the C4-2B4 cytosolic than p130. We, therefore, expected that the p130 extended N-terminal would not mediate Amot/Cad11 interaction and that the p80 isoform be the most
prominent Cad11 interacting protein. We further postulated that Amot would bind somewhere other than the PDZ motif, thereby allowing interaction with the PatJ/Syx/RhoA migration complex\textsuperscript{35}, or the C-C domains, to allow Cdc42 or Rac1 activation through the Amot/Rich1 interaction\textsuperscript{132, 145}.

**Results**

GST constructs expressing two copies of full-length Cad11-cyto, Cad11-cyto with a JMD deletion (ΔJMD) or a CBS deletion (ΔCBS) in both copies were used to identify the Amot binding domain (Fig. 9A). The GST fusion constructs were tested to ensure that p120 and β-cat bind to their respective domains and do not bind when those domains are deleted (Fig. 9B). As expected, Cad11-cyto-WT-2X was able to bind both p120 and β-cat, while Cad11-cyto-ΔJMD-2X only bound β-cat and Cad11-cyto-ΔCBS-2X only bound p120 (Fig. 9B). These GST fusion proteins were incubated with HEK293T lysate expressing Amot-YFP and we observed that Amot-YFP was unable to bind in the absence of the CBS region (Fig. 9C). To ensure that this binding pattern was due to Amot and not due to change in protein conformation by the YFP tag or nonspecific interaction with the YFP tag, we repeated the GST pull-down experiment using HEK293T lysate expressing p80-Amot-Flag. Again we found that in the Cad11-ΔCBS construct was unable to bind Amot (Fig 9D). To further show that Amot needed the CBS domain for its direct interaction with Cad11, we used bacterially expressed Amot-His\textsubscript{7} protein to bind GST(-), GST-Cad11-WT, or GST-Cad11-ΔCBS and found that the CBS domain is indeed needed to mediate Cad11/Amot interaction (Fig. 9E).
The CBS domain expressed by classic cadherins commonly binds β-cat suggesting a role for β-cat in the Amot/Cad11 interaction or Amot in the β-cat/Cad11 interaction. We generated CBS deletion mutants to identify the residues that mediate Amot binding to the Cad11 CBS domain and compare its location to the β-cat binding site within this domain (Fig. 10A). Cell extracts from HEK293T cells transfected with Amot-YFP were incubated with the Cad11 CBS mutants. As shown in Fig. 9B, deletion of the last 10 aa from Cad11-cyto (Cyto 146-156) resulted in loss of Amot binding, suggesting that the Cad11 CBS sequence GSKDTFDDDS (Fig. 10D) is involved in binding Amot. Sequence alignment comparing Cad11 and E-Cad cyto showed that the GSKDTFDDDS sequence is unique to Cad11 (Fig. 10D). This finding may explain the observation that Amot binds to Cad11-cyto but not E-Cad-cyto.

β-cat, however, was able to bind to all Cad11-cyto mutants except Cad11-cyto-ΔCBS (1-99), suggesting that the β-catenin binding site is near the aa 100-106 (Fig. 10C). The corresponding sequence, DSIQIYGYEG (Fig. 10D), coincides with the β-cat binding sequence determined by E-Cad deletion analysis and by crystal structure studies. These observations showed that β-cat and Amot bind to adjacent but non-overlapping sequences in the Cad11 CBS domain.

We then studied the known Amot protein binding domains to identify the Cad11 binding site. Amot contains two amphipathic helix rich C-C domains, CC1 and CC2, in the N-terminus and a PDZ binding motif, YLI, at the end of the C terminus. These domains are present in both p130 and p80. The extended p130 N-terminus known as the F-actin binding domain consists of two PPEY and one LPTY motifs. A series of 3xFlag tagged
Amot mutants were transfected into HEK293T cells (Fig. 11A). The lysates from the transfected cells were incubated with GST-Cad11-cyto-2X bound on agarose beads (Fig. 11B). We found that Cad11 preferentially binds the p80 isoform than p130 (Fig. 11B), consistent with the observation in Fig. 6B. Truncations of the CC1 domain (Mutant 1), deletions of both CC1 and CC2 domains (Mutant 2), or deletion of the PDZ motif (Mutant 5) did not affect Amot binding to Cad11-cyto-2X (Fig. 11B). These findings suggest that the Amot/Cad11 interaction is not through either of the CC domains. In support of this possibility, Amot mutant proteins containing only CC1 (Mutant 3) or CC1+CC2 (Mutant 4) domains were not able to interact with Cad11 (Fig. 11B). These results suggest that Cad11 does not interact with the Amot N-terminus CC domains (Fig. 11C).

As we expected, deletion of the C-terminus PDZ motif or the N-terminus C-C domains did not affect Cad11 binding. This suggests that though Amot binds to Cad11, the Amot protein interacting domains are left free to bind to the PatJ/Syx/RhoA migration complex\textsuperscript{35} or Rich1/Cdc42 complex\textsuperscript{132,145} (Fig 11C). However, deletion of the Amot middle domain resulted in loss of Cad11/Amot interaction (Fig. 11C). The p80 middle domain has not been extensively studied. Analysis of the p80 middle domain using the Simple Modular Architecture Research Tool (SMART smart.embl.de) program did not reveal a recognizable functional motif and was characterized as low complexity. Thus, we hypothesized Cad11 may interact Amot through an unknown motif in the middle domain.
Figure 9. Identification of the Amot binding domain on Cad11-cyto. (A) GST fusion protein constructs. Two copies of Cad11-cyto domain with deletion in the JMD or CBS were inserted after a GST tag. (B) GST fusion protein constructs tested to show they interacted with respective common cadherin interacting proteins p120 and β-cat. (C) Lysate from cells expressing Amot-YFP was incubated with the GST fusion constructs. (n=4) (D) Lysate from cells expressing p80-Amot-Flag was incubated with the GST fusion constructs. (n=4) (E) Purified Amot from bacterial expression was used to show that the CBS domain is needed to mediate Amot/Cad11 direct interaction. (n=3)
Figure 10. Identifying the location of the Amot and β-cat binding sequences within the Cad11 CBS domain. (A) Diagram of Cad11 deletion mutants within the CBS domain and summary of Amot and β-cat binding to the deletion mutants. (B) Deletion of the last 10aa from the C-terminus of Cad11-cyto (Cyto 1-146) resulted in loss of Amot binding. (n=4) (C) β-cat was found to bind to all cyto mutants except cyto-ΔCBS (Cyto 1-99). (n=4) (D) Sequence comparison of Cad11 and E-Cad cyto domains shows that while the β-cat binding motif is conserved between the two cadherins, the Amot binding motif is not found in the E-Cad cyto domain.
Figure 11. Cad11 binds to Amot middle domain. (A) Diagram showing the protein interacting domains in the Amot isoforms and mutant constructs and a summary of their binding to Cad11-cyto domain. These Amot constructs have 3x-Flag tag. (B) N-terminal and C-terminal deletions shown in the top two panels were compared to wild-type p80-Amot. The pull-down assays with GST-Cad11-cyto-2X are shown in the bottom two panels. Deleting the known protein binding domains on Amot did not affect Cad11 binding. Deletion of the middle domain results in loss of Cad11/Amot interaction. (n=3) (C) A schematic showing the uncharacterized Amot middle domain binding the last ten residues of the Cad11 CBS domain.
Chapter III, Section III: Endogenous Angiomotin/Cadherin-11 interaction within Prostate cancer and non-cancer cell lines

Rationale

Identification of Amot as a Cad11 interacting protein used endogenously expressed Amot in a GST pull-down assay utilizing a Cad11 fusion protein expressing two copies of the cytoplasmic region. Subsequent confirmation and mapping assays used overexpression of Amot or purified Amot protein expressed in bacteria. While these experiments validate Amot as a candidate Cad11 interacting protein, we needed to test whether these proteins indeed formed a complex when expressed at endogenous levels in mammalian cells. Furthermore, previous studies from our laboratory have shown Cad11 is expressed in clinical samples\textsuperscript{30}; therefore we need to confirm that Amot is expressed in clinically relevant tissue. We, thus, postulated that endogenous Amot expression is prostate cancer cell lines can form a complex with Cad11 in immunoprecipitation assays. We further surmised that the p80 Amot isoform is highly expressed in PCa patient derived samples.

