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PROFESSIONAL PUBLICATIONS

Balzer, E.M., Whipple, R.A., Thompson, K., Boggs, A.E., Slovic, J., Cho, E.H., Matrone, M.A., Yoneda, T., Mueller, S.C., Martin, S.S. (2010) c-Src differentially regulates the functions of microtentacles and invadopodia. Oncogene 29, 6402–6408.

Balzer, E.M., Tong, Z., Paul, C.D., Hung, W.C., Stroka, K.M., Boggs, A.E., Martin, S.S., Konstantopoulos, K. (2012) Physical confinement alters tumor cell adhesion and migration phenotypes. FASEB Journal 26, 4045-4056.

Vitolo, M.I., Boggs, A.E., Whipple, R.A., Yoon, J.R., Thompson, K., Matrone, M.A., Cho, E.H., Balzer, E.M., Martin, S.S. (2013) Loss of PTEN induces microtentacles through PI3K-independent activation of cofilin. Oncogene 32, 2200-2210.

Whipple, R.A., Vitolo, M.I., Boggs, A.E., Charpentier, M.S., Thompson, K., Martin, S.S. (2013) Parthenolide and costunolide reduce microtentacles and tumor cell attachment by selectively targeting detyrosinated tubulin independent from NF-κB inhibition.
Breast Cancer Research. 15:R83.

Charpentier, M.S., Whipple, R.A., Vitolo, M.I., Boggs, A.E., Slovic, J., Thompson, K.N., Bhandary, L., Martin, S.S.. (2014) Curcumin Targets Breast Cancer Stem-like Cells with Microtentacles That Persist in Mammospheres and Promote Reattachment. Cancer Research 74(4):1250-60.

Boggs, A.E., Vitolo, M.I., Whipple, R.A., Charpentier, M.S., Ioffe, O.B., Tuttle, K.C., Goloubeva, O.G., Lu, Y., Mills, G.B., Martin, S.S.. (2014) Metastatic and basal-like breast tumors exhibit elevated α-tubulin acetylation that promotes microtentacles, tumor cell reattachment, and migration. *Under Review at Cancer Research.*

INVITED TALKS

Boggs, A.E. *Investigating the role of tau-induced cytoskeletal alterations in microtentacle formation and breast cancer cell re-attachment.* Invited by Dr. Zev J. Gartner, Assistant Professor, University of California, San Francisco, February 2012.

Boggs, A.E. *Investigating the role of α-tubulin acetylation in microtentacle formation and reattachment of breast tumor cells.* Presented at the Third Annual Cancer Biology Research Retreat, Baltimore, MD, May 2013. Abstract was blindly selected for a pre-doctoral presentation.

PROFFERED COMMUNICATIONS / PRESENTATIONS

Boggs, A.E., Vitolo, M.I., Whipple-Bettes, R.A., Balzer, E.M., Yoon, J.R., and Martin, S.S. *Targeting activation of non-muscle myosin II to prevent breast cancer metastasis.* Poster Presentation at First Annual Cancer Biology Research Retreat, Baltimore, MD, May 2010.

Boggs, A.E. and Chabasse, C. *Co-Chair: Pre-doctoral Oral Session*. The First Annual Cancer Biology Research Retreat, Baltimore, MD, May 2010.

Boggs, A.E. and Martin, S.S. *Targeting cortical contractility to prevent breast cancer metastasis: Inhibiting activation of non-muscle myosin II*. Abstract Submitted to NCI-UMD Partnership for Cancer Technology Workshop, Bethesda, MD, June 2010.

Vitolo, M.I., Boggs, A.E., Thompson, K., Whipple, R.A., Yoon, J.R., Slovic, J., Charpentier, M.S., Matrone, M.A., and Martin, S.S. *PTEN loss disrupts the actin cortex to promote tubulin microtentacle formation and increased metastatic potential.* Poster Presentation at the Annual American Association for Cancer Research Meeting, Orlando, FL, April 2011.

Boggs, A.E., Vitolo, M.I., Whipple-Bettes, R.A., Matrone, M.A., Slovic, J., Charpentier, M.S., Yoon, J.R., and Martin, S.S. *Tau induced α-tubulin acetylation in microtentacle formation and breast cancer cell attachment.* Poster Presentation at the Second Annual Cancer Biology Research Retreat, Baltimore, MD, May 2011.

Boggs, A.E., Vitolo, M.I., Whipple-Bettes, R.A., Slovic, J., Charpentier, M.S., and Martin, S.S. *Investigating the role of a-tubulin acetylation in microtentacle formation and re-attachment of suspended breast tumor cells.* Poster Presentation at the Third Annual Cancer Biology Research Retreat, Baltimore, MD, May 2012.

Boggs, A.E., Vitolo, M.I., Whipple-Bettes, R.A., Slovic, J., Charpentier, M.S., and Martin, S.S. *Acetylation of α-tubulin contributes to microtentacle formation and re-attachment in suspended breast tumor cells.** Poster Presentation at the Annual American Association for Cancer Research Meeting, Washington, D.C., April 2013.
*Abstract selected for Women in Cancer Research Scholar in Training Award.

PROFESSIONAL POSITIONS HELD

Undergraduate Research Assistant 2006-2008
Villanova University, Department of Biology: Villanova, PA
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Laboratory Intern 2007-2008
Fox Chase Cancer Center: Philadelphia, PA
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ABSTRACT

Title of Dissertation: Acetylation of α-tubulin and its Role in Aggressive and Metastatic Breast Cancer

Amanda E. Boggs, Doctor of Philosophy, 2014

Dissertation Directed by: Stuart S. Martin, Ph.D., Associate Professor of Physiology

Metastatic breast cancer presents a therapeutic challenge, since existing treatments largely target primary tumor growth instead of metastatic spread. Because metastasis is the leading cause of breast cancer-related death, we must investigate new targets for the treatment of disseminated disease. This study describes a novel role for α-tubulin acetylation in metastatic breast cancer. We report that metastatic breast cancer cells have high α-tubulin acetylation that is maintained under suspended conditions and extends along microtentacles, tubulin-based protrusions that promote cell-cell and cell-substrate reattachment. Mutation of the acetylation site on α-tubulin and enzymatic modulation of this post-translational modification has a significant impact on microtentacle frequency and suspended tumor cell reattachment. Reducing α-tubulin acetylation in metastatic breast tumor cell lines also significantly inhibits migration, but does not affect proliferation. Investigating the translational importance of this modification in over 140 breast cancer patients' matched primary and metastatic tumors, we find that α-tubulin acetylation is maintained and in many cases increased in nodal metastases, relative to the primary tumor. We also discover a strong correlation between α-tubulin acetylation and the aggressive basal-like breast cancer subtype in a large cohort

of breast cancer patients. These data suggest α-tubulin acetylation may promote a more metastatic phenotype through its effects on reattachment and migration while serving as a marker of an aggressive breast cancer subtype.

Acetylation of α-tubulin and its Role in Aggressive and Metastatic Breast Cancer

by
Amanda E. Boggs

Dissertation submitted to the Faculty of the Graduate School of the
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Doctor of Philosophy
2014

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MomMom and PopPop for being my biggest cheerleaders. I owe everything I am and everything I will become to all of you. I am eternally grateful for everything you have done to support me and for your unconditional love through it all. I love you. Thank you.