Results

We examined the interaction of Amot and Cad11 in mammalian cells. We expressed two copies of Cad11-cyto in a 3xFlag-tagged vector (Cad11-2X-flag) and Amot-YFP in
HEK293T. Immunoprecipitation of Cad11 with anti-Flag antibody pulled down Amot-YFP while control IgG did not (Fig. 12A), confirming that Amot can form a complex with Cad11 in mammalian cells.

We then examined the level of endogenous Amot in PCa cells and HEK293T cells, which express both p80 and p130. As shown in Fig. 11B, C4-2B4 expressed both p130 and p80 with levels similar to those found in HEK293T. In contrast, PC3-mm2 expressed very low levels of Amot that can be seen at a longer exposure time (Fig. 12B).

C4-2B4 cells expressing exogenous Cad11 (C4-2B4/Cad11) were used for immunoprecipitation (IP) with anti-Cad11 mAb 1A5. p80, but not p130, was found in the 1A5 IP complex (Fig. 12C, top left), consistent with the observation that GST Cad11 constructs preferentially pulled down p80 (Figs. 5B and 11B). Furthermore, IP of Cad11 from PC3-mm2 cells, which endogenously express Cad11 but very low levels of Amot, also brought down p80 (Fig. 12C, top right).

Reciprocal IP using affinity-purified polyclonal anti-Amot Abs and C4-2B4/Cad11 or PC3-mm2 lysates were done to verify Amot/Cad11 interaction. Cad11 was found in the Amot immune complex from C4-2B4/Cad11 cells and PC3-mm2 (Fig. 12D, top). As both p130 and p80 are expressed in C4-2B4/Cad11 cells, anti-Amot Abs pulled down both Amot isoforms as expected (Fig 12D, bottom left). In PC3-mm2 cells, which express very low levels of Amot on Western blot (Fig. 12B), anti-Amot Abs immunoprecipitated proteins with a molecular mass of approximately 100 and 80 kDa (Fig. 12D, bottom right). It has been observed that the p130 Amot isoform is susceptible to proteolysis, resulting in generation of a band around the 100-kD mark. Thus, it is possible that the ~100-kDa protein
observed in the Amot immune complex in PC3-mm2 cells may be a p130 breakdown product. Together, these observations suggest that Amot associates with Cad11 in PCa cells.

To show that the p80 Amot isoform may also play a role in clinically relevant samples, we use isoform specific oligonucleotides to observe differential expression of the Amot isoforms in cDNA extracted from PCa patient derived xenograft (PDX) tissue taken from primary and metastatic sites, and possess a histopathology of either AdenoCa or SCPCa. We found that the PDX samples express higher levels of the p80 isoform than p130 (Fig. 13). Interestingly, the PDX that have been characterized as AdenoCa have overall higher AMOT expression compared to PDX characterized as SCPCa (Fig.13). SCPCa is a rare pathology of PCa with neuroendocrine differentiation, but can often be mistaken for AdenoCa. Furthermore, we found that the expression level of the Amot isoforms varies within groups that are either androgen receptor (AR) positive or negative suggesting AR expression does not affect or is not affected by the expression of AMOT (Fig 13). The AdenoCa PDX MDA-PCa-118b tumor\textsuperscript{36, 63} shows almost equal levels of the Amot p130 and 80 isoforms in the absence of functional AR, which is not observed in SCPCa samples, which may suggest a role of AR in regulating the p80/p130 ratio in the context of AdenoCa (Fig 13). We also found no correlation between PSA and AMOT levels as MDA-PCa 144-13 is derived from a patient who experienced decreasing PSA levels following androgen deprivation yet still developed bladder outlet obstruction and nodular prostatic mass invading the bladder\textsuperscript{9}. 
Figure 12. Endogenous Interaction of Amot and Cad11 in cell lines. (A) Mammalian expression of both Amot and Cad11 was used to show the proteins can form a complex in mammalian cells. (B) Expression of the Amot isoforms in the cell lines used for our study. PC3-mm2 has comparatively less Amot than other cell lines that can only be observed at long exposure or when the protein is concentrated using IP. (C) C4-2B4/Cad11 and PC3-mm2 cells were used for anti-Cad11 IP to show Cad11/Amot form an endogenous complex in PCa cells. (D) The C4-2B4/Cad11 and PC3-mm2 cells were used for anti-Amot IP to confirm Amot and Cad11 do indeed form a complex in PCa cells.
Figure 13. Differential Expression of Amot Isoforms in Patient Derived Xenografts. We compared PDX cDNA of different PCa type, tissue origin, and AR status to observe changes in Amot expression and differential Amot isoform expression using qPCR. The data suggests that the AdenoCa PDX samples have overall more Amot than SCPCa. And that overall, there is more p80 than p130 in most PDX samples despite PCa type, tissue origin, or AR status. qPCR done by Dr. Yu-Chen Lee, PhD.
Chapter IV: Elucidating the role of Angiomotin in Cadherin-11 mediated functions

Chapter IV, Section I: Study the effects of Angiomotin/Cadherin-11 interaction on Cadherin-11 mediated cell aggregation

Rationale

Previous experiments have shown that expression of Cad11 in L-cells results in increased cell-cell aggregation\(^{64}\). This phenotype allows for PCa cell and osteoblast interaction at the metastatic site\(^{47,64}\). Moreover, homophilic cadherin interaction plays a role in collective cell migration and possibly the formation of cancer cell aggregates called circulating tumor microemboli (CTM) that may aid in survival within the blood circulation\(^{40,46,119}\). In 2013, Oas \textit{et al} found that the CBS domain on VE-Cadherin was necessary to maintain cadherin-mediated cell adhesion strength\(^{89}\). However, since the entire CBS domain was deleted, it is unclear if \(\beta\)-cat binding or another CBS domain binding protein was responsible for conferring the cell adhesion strength. Bratt \textit{et al} used CHO cells to show that as cells reach confluency on a plate, Amot is upregulated and localizes to the cell-cell junction suggesting a possible role for Amot in strengthening cell-cell interaction\(^{21}\). As
Amot binds to the Cad11 CBS domain, which is needed to confer cadherin cell adhesion, we postulated that Amot may play a role in Cad11-mediated increased cell aggregation.