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DCIS: Ductal Carcinoma in Situ

LCIS: Lobular Carcinoma in Situ

TNM: Tumor Size, Nodal Status, Metastasis

HER2: Human Epidermal Growth Factor Receptor 2

SCMGENE: Subtype Classification Model (utilizing three genes)

PAM50: Prediction Analysis of Microarray

qRT-PCR: quantitative Reverse Transcriptase-Polymerase Chain Reaction

ER: Estrogen Receptor

PR: Progesterone Receptor

IHC: Immunohistochemistry

CTC: Circulating Tumor Cells

EMT: Epithelial-to-Mesenchymal Transition

ECM: Extracellular Matrix

McTNs: Microtentacles

MAP: Microtubule Associated Protein

PTM: Post-translational Modifications

TCP: Tubulin Carboxypeptidase

TTL: Tubulin Tyrosine Ligase

HDAC6: Histone Deacetylase 6

SIRT2: Sirtuin 2

HAT: Histone Acetyltransferase

MEC-17: Mechanosensory abnormality 17

αTAT1: α-tubulin N-acetyltransferase 1

FA: Focal Adhesions

EGF: Epidermal Growth Factor

BM: Basement Membrane

GFP: Green Fluorescent Protein

K40R: Lysine (K) 40 to Arginine (R) mutant

CIM-plate: Cellular Invasion/Migration-plate

TCGA: The Cancer Genome Atlas

RPPA: Reverse Phase Protein Array

IDC: Invasive Ductal Carcinoma

ILC: Invasive Lobular Carcinoma

CHAPTER 1: INTRODUCTION

Breast Cancer

Breast cancer is the most commonly diagnosed cancer among women. In 2013, an estimated 232,340 new cases of invasive breast cancer were diagnosed and about 40,000 deaths were attributed to this disease in the United States (2, 11). Among women world-wide, breast cancer is the main cause of cancer-related mortality (2). Breast cancer is a complex and heterogeneous disease in which each patient's tumor contains genetic and epigenetic alterations that makes treating each patient difficult (12). Despite a number of clinical challenges, we must continue to study this disease so that mortality rates can be lowered through new or improved targeted therapies.

The normal breast contains lobules and ducts that are lined with epithelial cells separated from surrounding tissue by a basement membrane (13). If abnormal cells have grown but not breached the basement membrane, this is considered ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS) (Figure 1), depending on the cellular

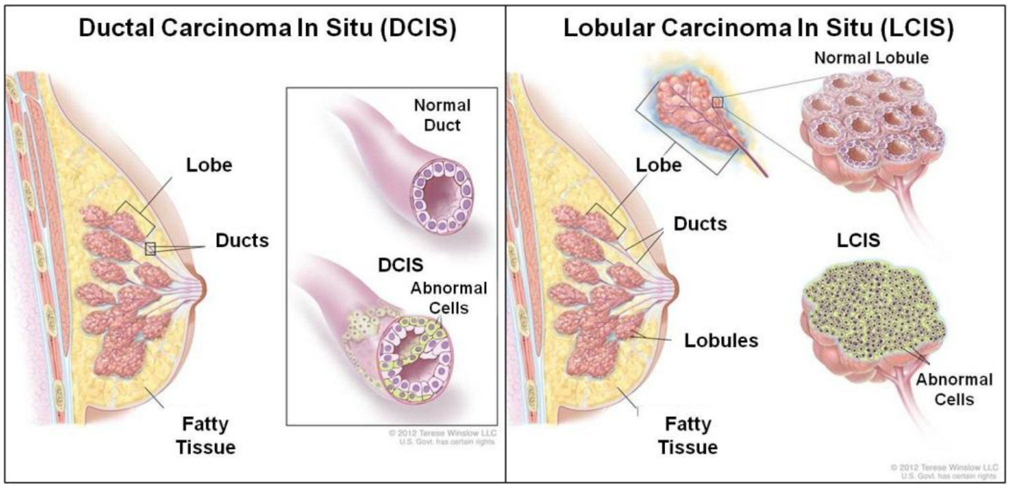

Figure 1: Ductal and lobular carcinoma in situ. Epithelial cells in the ducts or lobules of the breast can become transformed. If abnormal cells have grown but not invaded the basement membrane, this is considered ductal (left) or lobular (right) carcinoma in situ. In situ carcinoma may or may not develop into invasive cancer. Image modified from: http://www.cancer.gov

origin. DCIS and LCIS are noninvasive forms of breast cancer that may or may not develop into invasive cancer (11). If abnormal epithelial cells grow beyond the confines of the basement membrane, this is considered invasive carcinoma. Invasive breast cancer most commonly originates from the ducts or lobules and are considered ductal or lobular, respectively (11). Other less common histopathologic classifications of invasive breast cancer include, but are not limited to: medullary, neuroendocrine, tubular, apocrine, metaplastic, mucinous, sebacious, and micropapillary (13, 14). Each form of breast cancer presents a unique set of clinical challenges.

Breast cancers are initially staged to help determine patient prognosis. The TNM classification system is most commonly used to stage the tumor by size (T), lymphatic spread (N), and presence of distant metastases (M) (11). Stages are then assigned according to the aforementioned parameters. Stage 0 signifies in situ carcinoma. Stage I is localized invasive cancer, meaning it has not yet left the breast but has invaded the breast tissue. Stages II-III encompass larger primary tumors or those that have spread to regional lymph nodes. Stage IV signifies metastatic disease, indicating disseminated breast cancer cells have grown into a secondary tumor at a distant site (11).

Along with tumor size and lymph node positivity, histological grade also helps determine patient prognosis (15). Once a tumor sample has been taken, a pathologist studies the sample to determine the degree of cellular differentiation. There are three major morphological features examined to determine tumor grade: the extent of tubule/gland formation, nuclear pleomorphism, and mitotic count (15, 16). A value of 1-3 is given for each of these three features, with higher combined scores indicating a less differentiated tumor and poorer patient prognosis (17). A high percentage of tubule or gland formation is indicative of a well-differentiated tumor, since it more closely resembles the normal breast and would receive a low score. The size and shape of nuclei (nuclear pleomorphism) as well as a high mitotic count can indicate aberrant cell division, and thus, a less differentiated tumor with a high score and poorer patient prognosis (18).

Breast Cancer Subtypes

Patient treatment decisions are based on both clinical presentation, like TNM staging and tumor grade, as well as molecular classification. There are four main breast cancer subtypes: luminal A, luminal B, human epidermal growth factor receptor 2 (HER2+), and basal-like (11). These subtypes help determine if specific targeted therapies can be used and are linked to patient survival rates and specific sites of metastatic spread. While the eventual site of metastatic recurrence could be due to many factors, subtype-specific activation of pathways could promote metastatic homing to a particular microenvironment (13).

Molecular subtyping can be accomplished by using a number of classification models. One such model, SCMGENE, is a Subtype Classification Model utilizing three genes: estrogen receptor, HER2, and Aurora Kinase A (implicated in mitosis) (19). The PAM50 assay (Prediction Analysis of Microarray) is another model using a 50 gene signature that can be analyzed utilizing qRT-PCR (quantitative Reverse Transcriptase-Polymerase Chain Reaction) (20). Each of these subtyping methods has utility in determining patient prognosis, but the PAM50 method has also been used to predict treatment response as well as highlight the biological diversity of breast cancer with a larger gene set (21).

Luminal A and B subtypes are estrogen receptor (ER) and progesterone receptor (PR) positive and account for the majority of breast cancer diagnoses (Figure 2). Luminal A and B tumors also tend to express luminal cytokeratins and genes activated by the estrogen receptor (22). Patients diagnosed with the luminal A subtype respond best to endocrine therapies, have the best overall prognosis, and present the lowest risk of metastatic spread (23). The major differences between the two luminal subtypes are that luminal B cancers have lower expression of ER-regulated genes, higher expression of genes associated with proliferation, and tend to have poorer prognosis than luminal A tumors (22). Bone is the most common site of metastasis for both luminal A and luminal B subtype tumors (23).

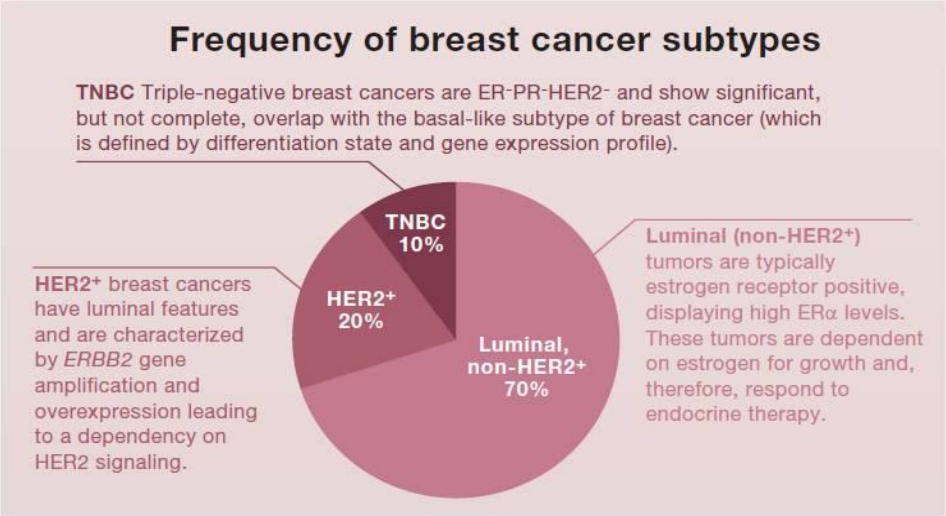

Figure 2: The four major breast cancer subtypes by frequency. The luminal (A and B) subtypes account for the majority of breast cancer diagnoses. These cancers respond best to anti-estrogen therapies. HER2+ cancers are dependent on HER2 signaling which presents a therapeutic target for management of this subtype, like the anti-HER2 monoclonal antibody trastuzumab. Many basal-like breast cancers are triple-negative, meaning they lack ER/PR/HER2. However, not all triple negative cancers molecularly classify as basal-like. Figure source: (2)

Roughly 20% of breast cancers diagnosed overexpress HER2 (Figure 2) (24). These tumors are found to be positive for HER2 via immunohistochemistry (IHC) and/or are found to have ERBB2 (encodes HER2) gene amplification by a technique called Fluorescence In Situ Hybridization (FISH) (13). Prior to targeted therapies, prognosis of HER2+ patients was poor, since HER2 signaling can affect many survival and differentiation pathways. However, recent introduction of targeted therapies like trastuzumab, a HER2-targeted monoclonal antibody, and lapatinib, a small molecule inhibitor of tyrosine kinases, have greatly increased patient survival rates (25). Although breast tumor cells can disseminate to many distant sites, HER2+ tumors preferentially metastasize to the brain (23, 24). Unfortunately, targeted therapies against HER2 cannot cross the blood-brain barrier and thus, are currently unable to affect the most commonly metastasized site (24).

Patients diagnosed with basal-like breast cancer are usually lacking expression of ER, PR and HER2-regulated genes (considered triple-negative) and have the poorest clinical outcome (26). Basal-like breast tumors often overexpress epidermal growth factor receptor (EGFR) and basal cytokeratins (CK) 5, 6, 14, and 17 (27). It is important to note that 70-80% of basal-like breast cancers are triple-negative, however, not all triple-negative cancers molecularly classify as "basal" and can fall under other subtypes (Figure 2) (23, 26). "Triple-negative" is a type of tumor that lacks expression of ER and PR, and does not show amplification of HER2. The basal-like subtype is molecularly determined by several different methods (like SCMGENE and PAM50), but is generally classified by overexpression of genes like EGFR and basal-specific cytokeratins (27).

Despite the fact that there are a number of overexpressed proteins associated with basal-like breast cancer, there is still little consensus on positive biomarkers. Basal-like tumors are highly proliferative, have high rates of metastatic spread, and tend to metastasize to the lung, bone, and distant lymph nodes (23, 27). Unlike ER+ or HER2+ cancers, there are currently no targeted therapies for the basal-like subtype. A better understanding of positive markers for this subtype will aid in more successful treatment of patients with basal-like tumors to manage or prevent metastatic disease.

Metastasis

Advances in detection and treatment over the last few decades have greatly increased survival rates of patients diagnosed with localized or regional breast cancer. The 5-year survival estimates are around 98-99% for patients diagnosed with localized disease, meaning the cancer has not left the breast (Figure 3). When the cancer has

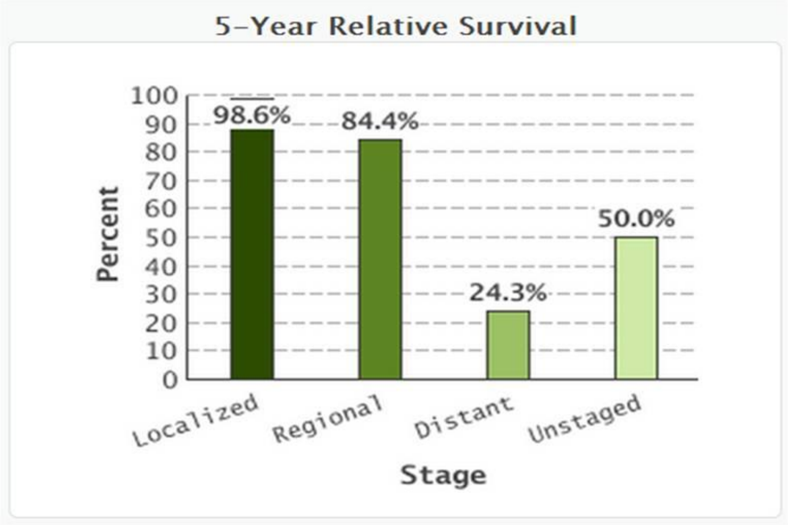

Figure 3: 5-year survival of breast cancer patients by tumor stage. Localized and regional breast cancer have high 5-year survival rates, possibly due to major advancements made over the last decades in detection and treatment. However, when breast cancers are diagnosed as "distant", or positive for metastatic disease, survival rates fall to around 24%. Image source: http://seer.cancer.gov

become "regional" and has spread to local lymph nodes, the 5-year survival rates are around 84% (11, 28). However, for women diagnosed with metastatic breast cancer, cancer that has left the breast and grown out at a distant site, survival rates drop dramatically (Figure 3). 5-year relative survival of patients diagnosed with metastatic or distant disease falls to around 24% (28). Because most breast cancer deaths are due to secondary disease, finding targets to treat or prevent metastasis is an important therapeutic priority.