Results

To examine the role of Amot in Cad11-mediated adhesion in cell aggregation, Cad11 with deletion of the 10 aa Amot-binding sequence GSKDTFDDDS located at the very C-terminus (Cad11-ΔAmot) or the 10 aa β-cat binding sequence DSIQIYGYEG (Cad11-Δβ-cat) in the cyto domain was generated and expressed in L-cells (Fig. 14A). Western blot of L-cells with Cad11-WT, Cad11-ΔAmot or Cad11-Δβ-cat constructs showed the Cad11 proteins were expressed; however, lower Cad11-Δβ-cat levels were observed (Fig. 14B). Membrane expression of Cad11 proteins was further shown using immunofluorescence imaging (Fig. 14C). Immunofluorescence analysis showed that both Cad11-WT and Cad11-ΔAmot were mainly localized at the cell-cell junction in L-cells, consistent with a role of Cad11 in mediating cell-cell adhesion. Interestingly, an increase in intracellular accumulation of Cad11-Δβ-cat was observed in L-cells (Fig. 14C). FACS analysis on non-permeabilized cells using monoclonal antibody mAb 2C7 ⁶⁴ that recognizes the extracellular domain of Cad11 showed cell surface expression of the Cad11 proteins, but a peak of less intensity was observed for cells expressing Cad11-Δβ-cat (Fig. 14D). It has been reported that β-cat plays an important role in membrane targeting of cadherin proteins²⁸. The inability of β-cat to bind Cad11-Δβ-cat may result in the decreased Cad11-Δβ-cat expression observed in Western blot (Fig. 14B), immunofluorescence imaging (Fig. 14C), and FACS (Fig. 14D). Significant changes in cell morphology were also observed in L-cells transfected with Cad11-Δβ-cat, likely due to a role of β-cat in linking cadherin to cytoskeletal structure³², ⁸⁹.
However, there was no observable difference in cell-cell aggregation between Cad11-WT and Cad11-Δβ-cat (Fig 15), though the Cad11-Δβ-cat aggregates were not as large and appeared to be more fragile to agitation. Expression of the Cad11-ΔAmot construct did not affect cell surface expression/localization or cell morphology. Furthermore, the cell-cell aggregation assay showed that both Cad11-ΔAmot was also able to confer cell aggregation activity similar to that of Cad11-WT and Cad11-Δβ-cat (Fig. 15). These observations showed that Amot does not play a role in regulating Cad11-mediated cell aggregation.
Figure 14. Characterization of L-Cells. (A) Cad11 constructs were made to express wild-type (WT), ΔAmot, Δβ-cat in L-cells. (B) Western blot analysis showed the Cad11 constructs were stably expressed in L-cells and that these cell lines also expressed Amot. (C) Immunofluorescence analysis shows the Cad11 constructs localize to the plasma membrane. (D) Flow cytometry also showed that all the Cad11 constructs are expressed on the cell surface. This figure was completed with the help of Drs. Guoyu Yu and Song-Chang Lin.
Figure 15. Amot does not affect Cad11-mediated cell-cell aggregation. (A) Live cell image comparing the ability of the Cad11 constructs to confer cell-cell aggregation after 3 hours. (B) A graph quantifying cell-cell aggregation by counting single cells after 3 hours. (n=2) This figure was completed with the help of Dr. Yu-Chen Lee.
Chapter IV, Section II: Elucidate the role of the Angiomotin/Cadherin-11 interaction on Cadherin-11 mediated cell migration

Rationale

Expression and function of Amot were first studied in endothelial cells, where it was observed that the differential expression of the Amot isoforms was necessary for regulating the various steps in neovascularization during embryogenesis. Experiments showed that in the presence of p80 expression, RhoA activity was mainly expressed at the leading edge of a migrating cell. However, when Amot was not expressed, RhoA activity was dispersed in the periphery of the cell resulting in a stationary cell. IP analysis showed that Amot was a necessary component in the PatJ/Syx/RhoA migration complex. IF analysis showed Amot localized to adherence junctions but IP and GST pull-down assays failed to show direct interaction or co-localization with E-cad or vascular endothelial-cadherin (VE-cad). Our biochemical analysis found that Amot is able to bind specifically and directly to Cad11 via a ten residue binding site that is uniquely expressed the Cad11 CBS domain and is not found in the E-Cad cyto (Fig. 10D). We, therefore, postulated that the Cad11/Amot interaction may be used to promote Cad11-mediated migration in PCa cells.
Results

To examine the role of Amot on Cad11-mediated migration in PCa cells, C4-2B4 cells were transduced with Cad11-WT, Cad11-ΔAmot, or Cad11-Δβ-cat retroviral vectors. Western blot showed that Cad11-WT, Cad11-ΔAmot, or Cad11-Δβ-cat proteins were expressed (Fig. 16A). Immunoprecipitation of Cad11 proteins in C4-2B4 showed that Cad11-WT and Cad11-Δβ-cat bound p80, while Cad11-ΔAmot did not (Fig. 16B). Cad11-WT and Cad11-ΔAmot bound β-cat while Cad11-Δβ-cat did not, as expected (Fig. 16B). FACS analysis on non-permeabilized cells using mAb 2C7 showed that the Cad11 mutants were localized at the plasma membrane, although the mean fluorescence intensity of Cad11-Δβ-cat was lower than that observed in Cad11-WT and Cad11-ΔAmot (Fig. 16C).

Immunostaining further confirmed that all three Cad11 proteins were localized on the plasma membrane in C4-2B4 cells (Fig. 16D). We noted high levels of intracellular Cad11-Δβ-cat protein, similarly observed in L-cells expressing Cad11-Δβ-cat (Fig. 16D). Interestingly, differences in cell morphology were observed among C4-2B4 cells with Cad11 mutants. The Cad11-ΔAmot possesses multiple filopodial projections already suggesting they may be less motile (Fig 16D). The Cad11-Δβ-cat mutants, however, are more bipolar, which may be more conducive to directional movement (Fig 16D). How these mutations elicit these morphological changes is not yet clear. It may be that deletion of the Amot binding site on Cad11 results in loss of the Amot/PatJ/Syx complex to generate a leading edge resulting in loss of RhoA localization of a single focal point resulting in a flat, stellate cell with limited migratory potential. The Cad11-ΔAmot cells also appear to stain for Amot localization near MTOC, which is also a site of RhoA activity suggesting that when Amot is not bound to Cad11, it may localize to the MTOC. To test this possibility, the
FRET analysis done by Ernkvist et al. used to observe changes active RhoA localization can be done using the Cad11 mutants in conjunction with MTOC staining. We then examined the effects of Cad11-WT, Cad11-ΔAmot or Cad11-Δβ-cat on C4-2B4 cell migration. As shown in Fig. 16E, Cad11-ΔAmot expression significantly reduced Cad11-mediated migration in C4-2B4 cells compared to Cad11-WT cells.

To further examine the effect of Amot in Cad11-mediated migration, we expressed Cad11-WT, Cad11-ΔAmot, or Cad11-Δβ-cat in PC3-shCad11 cells. Western blot showed that Cad11 knockdown in PC3-shCad11 significantly reduced the endogenous level of Cad11 compared to the control PC3-sh-vec cells (Fig. 17A). The PC3-shCad11 cells with Cad11-WT, Cad11-ΔAmot, or Cad11-Δβ-cat bicistronic retroviral vector containing GFP were analyzed using Western blot to confirm Cad11 expression (Fig. 17A). PC3-shCad11 transduced with empty pBMN-I-GFP was used as a control (PC3-shCad11-GFP) (Fig. 17A). Immunostaining showed that the Cad11 proteins again localized to the plasma membrane (Fig. 17B). These cells were then used to study Cad11-mediated migration. Similar to the observed phenotypes in C4-2B4 cells, re-expression of Cad11-WT in PC3-shCad11 cells significantly increased the migratory activity compared to PC3-shCad11-GFP (Fig 17C). Expression of Cad11-Δβ-cat in PC3-shCad11 cells also increased migration compared to PC3-shCad11-GFP (Fig 17C). In contrast, expression of Cad11-ΔAmot did not promote the migration in PC3-shCad11 cells (Fig 17C).