Metastatic dissemination itself is a complex yet inefficient process. Animal models have suggested that only 0.01% of cancer cells that leave the primary tumor site can form distant metastases (1). Primary tumors in patients can also shed millions of cells into the bloodstream that never develop into overt disseminated disease (1, 29-31). Despite this, a significant percentage of breast cancer patients develop metastatic disease (32) and it is still responsible for approximately 90% of cancer-associated deaths (29). Another clinical challenge is that many breast cancers can form detectable metastases decades after removal of the primary tumor (31). A better understanding of how and why metastases occur may elucidate new opportunities for therapeutic intervention.

In order for a primary tumor to metastasize, cancer cells must leave the breast and enter the blood or lymphatic system. Once detached, these circulating tumor cells (CTCs) attach and/or arrest at secondary sites before extravasation and metastatic outgrowth. Micrometastases must then survive and grow out at a secondary site, possibly overcoming dormancy (1, 31). Although the aforementioned cascade is a greatly simplified model of metastasis, each one of these steps has the potential to become a therapeutic target in preventing or treating disseminated disease (outlined in Figure 4).

The specific processes of CTC reattachment from circulation, migration, and invasion will be examined in further detail.

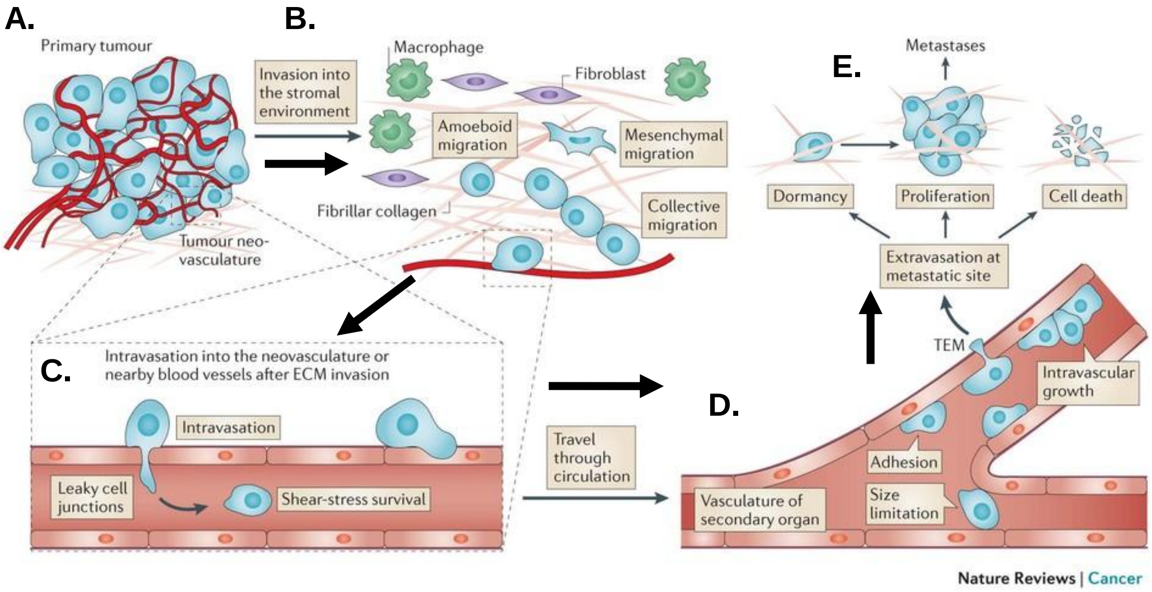

Figure 4: The metastatic cascade. (A) A primary tumor recruits new blood vessel formation (neovasculature) to enhance tumor growth. (B) Once this tumor grows beyond the confines of the basement membrane, it can invade into the local stroma where a number of different cell types can promote migratory capability. (C) After initial invasion into the surrounding tissue, cells can enter the bloodstream, circulate throughout the body, and survive a number of factors. (D) Disseminated tumor cells must then attach and/or arrest at secondary sites before extravasation. (E) Even after tumor cells arrest at a secondary site, they must overcome dormancy and cell death to proliferate and form detectable metastases.
Figure modified from: (1)

Migration and invasion are necessary steps for an individual tumor cell or group of tumor cells to leave the breast as well as enter and exit the blood or lymphatic system. Cancer cell migration is typically classified as mesenchymal (elongated) or amoeboid (rounded) (Figure 4B), although tumor cells can switch between these migratory methods depending on the local microenvironment (1). Intravasation, the invasion of tumor cells from the primary tumor towards/into blood vessels (Figure 4C), can be facilitated by immature blood vessels within the tumor (1, 33). This occurs because tumors induce localized angiogenesis (blood vessel formation) to grow and survive. Blood vessels in the tumor are usually quickly formed and have weak cell-cell junctions through which cancer cells can enter the bloodstream (1). The process of intravasation could also utilize a complex coordination of the cytoskeleton for migration and proteases to disrupt endothelial junctions and remodel the stroma (1, 31, 33). The epithelial-to-mesenchymal transition (EMT) of primary tumor cells can also be involved in cancer cell motility and invasion (34). An EMT promotes tumor cell dissemination through the loss of epithelial markers that could prevent improper growth and invasion. With a gain of mesenchymal markers that do not require proper cell-cell contacts, an EMT can also promote cellular movement and tumor dissemination (35). The role EMT plays in cancer cell dissemination and CTC character is developing into a very active area of investigation (35-38).

Once primary tumor cells leave the breast, they enter a harsh environment within the microvasculature (Figure 4C, D). Many epithelial cells will be ruptured due to the small diameter of capillary beds and blood-flow shear forces. CTCs must also avoid targeting from the immune system, as well as anoikis, death due to detachment (29). It is suggested that CTCs with specific traits can better evade the immune system and survive deformability within circulation (29, 33). This process could be aided by cell-cell contacts between tumor cells and/or other host cells, as well as a more deformable cytoskeleton to squeeze through small capillaries (29).

Detached and circulating tumor cells must reattach or arrest at distant sites before they are able to extravasate (Figure 4D). It is unclear whether initial arrest/adhesion is a passive or active process, but most likely, both occur in breast cancer patients. As stated before, capillary beds have very small diameters that can trap circulating cells within minutes of dissemination (39). Since the lung capillaries are the first small-diameter blood vessels to be encountered by exiting breast tumor cells, physical restriction could keep these cells in one site long enough to extravasate into the lung (29, 39). However, breast cancer can still metastasize to more distant areas and extravasate through large diameter blood vessels, so selective reattachment and adhesion of tumor cells to the vasculature must also depend on other factors (1).

Extravasation, movement out of circulation and towards a secondary site (Figure 4D, E), requires similar cytoskeletal coordination as intravasation. Specifically, extravasation requires the formation of cytoskeleton-based protrusive structures to invade cellular junctions and promote degradation of the extracellular matrix (ECM) (9). The site of extravasation can be dependent upon a number of things, but secreted factors have been found to aid in recruitment of cancer cells to a distant site (29, 40). Chemokines like CXC-chemokine ligand 12 (CXCL12) and its chemokine receptor CXCR4 have been frequently implicated in breast cancer extravasation and metastasis (41, 42) while CXCLs 9, 10, 11 and the corresponding receptor CXCR3 are linked to poor patient prognosis and metastatic colonization of the lung (43). Secreted chemokines from distant organs can help attract tumor cells that express the appropriate chemokine receptors. This can promote extravasation, migration, and re-adhesion of tumor cells to a distant site (1, 44).

Each one of the aforementioned steps could present a therapeutic target for treating or preventing disseminated disease. We will begin our investigation by focusing first on the transit of disseminated and circulating tumor cells from the primary tumor to the distant metastatic site.

Circulating Tumor Cells

CTCs are powerful instruments for breast cancer research (45). Clinically, it is far more feasible to collect a blood sample from a breast cancer patient than biopsy multiple tissues for solitary, disseminated tumor cells. Once isolated, CTCs could undergo a number of analyses, like genomic sequencing or immunofluorescence, to help characterize disseminated cells (Figure 5) (8). For example, studies have suggested specific cell-surface markers on CTCs could help predict disease-free survival in patients (38, 46). Since CTCs have already left the primary tumor, they may present

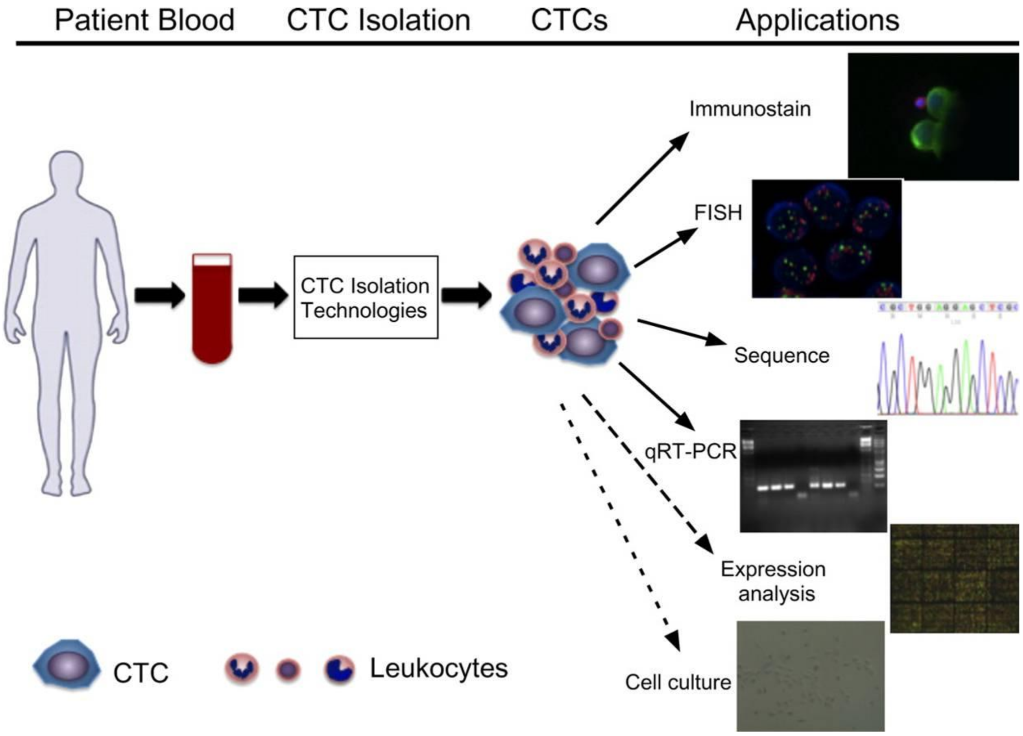

Figure 5: Applications of CTCs in cancer research. Circulating tumor cells (CTCs) can be isolated from a patient's blood and separated from other circulating cell types (like leukocytes). Once CTCs are isolated, there are many ways to study these cells to gain a better understanding of disseminated disease. Cells can be stained for certain prognostic markers (immunostain) and can undergo Fluorescence In Situ Hybridization (FISH) to determine if genomic amplification or translocation has occurred. CTCs could also be sequenced to investigate DNA mutations or undergo qRT-PCR to determine mRNA changes that could affect tumor growth. The hope is that CTCs would provide a certain expression profile that could aid in diagnosis and treatment of these patients. Figure source: (8)

characteristics that could aid in a better understanding of factors influencing metastatic spread and possibly elucidate therapeutic targets (45).

CTCs in a patient's blood have been linked to patient prognosis, treatment efficacy, and the potential for metastatic progression (47). A threshold of 5 or more CTCs in a breast cancer patient's blood sample is correlated with increased risk of metastasis as well as reduced disease-free and overall survival (48-50). Other studies have suggested CTCs can be used as a predictor of therapeutic response. An increase of CTCs during systemic chemotherapy could suggest a treatment is not effective, while a decrease in CTCs could indicate a positive therapeutic response (51-54). A very recent study has shown CTCs in the blood only 3-5 weeks after the start of chemotherapeutic treatment can distinguish patients with treatment-sensitive disease from those with treatment-resistant disease (50). This could speed up the process of selecting therapies that work best for individual patients, instead of waiting longer periods of time and monitoring a patient while the treatment is ineffective.

CTCs can aggregate with other tumor cells as well as other cell types to promote cell survival in circulation (29). As stated previously, the vasculature is a harsh environment for CTCs since they can be targeted for destruction by the immune system or can burst in small diameter capillaries due to mechanical forces (1). It is believed that CTC aggregation can abrogate anoikis (cell death due to detachment from the basement membrane) and aid in resistance to the blood flow shear forces in circulation, since CTC clusters promote cell-cell contacts and help protect individual cells from shearing (8). It is also suggested that clustering with other cell types in circulation, like platelets, can "hide" tumor cells from the immune system while in transit in the vasculature (55).

Adhesion of CTCs to the microvasculature in an *in vivo* metastasis model has been shown to be dependent upon the integrity of the microtubule network (56). Additional studies have shown suspended cell aggregation and reattachment is dependent upon microtubule-based structures and is inhibited by microtubule depolymerizing agents (6, 7). It is believed that microtubule-based membrane protrusions formed upon suspension, termed microtentacles (McTNs), promote cell-cell and cell-substrate attachment (6). This highlights a potential therapeutic target to prevent CTCs from adhering to other CTCs or the endothelial layer in circulation (7, 56-59).

Microtentacles

Breast tumor cells produce long and dynamic microtubule-based membrane protrusions, termed microtentacles (McTNs), upon detachment (Figure 6) (6, 7, 60, 61). These protrusions were originally thought to be actin-based, similar to well-known protrusive structures like podosomes and invadopodia. However, early studies found that treatment with an actin depolymerizing agent, Cytochalasin D, as well as treatment with

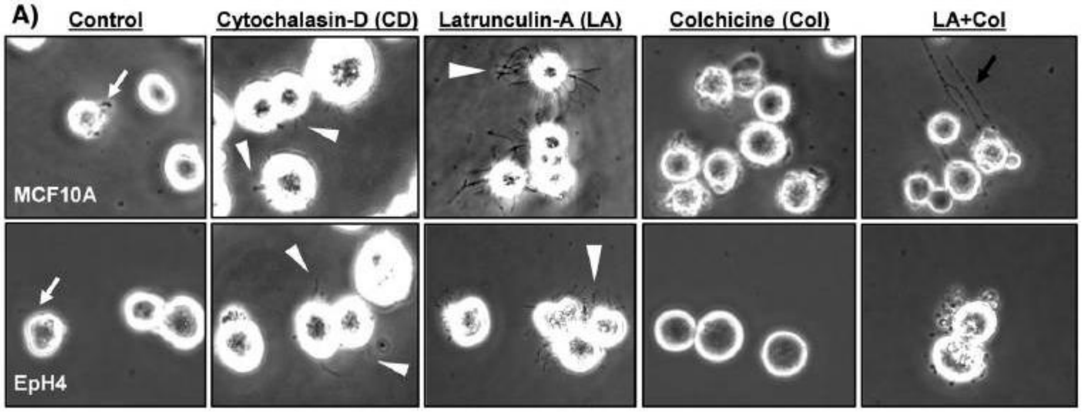

Figure 6: Microtentacles (McTNs). McTNs are tubulin-based structures formed upon cell suspension. When cells were treated with actin depolymerizing or destabilizing agents (CD and LA), McTNs were enhanced, suggesting these structures were not actin-based. When cells were treated with a tubulin depolymerizing agent (Col), McTNs were dramatically reduced. Treatment with both agents greatly disrupted these structures. Figure source: (6)