The Cad11-WT, Cad11-ΔAmot, or Cad11-Δβ-cat constructs were also expressed in the non-PCa cells HEK293T, which endogenously express Amot but not Cad11 (Fig. 18A). Deletion of the Amot binding site abolished Cad11-mediated migration in HEK293T-Cad11
cells (Fig. 18B), as was observed in PCa cell lines (Fig. 16E, Fig. 17C). Together, these observations indicate that Amot plays a role in Cad11-mediated cell migration.
Figure 16. Amot binding needed for Cad11-mediated migration in C4-2B4 cells.
(A) Western blot of Cad11 constructs used to infect L-cells were expressed in C4-2B4.
(B) IP assays were done to test that the deletion mutants were able to interact with the proteins as expected. (C) FACS analysis showed the Cad11 constructs were expressed on the cell surface. (D) IF analysis showed the localization of the Cad11 constructs and Amot. (E) Cells (green circles) were suspended in serum-free media (light blue) within a migration insert placed in serum containing media (purple) for 8 hours before counting the cell that migrated across the insert barrier. The bar graph quantifies the results of the assay showing that the migration phenotype is lost when the Amot binding site is deleted (n=4). Panels C and D were done with the help of Dr. Song-Chang Lin.
Figure 17. Amot binding needed for Cad11-mediated migration in PC3-mm2 cells. (A) Western blot analysis showing the knockdown of the endogenous Cad11 and expression of the new Cad11 constructs. (B) IF analysis shows expression and localization of Cad11 constructs and Amot in PC3mm2 cells. (C) PC3-shCad11 cells expressing the new Cad11 constructs were used for a migration assay. The bar graph quantifies the results of the migration assay showing the loss Cad11-mediated migration when the Amot binding site is deleted. (n=3) This figure was completed with the help of Grace Liu and Dr. Song-Chang Lin.
Figure 18. Cad11/Amot mediated migration in HEK293T cells. (A) The Cad11 constructs were transduced into HEK293T cells and Western blot analysis shows stable expression of the Cad11 proteins. Expression of Cad11-WT and Cad11-Δβcat also resulted in increased p80 compared to vector and Cad11-ΔAmot. (B) The cells were used to show that in non-PCa cells, the Amot binding site is needed for Cad11-mediated cell migration (n=2).
Chapter V: Conclusions

1. Using a protein subtractive method, we were able to ascertain a unique subset of Cad11 cytoplasmic interacting proteins that may aid to elicit Cad11 unique functions in PCa cells (Table 2).

2. Two peptides corresponding to Amot were identified through mass spectrometry analysis as a novel Cad11 (Fig. 8A).

3. Biochemical experiments found that Amot bound directly and uniquely to Cad11 and not to E-Cad (Fig. 8C).

4. The Amot/Cad11 interaction is mediated by the Cad11 CBS domain (Fig. 9C, D, and E).

5. The formation of the Amot/Cad11 complex requires the last ten residues of Cad11 CBS domain while β-cat binds to the first ten residues of the CBS domain. Thus, Amot and β-cat do not share a binding site on Cad11 CBS (Fig. 10B).

6. The last ten residues of Cad11 CBS domain are unique to Cad11 and not found of E-Cad, which may be why Amot does not bind to E-Cad and also suggests that Amot may not bind to other classic cadherins (Fig. 10D).

7. Cad11 does not bind to any known and characterized Amot interacting protein domains (Fig. 11B).

8. Cad11/Amot interaction requires an unknown domain in the uncharacterized Amot middle domain (Fig. 11B).

9. Amot is endogenously expressed in the PCa cell lines C4-2B4 and PC3-mm2. Though PC3-mm2 cells express less Amot protein than C4-2B4 (Fig. 12B).
10. Cad11 and Amot are able to form an endogenous complex in PCa cells (Fig. 12C and D).

11. PDX cDNA samples show p80 expression is greater than p130 overall (Fig. 13).

12. PDX cDNA samples show that AdenoCa samples have more Amot than SCPCa (Fig. 13).

13. Deletion of the Amot and β-cat binding sites on Cad11 CBS do not affect Cad11-mediated cell-cell aggregation (Fig. 15).

14. Expression of Cad11-ΔAmot in PCa and non-PCa cells results in loss of Cad11-mediated cell migration suggesting Cad11/Amot interaction promotes PCa cell migration (Figs. 16 E, 17C, and 18B).

15. The Cad11/Amot interaction is needed to promote Cad11-mediated cell migration, which may utilize the Amot/PatJ/Syx migration signaling complex observed in endothelial cells to localize RhoA activity and actin polymerization to the leading edge.

Figure 19. Model for Amot/Cad11 interaction and its role in Cad11-mediated PCa cell migration. Amot uniquely binds to Cad11 and not E-Cad. This interaction results in promoting Cad11-mediated migration likely by recruiting the Amot/PatJ/Syx-RhoA activating complex to the cell membrane to localize actin polymerization where Cad11 is bound.
Chapter VI: Discussion

Cadherin Switching in Prostate Cancer Cell Migration

The functions of cadherins include but surpass that of cell adhesion alone. Through homophilic interactions, cadherins establish cellular identity to maintain tissue integrity as well as coordinate collective cell migration\textsuperscript{42,119}. The contribution of these functions to the process of cadherin switching is an important aspect of cell plasticity that mediates key processes during normal human development, such as the migration of the mesoderm during embryonic gastrulation\textsuperscript{42}. Similarly in cancer, cadherin switching affects not only the polarity and adhesive properties of a cancer cell, but also the interaction with its intracellular and extracellular environment\textsuperscript{99,133}.

Cancer associated cadherin switching results in the expression of a new cadherin interacting with a new subset of cytosolic proteins to affect intracellular signaling. This exhibits changes to cellular morphology and function, such as loss of polarity and increased cellular migration\textsuperscript{8,88,133}. A common cadherin switch in the metastasis of multiple cancers is the E-Cad to N-Cad switch\textsuperscript{88}. However, PCa metastasis instead seems to rely on the upregulation of the mesenchymal cadherin, Cad11\textsuperscript{30,62}. Though PCa cells also express N-Cad, the knockdown of Cad11 using shRNA was sufficient to decrease cell migration\textsuperscript{47} and incidence of bone
metastasis\textsuperscript{30}. Furthermore, targeting the Cad11 extracellular region with a monoclonal antibody was able to suppress Cad11-mediated metastasis to bone\textsuperscript{64}. Together, this suggests that the cadherin switch to Cad11, and Cad11-specific interacting proteins, may provide insight into the mechanism of Cad11-mediated PCa metastasis to bone.

This study identified Amot as a novel Cad11 interacting protein whose interaction plays a role in regulating Cad11-mediated cell migration in PCa cells. \textit{In vitro} assays showed that Amot was able to bind to Cad11 but not E-Cad (Fig. 8B). Further study using bacterially expressed and purified protein showed the Amot/Cad11 interaction did not require an adaptor protein (Fig. 8C). This novel and direct interaction between Cad11 and Amot provides a molecular basis for the differences in cell migration and metastatic potential observed in cells expressing Cad11 versus E-Cad. Because loss of E-Cad expression is a noted characteristic of EMT and upregulation of Cad11 is needed to promote PCa metastasis\textsuperscript{133}, our study provides one of the mechanisms in PCa by which cadherin switching promotes cell migration.

How the Cad11/Amot interaction increases cell migration is yet to be determined. Studies on endothelial cell migration by Ernkvist \textit{et al} \textsuperscript{35} showed that Amot uses its C-terminus PDZ motif, specifically the sequence EYLI, to bind to the third PDZ domain of the scaffold protein PatJ. Once Amot binds to PatJ, the guanidine exchange protein Syx is recruited to bind to the tenth PDZ domain on PatJ to form a signaling complex that regulates endothelial cell migration by localizing RhoA activity to the leading edge of the cell\textsuperscript{35}. Similarly, Wu \textit{et al} \textsuperscript{136} showed that Amot can also interact with Mupp1, another multiple PDZ domain protein, to recruit Syx, which activates RhoA to increase endothelial cell migration. Though Amot and PatJ localize to the plasma membrane, neither have transmembrane domains nor motifs to bind phospholipids to mediate this phenomenon. So how does the Amot/PatJ/Syx/RhoA signaling
complex get recruited to the plasma membrane? Herein may be the function of the Cad11/Amot interaction within PCa cells.