the actin disrupting agent Latrunculin A, actually enhanced the frequency of protrusions in suspended cells (Figure 6) (6). When suspended cells were treated with the microtubule depolymerizing agent, Colchicine, these cellular protrusions were greatly reduced, indicating that these protrusions were actually microtubule-based (6). Data support a model in which McTNs are generated when the physical force generated by outwardly expanding microtubules overcomes the contractile force of the actin cortex underlying the plasma membrane (Figure 7A) (7, 60).

It is thought that McTN formation may be a normal response to detachment for epithelial cells, since cell-cell and cell-substrate contacts are disrupted. However, it is important to note that invasive breast tumor cells produce significantly higher frequencies of McTNs compared to non-invasive cell lines (62). It is suggested that more metastatic

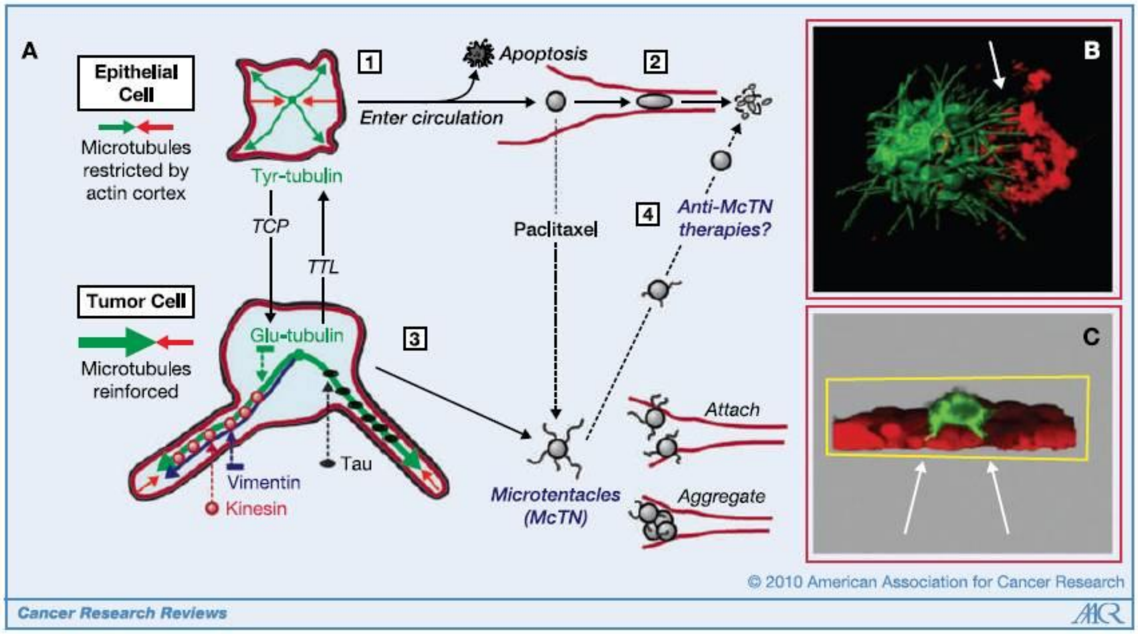

Figure 7: Microtentacles (McTNs) and suspended cell reattachment. (A) McTNs are formed when the outward force of expanding microtubules overcomes the inward force of the actin cortex. Tubulin in McTNs can be further stabilized by post-translational modifications (glu-tubulin), or binding to microtubule associated proteins like tau or kinesins/vimentin. McTNs promote both (B) cell-cell and (C) cell-substrate aggregation to enhance reattachment efficiency of suspended cells. Figure source: (7)

breast tumor cells with higher McTN frequency can more efficiently reattach to other CTCs (61). These protrusions encircle adjacent cells to promote cell-cell aggregation and clustering (Figure 7B) (7). It has been shown that tumor cell lines that can cluster efficiently metastasize at a higher frequency than those that cannot (1, 29). As previously noted, CTC aggregates may enhance the survival of disseminated cells and help promote trapping and eventual extravasation at a distant site. This aggregation could be either homotypic (other CTCs) or heterotypic (platelets, white blood cells) that could aid in evading the immune system or promoting motility out of the bloodstream (7).

McTNs can also facilitate reattachment of tumor cells to an extracellular matrix and endothelial monolayer (Figure 7C) (6, 61, 62). In experimental metastasis assays in which luciferase-expressing breast tumor cells were injected into the tail vein of mice, it was found that cells with higher frequencies of McTNs were retained more efficiently in the lung (59, 63). It is hypothesized that McTNs promote the initial reattachment of CTCs in the bloodstream prior to extravasation since McTN structures can penetrate an endothelial monolayer (7).

Cytoskeletal Components of McTNs

The cytoskeleton, composed of actin microfilaments, microtubules, and intermediate filaments, plays a vital role in McTN formation and function (7). Although the focus of subsequent studies will be on the microtubule network, a brief background of other contributing cytoskeletal elements must be highlighted.

Actin in its monomeric form (G-actin) is expressed in all eukaryotic cells. When actin monomers bind ATP, actin can polymerize into filamentous actin (F-actin) (64). An actin filament consists of two parallel protofilaments that wrap around each other to create structures with distinct structural polarity (65, 66). Actin can be found throughout the cell but concentrates beneath the plasma membrane in a network of filaments called the actin cortex (65). Interestingly, loosening of the actin cortex is a frequent occurrence in more metastatic breast cancer cells (67). As cancer progresses, the ratio of F-actin: G-actin decreases, resulting in a weakened actin cortex (7). This could promote cellular deformability, help disseminated cells migrate through cellular junctions, and even survive the small diameter of capillary beds (68). Actin depolymerization has also been shown to enhance McTN frequency, since outwardly expanding microtubules are no longer restricted by an inward cortical contractile force (Figure 7A) (60, 61).

McTNs can be further stabilized by vimentin intermediate filaments (Figure 7A-bottom panel) (36, 60-62). Intermediate filaments are elongated molecules that, along with another monomer, form parallel coiled-coil dimers. These dimers stagger and form antiparallel tetramers that can pack together to form strong filaments that do not have specific polarity (65). Intermediate filaments help confer mechanical stability to animal cells through their interaction with sites of cell-cell and cell-matrix contact as well as other cytoskeletal elements (7, 65). Importantly, vimentin intermediate filaments have been shown to cross-link and stabilize microtubules (69). High vimentin protein levels are associated with more McTNs, while dominant-negative expression of vimentin has also been shown to decrease McTNs in highly metastatic breast cancer cell lines (62). It is hypothesized that vimentin's association with a stable form of tubulin (detyrosinated tubulin) through binding of kinesin motor proteins, supports McTN formation and function (7, 62, 70).