The upregulation of Cad11 during the EMT cadherin switch, the mesenchymal cadherin could induce recruitment of Amot to the plasma membrane and function as anchor for the migration complex. Using mutants with deletions of the known protein interacting domains on Amot, we found that Cad11 binds to Amot through the middle domain, which does not appear to have known protein binding motifs (Fig. 11). We predicted that Cad11 would not bind to the PDZ motif, which would allow Amot to continue its known interaction with the PatJ/Syx/RhoA migration complex. As we did not find any amphipathic helices in the CBS domain, we did not expect Cad11 to bind Amot through the C-C domains. Therefore, it is also possible that the Cad11/Amot interaction may increase migration by leaving the N-terminal C-C domains free to form the Rich1/Amot complex that could result in activation of a GTPases: Rac1 or Cdc42. It has been shown that the Amot/Rich1 complex promotes Rac1-mediated activity. It is therefore possible that Cad11 functions to recruit the Amot and its associated small GTPase to the plasma membrane to create the leading edge of a migrating cancer cell. Further study into the how Cad11 expression affects RhoA, Cdc42, and/or Rac1 is required.

**Cad11 and Small GTPases**

The relationship between classic cadherins and small GTPases has been a subject of study in development and cancer. Studies have shown that the activity of the Rho GTPases functions not only to induce actin polymerization and microtubule activity in promoting cell
motility\textsuperscript{115, 135}, but also actin contraction at focal adhesion sites to stabilize cadherin cell-cell contact\textsuperscript{19}. Expression of wild-type E-Cad has been shown to promote polarity and impede cellular migration by reducing the levels of active RhoA and Cdc42\textsuperscript{10}. In addition, N-Cad expression in neuronal cells is associated with the small GTPase Rap1 in order to orient cell migration toward the cortical plate\textsuperscript{53}. However, in as much as cadherin expression affects activity of the small GTPases, GTPase activity also affects cadherin. RhoA activity, for instance, is suggested to participate in clustering cadherin molecules at the site of cell-cell contact\textsuperscript{7}. Therefore, understanding the functions pertaining to the relationship between classic cadherins and small GTPases can lend further insight as to how that relationship varies with Cad11 to elicit Cad11-specific phenotypes in PCa cells. But the first question would be: what small GTPase is responsible for facilitating Cad11-mediated PCa cell migration?

There are a few possible small GTPases that could be activated and localized due to Cad11 expression in PCa cells. As a result of Amot interaction with Rich1\textsuperscript{145} and the PatJ/Syx complex\textsuperscript{35}, Amot may serve as an adaptor protein facilitating Cad11-mediated activation of RhoA, Cdc42, or Rac1 at the plasma membrane to mediate a variety of changes to the PCa cell cytoskeleton. The Cad11/Amot interaction may recruit active Cdc42 and/or Rac1 to aid in the extension of filopodia and actin contraction at adherence junctions to facilitate collective migration, or Rho to induce changes in cell shape and contraction/compaction to aid in extra- and extravasation\textsuperscript{18, 19, 43}. Moreover, it is possible that the Cad11/Amot interaction could affect localization of more than a single small GTPase as Cad11 binds to the middle domain leaving both the C-C and PDZ domains free to bind to Rich and PatJ (Fig. 11). Considering Rho participates in actin contraction for stabilizing cadherin adherence cell-cell contacts, could any of these small GTPases affect Cad11-mediated cell aggregation?
The small GTPases likely do not affect at Cad11 mediated cell-cell adhesion sites as observed in other cadherins\textsuperscript{18}. When the Amot binding site was deleted on the Cad11 CBS and expressed in L-cells, there was no effect on Cad11-mediated intercellular contacts (Fig.15). This suggests that the Cad11/Amot recruitment of active Rho proteins may only contribute to migration without affecting cell aggregation for collective migration, or affect binding and interaction with bone stromal cells. Moreover, it suggests that the Cad11 interaction with JMD binding proteins, such as p120, and their downstream signaling could remain unaffected.

Studies have shown that p120, a common cadherin interacting proteins, is able to regulate activity of small GTPases in order to maintain the integrity of the adherence junction through a variety of mechanisms\textsuperscript{59}. When p120 is bound to the cadherin molecule, it functions to stabilize cadherin expression on the cell membrane and prevent endocytosis\textsuperscript{86, 89}. Unbound p120 is phosphorylated by Src in order to bind and suppress RhoA function\textsuperscript{59}. However, p120 binding to a small GTPase does not always suggest suppression of activity. In BCa, p120 knockdown resulted in loss of ErbB2 activation of Rac1 and Cdc42 to induce cell migration\textsuperscript{52}. This phenomenon was rescued by ectopic expression of p120\textsuperscript{52}. Moreover, the tumor associated Rac1 splice variant called Rac1b depends on binding p120 to direct cell migration\textsuperscript{93}. The ability of p120 to suppress RhoA while activating Rac1 and Cdc42 is important in the orientation of cell migration. RhoA promotes cell retraction to induce cell polarity while suppressing Rac1 derived protrusions at the leading edge of the cell\textsuperscript{91}. It is possible that p120 suppression of RhoA and activation of Rac1/Cdc42 results in the establishment of the leading edge\textsuperscript{24}. This suggests there are conditions in which p120 could function in accordance with GTPases to promote cell adhesion as well as migration. However, this would depend not only on the small GTPases recruited by the classic cadherin, but also the p120 isoform expressed by the cell. For example,
the mesenchymal p120 isoform 1 inhibits Rho activity\textsuperscript{143} while the epithelial p120 isoform 3 has no effect, and isoform 4 activates RhoA\textsuperscript{59}.

During cadherin switching, many mesenchymal molecules are upregulated while epithelial proteins are downregulated, therefore, I postulate two ways in which the cadherin/p120 relationship in this process changes GTPase activity. First, it is conceivable that the E-Cad to Cad11 switch also changes which p120 isoform is expressed. If this proves to be the case, identifying which of the p120 isoforms is expressed by PCa cells that express Cad11 will help understand changes in Cad11-associated GTPase activation. Also, the p120/GTPase signaling depends on unbound p120\textsuperscript{59}, therefore, it is possible that Cad11 affinity to the expressed p120 isoform will determine what GTPases are inhibited and which are available to mediate changes to the cytoskeleton. One question is if the Cad11/p120 Rho GTPase signaling has any functional relationship with the Cad11/Amot Rho GTPase signaling.

The Cad11/p120-mediated signaling of Rho GTPases could affect the Cad11/Amot signaling in different ways. If Cad11 expressing PCa cells express p120 isoform 3, Cad11/Amot recruitment of either RhoA via PatJ or Rac1/Cdc42 via Rich1 would not be affected. If the cells express Cad11 and p120 isoform 4, we may see that the unbound p120 would perhaps further enhance the Cad11/Amot activation of RhoA. However, if Cad11 is not the only mesenchymal protein upregulated by PCa cells and the p120 isoform 1 is expressed, the unbound protein may suppress Cad11/Amot activation of RhoA. If this is the isoform expressed, the Cad11/Amot/Rich1 complex in Cad11 expressing PCa cells may instead activate Rac1 or Cdc42. Another possibility is that Cad11 would maintain p120 binding to prevent inhibition of Rho GTPase activation via Amot binding. It is important to consider that a cell may express more than one of the p120 isoforms; therefore localization of the p120 isoforms may also help predict
what Rho GTPase will be activated in what part of the cell, i.e. Rac1/Cdc42 at the protrusion of the leading edge and RhoA actin contraction at the lagging end. Therefore, it is important to examine how these p120 isoforms interact with cadherin molecules to mediate GTPase-signaling.