Microtubules are long and hollow cylinders made up of α- and β-tubulin heterodimers. The microtubule polymer is built from 13 parallel protofilaments of alternating α- and β-tubulin forming a structure with distinct polarity and a hollow lumen (Figure 8) (65). Microtubules originate from the microtubule organizing center (MTOC), also known as the centrosome. Cytoplasmic microtubules are nucleated at the centrosome and grow outward toward the cell periphery and actin cortex (65).

Polymerization of microtubules requires GTP and can be highly dynamic due to quick hydrolysis of GTP into GDP on β-tubulin (Figure 8). GDP-bound β-tubulin has a weakened binding affinity and promotes depolymerization of the microtubule polymer, a process called treadmilling (65). Treadmilling occurs when there is a constant loss of GDP-bound tubulin from the minus end (shrinking end) while it is constantly being

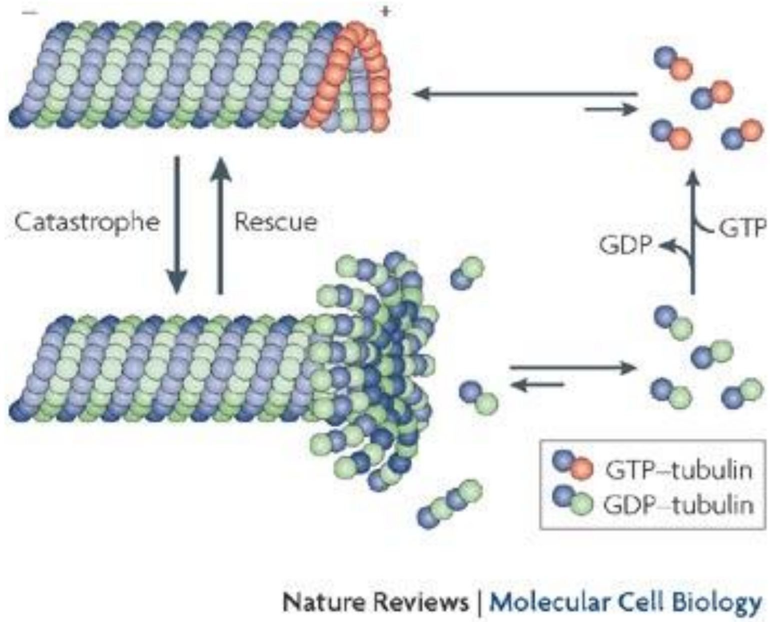

Figure 8: Microtubule polymerization and depolymerization. Microtubules are hollow cylinders that can grow (polymerize) and shrink (depolymerize) at varying rates, depending on a number of factors. Dynamic instability occurs when microtubules are actively polymerizing and depolymerizing at the same time. Rapid depolymerization is considered "catastrophe", while the reverse is considered a "rescue". Image source: (3)

replaced by GTP-bound tubulin at the plus (growing) end (71). Microtubule turnover can also be influenced by a process called dynamic instability (Figure 8). GDP-bound tubulin promotes depolymerization that is approximately 100 times faster than at the end containing GTP. A GTP "cap" can help microtubule growth while loss of the GTP cap and rapid disassembly promotes dynamic instability of the microtubule polymer (65). Despite the rapid turnover of cytoplasmic microtubules, there are many additional factors that can influence microtubule stability.

Stable microtubules are the main constituent of McTNs (6, 7). Overexpression of the microtubule associated protein (MAP) tau that stabilizes and bundles microtubules can enhance McTN frequency and reattachment of suspended cells *in vitro* and in an *in vivo* mouse model of metastasis (59). Inhibition of McTNs by microtubule-destabilizing drugs, like nocodazole, significantly reduces cell-cell and cell-substrate reattachment efficiency of a number of suspended breast tumor cell lines (6). Previous studies have also shown McTNs are enriched in a stable form of α-tubulin, known as detyrosinated α-tubulin (glu-tubulin), in which the C-terminal tyrosine is cleaved to expose a glutamic acid (69). Glu-tubulin has a half life of approximately 16h while the tyrosinated form of α-tubulin has a half-life on the order of minutes (69). This means the turnover of the microtubule polymer is much slower when α-tubulin is detyrosinated. Glu-tubulin is associated with poor patient prognosis in breast cancer (72) and high McTN frequency (6, 36). Decreasing detyrosination of α-tubulin (glu-tubulin) has been shown to decrease McTN formation and inhibit reattachment (36, 73). Detyrosination of α-tubulin will be discussed in further detail in the next section.

Post-translational Modifications of α-tubulin and McTNs

Post-translational modifications (PTMs) of α-tubulin can control diverse microtubule functions like trafficking and cellular tensegrity (4, 74), but we are only beginning to uncover the many functions that could impact cancer progression and metastasis. PTMs of α-tubulin include polyglyclation, polyglutamylation, detyrosination, generation of Δ2 tubulin, and acetylation (Figure 9) (4). Polyglutamylation, detyrosination, and acetylation of α-tubulin are enriched at the mitotic spindle during cell division and are also thought to aid in cytokinesis (4). Polyglyclation mainly occurs in ciliated cell types (75). Polyglutamylation is another poly-modification that can occur on both α- and β-tubulin in axonemes, centrioles, and basal bodies, but very rarely occurs on microtubules in the mitotic spindle or in the cytoplasm (76).

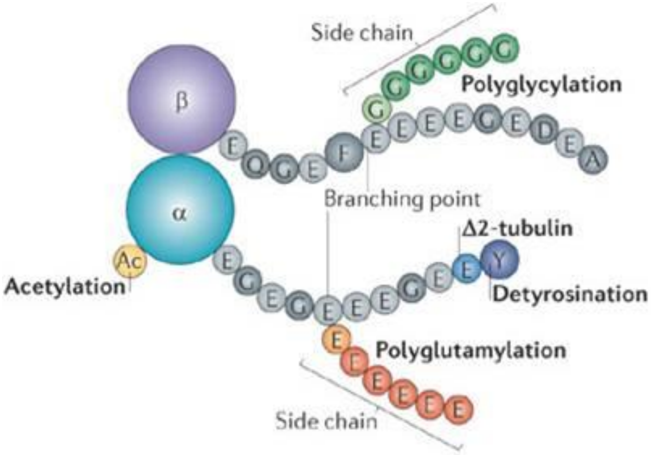

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Figure 9: Post-translational modifications of tubulin. α and β-tubulin can undergo polyglyclation and polyglutamylation, modifications that could affect microtubule function in ciliated cell types. α-tubulin can also undergo detyrosination and subsequent generation of Δ2 tubulin by removal of C-terminal amino acids. Acetylation of α-tubulin is unique in that it occurs on the inside (lumen) of the microtubule polymer and not at the C-terminus.
Image source: (4)

As noted previously, McTNs are enriched in a detyrosinated form of α-tubulin (glu-tubulin) (6), a post-translational modification promoted by an unidentified tubulin carboxypeptidase (TCP) that cleaves the C-terminal tyrosine (Figure 9). Detyrosinated α-tubulin is a very stable form of tubulin with a long half life. This modification can be reversed by tubulin tyrosine ligase (TTL) (69). TTL re-tyrosinates soluble tubulin dimers, so most newly assembled microtubules are made of tyrosinated tubulin (4). As noted previously, tyrosinated tubulin is the least stable form of α-tubulin with a half life on the order of minutes. Detyrosinated tubulin can be further converted to Δ2 tubulin by the removal of the final C-terminal glutamic acid residue (77). This process is irreversible because TTL cannot act on Δ2 α-tubulin. The function of Δ2 tubulin is largely unknown, but it is suggested to play a role in neuronal function and differentiation, since it is a permanent modification (4).