Something to consider is that Cad11 is not alone in PCa cells. For example, PC3-mm2 cells, which endogenously express Cad11, also express N-Cad. It is possible that Cad11 is changing Rho GTPase activation in two ways. One way is that Cad11 is able to sequester cytosolic p120 to maintain cell-cell contact, and to bind Amot for recruitment and activation of Rho GTPases to induce migration (Fig. 20A). If this is the case, deletion of the p120 binding site on Cad11, could show a potential change in Cad11/Amot-mediated migration as well as cell-cell adhesion. As Cad11 relies on binding p120 to stabilize expression on the plasma membrane, ectopic expression of p120 may be a better method to examine the effects of p120 on Cad11/Amot functions. The excess unbound p120 would help determine what Rho GTPases would be active at the cadherin cluster site by competing with the Amot/Rich or Amot/PatJ complexes for binding to RhoA or Rac1. Another possibility is that Cad11 acts in concert with other mesenchymal cadherins, such as N-Cad to regulate p120-mediated Rho GTPase activity. Any unbound p120 would be bound by N-Cad to maintain cell-cell contacts while Cad11 binds Amot to induce cellular migration (Fig. 20B).

To test this possibility, we would delete the p120 binding site on either Cad11 or N-cad, or delete the p120 binding site on both to compare changes in Rho GTPase activation. However, as both cadherins require binding p120 to help stabilize their expression on the plasma membrane, we could ectopically express p120. Again it would be important to know which p120 isoform is expressed to anticipate what Rho GTPases will be suppressed or activated.
Moreover, it would be important to examine the proximity of the Cad11 and N-cad cadherin clusters induced by GTPase activity as the unbound p120 of either cadherin would affect the GTPase signaling of the other. Therefore, the affinity between Cad11 and the different p120 isoforms should be compared to that of N-cad in order to better surmise what p120 binds to which cadherin in order to change Rho GTPase activity. Considering that the cadherin/p120 interaction is regulated through receptor and non-receptor tyrosine kinases\textsuperscript{59, 72, 73}, it would also be prudent to identify the tyrosine kinases associated with Cad11 in normal and cancerous cells. It is possible that there is a Cad11-associated kinase that may phosphorylate p120 to increase the concentration of unbound p120 which may affect Cad11/Amot-mediated GTPase activation (Fig. 20C).

Moreover, it would be of interest to examine if there are any Cad11-specific interacting intracellular proteins that may enhance Cad11/p120 interaction to prevent p120 phosphorylation that may increase the concentration of cytosolic unbound p120, which would alter GTPase activity. Considering Cad11 uniquely binds Amot to mediate the migration function, perhaps there are other Cad11-specific proteins that help enhance the potency of this interaction by affecting the interaction between Cad11 and the common cadherin interacting proteins.
Cell Migration via Cad11 versus Other Cadherins

Members of the Cadherin superfamily mediate multiple cellular functions through various signal pathways using a variety of intracellular proteins in different cell types and
cellular contexts. These interactions are tightly regulated by extracellular and intracellular changes. Atypical cadherins are able to interact with guanosine nucleotide-binding proteins to induce intracellular signaling cascades that affect transcription, motility, and secretion. Protocadherins, such as Fat and Dachsous, participate in downstream signaling promoting contact inhibition via the Hippo Pathway. Though classic cadherins have common interacting proteins, such as $\beta$-cat and p120, they also possess interacting proteins unique to them. However, some of those unique interacting proteins function to regulate cadherin interaction with $\beta$-cat or p120. For example, Xu et al. showed that dephosphorylation of $\beta$-cat by PTP1B is required for $\beta$-cat binding to N-cadherin. Besides $\beta$-cat, $\alpha$-catenin and p120, E-Cad has also been shown to interact with presenilin and Hakai. What is the function of regulating the stability of the cadherin/catenin complex?

Cell migration through most mesenchymal cadherins is mostly attributed to post-translational modifications to either the cadherin molecule or $\beta$-cat. For example, Qi et al. found that during transendothelial migration of melanoma cells, N-Cad was tyrosine phosphorylated resulting in the loss of $\beta$-cat binding and release of N-Cad from the cytoskeleton. Would phosphorylation of Cad11 or $\beta$-cat affect Cad11-mediated migration as observed in other classic cadherins?
If Cad11-mediated migration was equally dependent on its dissociation from β-cat to increase cell motility, cells expressing the Cad11-Δβ-cat construct would have shown a further increase in cell migration compared to cells expressing Cad11-WT. However, there was no statistically significant change in cell migration due to deletion of the β-cat binding site compared to wild-type Cad11 (Figs 16-18). This suggests that the mechanism of Cad11-mediated cell migration is unique when compared to other cadherins, thereby requiring a unique interacting protein to mediate migration. Furthermore, this Cad11-specific mechanism of migration may be due to its unique ability to bind Amot, especially since it contains the Amot-binding motif that is absent in E-Cad (Fig. 8D) and the CBS domain of other classic cadherins (Fig. 21).

**Figure 21. The Amot binding site is unique to Cad11.** A comparison of the cyto domains of different classic cadherins shows the ten residues that bind Amot are present only in Cad11.
Regulating Cad11/Amot Interaction

Though Amot is expressed as two different isoforms, p80 and p130, they possess distinct functions. Many of our studies indicate that even when p130 is abundantly expressed, Cad11 preferentially interacts with p80. First, mass spectrometry analysis of Cad11-cyto interacting proteins identified two peptides that belonged to Amot, not Amot-L1 or Amot-L2 (Table 2 and Fig. 8A). Then, GST-pull down assays using p130 or p80 expressed in HEK293T cells showed that Cad11-cyto interacts mainly with p80 (Fig. 11B). Furthermore, immunoprecipitation conditions preferentially bound p80, but not p130, co-immunoprecipitated with Cad11 in both PC3-mm2 and C4-2B4/Cad11 PCa cells (Fig. 12C). How p80 interacts with Cad11 remains to be determined. Considering the aspartic acid residues in the last ten amino acids of the Cad11 CBS that promote Amot binding, I examined the middle domain for areas rich in positively charged residues, such as arginine, that may participate to create a salt bridge. A salt bridge combines two noncovalent interactions, hydrogen bonding and electrostatic interactions, to form a stable interaction between proteins (Fig. 22).
During optimization of the Co-IP assays, we found higher protein levels of Amot in the Cad11 IP complex when the lysis buffer contained less than 100mM of NaCl compared to the standard 150mM of NaCl concentration. Cadherin molecules are known to form salt bridges to bind β-catenin in the first residues of the CBS\textsuperscript{140}, suggesting the possibility the Cad11/Amot interaction may be mediated by the same noncovalent interactions. This observation suggests the importance of electrostatic interactions in mediating Cad11/Amot interaction to form a Co-IP complex. The aspartic acids in the identified Amot binding motif on Cad11 would provide the negative charge for the electrostatic interaction; therefore, we needed to identify the corresponding residues that would provide the positive charges within the Amot sequence. Interestingly, there is a cluster of arginine and histidine residues in the beginning of Amot middle
domain, i.e. amino acids 337 to 342, 358-365, 404-411, which may contribute to Amot/Cad11 binding.

What regulates Cad11/Amot binding is not yet clear. It is possible that post-translational modifications such as phosphorylation/dephosphorylation of β-cat or Cad11 are involved in regulating the binding of Amot to Cad11. Our studies showed that Amot and β-cat bind to distinct amino acids sequences that are about 40 amino acids apart within the Cad11 CBS domain. Because the molecular weights of both Amot and β-cat are relatively large, it is also possible that the proximity of their binding sites may cause steric hindrance and that Cad11/Amot interaction may be regulated by Cad11/β-catenin status. How Amot binding to Cad11 is regulated is currently being investigated. However, we must consider that this unique interaction may be mediated by another Cad11-specific interacting protein. Therefore, we must also study the interaction between Cad11 and the other proteins identified in the mass spectrometry analysis (Table 2) that may lend insight into the mechanism of other Cad11-mediated cellular functions that contribute to PCa progression and metastasis.

**Function of Cad11/Amot Interaction in Development of Bone Metastasis**

Cancer metastasis is a multistep process by which cancer cells are able to change their cellular properties in context with the surrounding tissue architecture and adopt new cellular functions that allow them to home and colonize a distant site. Previous studies of Amot
characterized p80 as an oncoprotein in various contexts. Amot expression enhances endothelial invasion and stabilize established tubes\textsuperscript{66}. Knockdown of Amot in NF2 mutant cells inhibited the tumorigenicity of NF2 cells\textsuperscript{145}. Amot was also shown to be upregulated in aggressive breast cancer and high levels of Amot transcript were correlated with shorter overall survival of the patients\textsuperscript{66}. Though we did not focus on the tumorigenicity of Amot in PCa, we found Amot contributed to the metastatic potential of Cad11 expressing PCa cells by promoting cellular motility (Figs. 16E, 17C, 18B). Therefore, the role of Amot in cancer metastasis may be limited to cell migration in the context of Cad11.

Previous work showed that expression of Cad11 contributed to the development PCa metastasis by increasing cell aggregation, increasing migration, and increasing the incidence of metastasis to bone\textsuperscript{30, 47}. A recent publication from the Lin laboratory by Dr. Yu-Chen Lee et al found that Cad11-mediated cell adhesion was needed for PCa cells to home and colonize the bone\textsuperscript{64}. As my deletion studies showed, interaction with Amot did not affect Cad11 intercellular interaction (Fig. 15) and therefore may not affect the ability of PCa cells to target to the bone. Together our observations suggest that the Cad11/Amot migration complex may play a role in early stages of PCa metastasis, such as the escape from the primary site or in aiding with transendothelial migration via changes in Rho GTPase activity and actin reorganization. However, it would be of interest to observe other functions of Amot that are independent of Cad11.
The Amot Mediated Functions in Prostate Cancer

This study focused on the role of Amot in Cad11-mediated functions that promote PCa progression and metastasis. We found that in PCa cells that express Cad11, Amot is able to interact with Cad11 to promote cell migration. However, Amot is an adaptor protein that contributes to a variety of cellular functions through its ability to bind different proteins. Depending on the cell type and the proteins expressed in that cell type, the resulting Amot interactions can contribute to cell signaling mechanisms that increase cell migration, proliferation, and contact inhibition. Therefore, we must examine the Amot in PCa that may be independent of Cad11 or investigate the possible role of Cad11 in Amot-mediated functions.

The short isoform p80 utilizes its C-C domains to create hydrophobic protein-protein interactions with proteins, such as Rich1 and Merlin\textsuperscript{132, 145}. In epithelial cells, the p80/Rich1 interaction is needed to regulate Cdc42 activity in order to establish the apical polarity complex\textsuperscript{132}. Through the same domain, p80 can bind to the tumor suppressor protein Merlin, also called NF2\textsuperscript{145}. In Schwann cells, a study showed that Rich1 and Merlin compete for Amot binding\textsuperscript{145}. Under normal conditions, this interaction functions to suppress cell proliferation, but there are cases in neurofibromatosis when Merlin is mutated and the failure to bind Amot results in increased cell proliferation\textsuperscript{145}. Mutation or loss of Merlin in melanoma also enhances cell proliferation, as well as migration\textsuperscript{84}. It would be of interest to measure levels of Merlin expression in PCa cells and how its interaction with Amot or competition with Rich1 affects the role of Amot in Cad11-mediated cell migration. Though our study showed Cad11 preferentially bound p80 than p130, the long isoform is still expressed in PCa cells.
The initial experiments in which Amot was identified as a Cad11 interacting protein showed that p130 could bind to the Cad11 cyto GST construct (Fig. 8B). Moreover, this interaction was specific as p130 did not bind to the E-Cad GST construct (Fig. 8B). Though the interaction is not as prominent as the Cad11/p80 binding, there may still be conditions in which Cad11 could potentially interact with p130. Therefore, we must also look to examine the functions of p130 in PCa cells in the presence and absence of Cad11. Much like Merlin, the p130 Amot isoform has also been found to participate in promoting the Hippo tumor suppressor pathway. Through the extended 409 aa N-terminal, Amot is able to sequester the transcription factor YAP in order to facilitate Lats 1/2 mediated phosphorylation\textsuperscript{147}. The extended N-terminal also contains F-actin binding domains that lends to the p130 function of maintaining cell shape\textsuperscript{33}.

If under certain conditions, Cad11 could bind the p130 Amot isoform, perhaps this interaction would function to suppress the Hippo pathway and promote YAP nuclear translocation. Increased nuclear YAP translocation and transcription activity would be of great use to Cad11 expressing PCa cells. Many of the YAP transcription targets promote not only cell proliferation, but cell survival as well as secretion of bone morphogenic proteins that may function to aid in PCa colonization of the bone\textsuperscript{6, 54}. However, a similar outcome could be rendered through p80 binding Merlin in order to prevent the formation of the Merlin/Kibra complex to initiate phosphorylation of Mst 1/2, a key step in the Hippo pathway\textsuperscript{145}. Another possibility could be to remove p130 binding from F-actin in order to allow changes in the cell cytoskeleton to further promote cell migration. But what could be a Cad11 independent role of the p130 Amot isoform in PCa?

The extended N-terminal of p130 contains phosphorylation sites that regulate the p130 protein turnover through ubiquitination and protein degradation\textsuperscript{129}. It would be of interest to
examine changes in p130 expression during PCa progression and in metastatic lesions. The cDNA tested from the PDX samples showed that p130 mRNA levels are lower compared to p80 mRNA (Fig. 13). This suggests that p130 may be downregulated in PCa, though analysis of protein expression would ultimately provide better insight into the functionality of p130 in the PDX samples. How levels of p130 mRNA and protein are regulated could be used to identify new potential therapeutic targets.

**Diagnostic/Prognostic Marker or Therapeutic Target**

Rate of diagnosis for PCa has increased over time due to an increase in the population of the elderly and also due to the optimization of the PSA test\(^\text{127}\). The sensitivity of the PSA test has led to over-treatment of low-risk patients and non-essential radical prostatectomies\(^\text{81, 109, 110}\). Studies in proteomics using clinical samples and functional assays are needed to identify clinically relevant biomarkers that can function to accurately diagnose the stage and assess the prognosis as to the severity of the disease. Considering that even low levels of Amot are able to elicit migration in the presence of Cad11 as in the case of PC3-mm2 cells (Fig. 12B), Amot may not be as good a diagnostic or prognostic marker in PCa. However, examining the co-expression of Cad11 and Amot in patient samples would give a better gauge of metastatic potential. This is supported by the data attained using the C4-2B4 PCa cell line, which expresses more Amot than PC3mm2 (Fig. 12B) but has limited migratory potential until Cad11 is expressed via retroviral vector (Fig. 16E). Together this suggests that monitoring upregulation of Cad11 expression in
conjunction with Amot protein expression may serve as a warning that the PCa patient is at risk of metastasis.

Though the Cad11/Amot interaction may not affect the PCa/osteoblast interaction, we have yet to understand the conditions which promote Cad11/Amot interaction or what other functions this complex may possess once in the bone. Analysis of samples derived from primary tumor or bone metastatic lesions showed that despite changes in AR signaling or PSA levels, the Amot p80/p130 ratio remains favorable to the p80 isoform in PCa (Fig. 13). Examination of protein expression in these PDX samples may be warranted prior to elucidating p80 function. Therefore, investigation of the functional role of Amot in PCa independent of Cad11 interaction within the bone microenvironment compared to the primary site is warranted.

This study has shown that Amot is a novel Cad11 interacting protein, which functions to mediate PCa cell migration and may subsequently contribute to the metastatic potency of Cad11 expressing cells. As the mechanisms that regulate Cad11/Amot interaction are yet to be identified, it would be premature to suggest an agent that may target and inhibit this interaction or at what stage in the disease inhibiting the Cad11/Amot interaction would be of benefit. Lee et al found that treatment with an anti-Cad11 mAb (2C7) targeting the third extracellular domain was able to inhibit cell aggregation and PCa cell/osteoblast interaction thereby reducing incidence of bone metastasis. As loss of Amot binding did not affect Cad11-mediated adhesion or cell aggregation, it is uncertain the effect 2C7 treatment on the Cad11/Amot interaction. In the paper, the cells were injected into the left ventricle, essentially examining the effects of Cad11-mediated adhesion on homing and colonization rather than dissemination from the primary tumor site. Since I speculate that the Cad11/Amot interaction may function more in migrating out to escape the prostate, it would be of interest to inject PCa cells into the prostate
and then treat with 2C7 to observe changes in Cad11 and Amot expression and function. However, it may be that 2C7 treatment prevents the PCa/osteoblast interface to suppress PCa colonization of the bone rather than dissemination from the primary site. Therefore, we would need to inhibit the intracellular interaction of Cad11 and Amot.

While some protein-protein interactions use large binding surfaces, successful binding of Amot to the Cad11 CBS seems contingent on ten residues at the very C-terminus of Cad11 that could pose a challenge as a drug target. However, recent studies have tackled these “undruggable” and short binding motifs by designing short peptides that mimic the interface of protein-protein interaction\textsuperscript{25, 68}. These peptides tend to be small enough and thus permeable to the mammalian cell membrane\textsuperscript{68}. Treatments with these peptides do not affect gene transcription, therefore have minimal impact on non-cancerous cells\textsuperscript{25, 68}. Furthermore, unlike other small molecule inhibitors that must be administered intravenously, these peptides can be taken orally since they are metabolically stable\textsuperscript{68}. As the mechanisms regulating the Cad11/Amot interaction are not yet known, designs and screening for such a peptide would still be premature. However, considering the low complexity of the Amot middle domain where Cad11 binds, it is possible that this may be the most effective method of inhibiting the formation of the Cad11/Amot complex to prevent PCa cell migration.
Chapter X: Future Directions

This study, heretofore, has identified Amot as a functional component of the Cad11-mediated migration mechanism in PCa. Mutant constructs of Amot and Cad11 identified the domains that mediate their binding in order to confer the function of cellular migration. However, the forces that mediate and mechanisms that regulate the formation of the Cad11/Amot complex are not known. Protein folding and protein-protein interactions are sometimes mediated by hydrophobic or electrostatic interactions. Protein-protein interaction studies have shown the importance of post-translational modifications, such as phosphorylation or acetylation. Furthermore, understanding the mechanisms that mediate and regulate Cad11/Amot interaction in PCa may be applied to cancers that also express these proteins and have bone tropic tendencies.

Amot is known to interact with various proteins using hydrophobic interactions via its C-C domains. However, analysis of the Amot middle domain showed no further evidence of an amphipathic helix that could result in another C-C domain to mediate Amot/Cad11 interaction. To further study this possibility, new N-terminal mutants of Amot were generated to identify the positively charged amino acids in the middle domain that may form the Amot/Cad11 salt bridge.
Then site-directed mutagenesis can be used to delete or mutate those residues to show the importance of this salt bridge within the context of Cad11-mediated migration. Furthermore, the aspartic acid sequence expressed by Cad11 CBS will be expressed at the tail end of E-Cad to show that this sequence participates in the formation of a salt bridge to promote Amot/Cad11 interaction.

During optimization of the co-immunoprecipitation (Co-IP) assays to confirm the endogenous interaction of Cad11 and Amot, we also found that their interaction was better detected with the addition of sodium orthovanadate in the low salt lysis buffer. The addition of sodium orthovanadate to the lysis buffer suggested that phosphorylation/dephosphorylation of proteins are involved in the formation of the Cad11-Amot complex. Post-translational modifications play an important role in regulating signal transduction pathways. β-catenin tyrosine phosphorylation, for example, has been studied to release cadherin from the actin cytoskeleton resulting in cellular migration. Pervanadate treated and untreated cells prior to lysis will be used for Cad11 and Amot Co-IP experiments to study how changes in protein phosphorylation regulate their interactions. The tyrosine phosphorylation site of Amot and Cad11 will be determined by using phospho-tyrosine antibodies and mass spectrometry. These phospho-tyrosine sites will then be mutated to observe changes in Cad11 and Amot interaction as well as Cad11-mediated functions in PCa.

Development of osteoblastic lesions results in increased mortality of men with PCa. The increased osteoblast activity during bone formation results in increased PCa cell survival and growth. Relieving this aspect of disease burden can increase a PCa patient’s quality of life. To understand the role of Amot/Cad11-mediated migration in the context of metastasis, the PC3-mm2 cell lines generated for Fig. 17 will be used for intraprostatic and intracardial injections.
An intraprostatic injection will embed the luciferase tagged cells in the mouse prostate to observe invasion out of the primary site and incidence of metastasis to lymph node or even bone via bioluminescence. An intracardial injection will introduce the mutant cell lines into circulation that will allow us to study how the Amot/Cad11 interaction may contribute to PCa extravasation into the bone microenvironment. Understanding the role of the Amot-Cad11 interaction in cell migration as well as the regulatory mechanisms that govern it, may aid in the development of treatments to inhibit their interaction and perhaps prevent PCa metastasis to bone. Furthermore, independent studies of Cad11^{67, 97} and Amot^{51, 100} have shown their increased expression have been correlated with BCa progression. It is, therefore, possible that the Cad11-Amot interaction proposed in this study can be applied to BCa and perhaps other bone tropic cancers.
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Vita

Angélica Ortiz was born in Jamaica, New York on April 3, 1987, the daughter of Salvadora Angélica Gomez de Ortiz and Israel Villalobos Ortiz. After completing the science track in Asa Philip Randolph Campus High School, New York, New York in 2000, she entered Columbia University in New York, New York. She received her degree in Bachelor of Arts with a major in English and Comparative Literature with a concentration in Pre-Medical Studies in May, 2004. She then moved to London, England to attend Royal Holloway, University of London where she earned her Master of Arts in Medieval Studies in September, 2005 while working as a research assistant at the University College of London Ophthalmic Institute. She then worked several years in the Department of Molecular Pharmacology at McLean Hospital a Harvard University Affiliate in Belmont, Massachusetts. In May 2009, she entered The University of Texas Graduate School of Biomedical Sciences at Houston.

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