Detyrosination is increased on long-lived microtubules, but it is unclear if this modification helps stabilize microtubules on its own (4). This modification can promote microtubule stability through its preferential association with microtubule motor proteins, like kinesin-1, that can further initiate cross-linking to vimentin intermediate filaments (69). Kinesin overexpression as well as high vimentin expression have both been previously associated with high McTN frequency (62, 70). Inducing an EMT in experimental models has also been shown to increase detyrosination of α-tubulin, which promotes McTN formation and enhances reattachment of breast tumor cells to an endothelial monolayer (36).

Detyrosination is the only α-tubulin PTM associated with microtubule stability that has been found to play a significant role in McTN formation and function (6).

However, previous studies could not establish a correlation between cancer invasiveness and detyrosination of α-tubulin (62). Other PTMs like polyglyclation and polyglutamylation could also confer microtubule stability, but are not well-known for their role in stabilizing cytoplasmic microtubules (4). Because CTC reattachment is dependent upon stable microtubules *in vivo* (56), an alternative α-tubulin PTM associated with cytoplasmic microtubule stability was investigated in the present study.

Acetylated α-tubulin

Acetylation of α-tubulin is a well known marker of stabilized microtubules that occurs on lysine 40 (K40) (4). Acetylation is a unique modification of α-tubulin, since it occurs on the luminal side (inside) of the hollow microtubule polymer (75). It is thought that minor changes in microtubule structure form large enough pores for the acetyltransferase to enter the microtubule lumen or that it must enter at the microtubule plus (growing) end (78). Acetylation only occurs on polymerized microtubules (4), but is a very common modification found on stable microtubules in a number of different cell types (75).

Acetylation of α-tubulin has been shown to not only be an indicator of stabilized microtubules, but can also further stabilize microtubule polymers (79). A recent study utilizing electron microscopy has suggested acetylation at K40 actually strengthens lateral interactions of α-tubulins by forming stable salt bridges. This promotes interactions between microtubule protofilaments and bundling. This group also found that α-tubulin acetylation maintains the appropriate protofilament number within the microtubule lattice that can also affect microtubule stability (79).

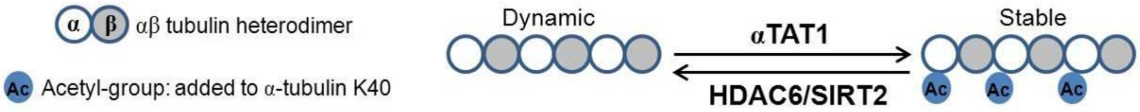

Figure 10: Acetylation of α-tubulin. Dynamic microtubules can be acetylated on lysine 40 (K40) of α-tubulin by the α-tubulin specific acetyltransferase (αTAT1). Acetylated microtubules are stabilized and play a number of roles in the cell. HDAC6 and/or SIRT2 can deacetylate α-tubulin to produce less stabilized and more dynamic microtubules.
Image credit: *Amanda Boggs-unpublished*

The enzymes responsible for α-tubulin deacetylation were discovered well before the tubulin acetyltransferase. Histone deacetylase 6 (HDAC6) and sirtuin 2 (SIRT2) have both been shown to deacetylate α-tubulin (Figure 10) (4). It has been shown that knocking down either HDAC6 or SIRT2 induces an increase in α-tubulin acetylation (80, 81), so it is believed that these two enzymes could be interdependent in the cytoplasm (75). However, a difficulty in studying these deacetylases is that HDAC6 and SIRT2 also have a number of other cytoplasmic substrates (p53, cortactin, etc.) that could contribute to a specific phenotype (82). Patient-centered studies suggest high HDAC6 levels and low acetylated α-tubulin are associated with good prognosis and increased survival of breast cancer patients (83, 84), but the mechanisms behind this correlation and the role of this PTM in metastatic breast cancer are not clear.

Acetyltransferase ARD1-NAT1 (arrest defective 1-amino terminal, α-amino acetyltransferase 1) (85) as well as the histone acetyltransferase (HAT) Gcn5 (86) can promote acetylation of α-tubulin, but tubulin is not the major substrate of these enzymes. More recently, it was shown that the main α-tubulin acetyltransferase in mammals is an orthologue of the *Caenorhabditis elegans* (*C. elegans*) protein called mechanosensory abnormality 17 (MEC-17) (87, 88). The introduction of MEC-17 promotes rapid acetylation of α-tubulin in cells with low basal levels while the loss of MEC-17 in cells with high acetylation dramatically reduces α-tubulin acetylation (87, 88). MEC-17 loss has also been shown to promote microtubule instability, impair touch receptors, and cause degeneration of axons in *C. elegans* (88, 89).

MEC-17 was recently named αTAT1 (α-tubulin N-acetyltransferase 1), the primary α-tubulin acetyltransferase that promotes α-tubulin acetylation in the cytoplasm (Figure 10) (87). αTAT1 transfers an acetate group from acetyl-coA to the ε-amino group of K40 of α-tubulin (4). In mammalian cells, overexpression of αTAT1 was sufficient to promote acetylation, but the catalytically inactive form (D157N) could not elevate α-tubulin acetylation (87). This group also found that two distinct siRNA duplexes against αTAT1 reduced αTAT1 protein and mRNA levels and also decreased acetylation of α-tubulin by more than 80% in human cells (87). This suggests αTAT1 is the major α-tubulin K40 acetyltransferase in not only *C. elegans* but also in mammalian cells (87, 90). Despite the fact that this protein is closely related to the Gcn5 family of histone acetyltransferases, αTAT1 does not have acetyltransferase activity against histones (87).

Acetylation of α-tubulin and McTNs

Previous studies have shown increases in detyrosinated α-tubulin promote McTN formation through increasing microtubule stability while inhibiting detyrosination decreases McTNs (6, 36, 73). Acetylation of α-tubulin, like detyrosination, is a marker of stabilized microtubules and could actually further stabilize the microtubule polymer (4, 79). Also, overexpression of the MAP tau promotes bundling and stabilization of microtubule polymers, increases McTNs, and promotes reattachment of suspended breast tumor cells *in vitro* and in an *in vivo* mouse model of metastasis (59). Interestingly, along with increasing McTNs and reattachment, tau overexpression has also been shown to increase acetylation of α-tubulin (91, 92). These studies indicate stabilization of the microtubule network can enhance McTN formation and function in breast tumor cell lines, but it was initially unknown if acetylation of α-tubulin could affect McTNs. Aim 1 investigates the specific role of α-tubulin acetylation in McTN formation and function.

Reattachment from suspension is only one step in the metastatic cascade that could be affected by acetylation of α-tubulin. Next, we will discuss two additional steps of the metastatic cascade that could also be affected by changes in microtubule stability, migration and invasion.

Acetylated α-tubulin and Cellular Migration

Actin-based structures like lamellipodia (sheet-like membrane ruffles filled with branched actin filaments) and filopodia (thin protrusions filled with bundled actin filaments) are most commonly studied in the process of cellular migration (Figure 11) (66, 93). Migration occurs when actin polymerization generates protrusive activity at the leading edge of a migrating cell, while actinomyosin activity at the sides and rear drive contractility at the back of the cell (94). Although actin and actin-regulating proteins play

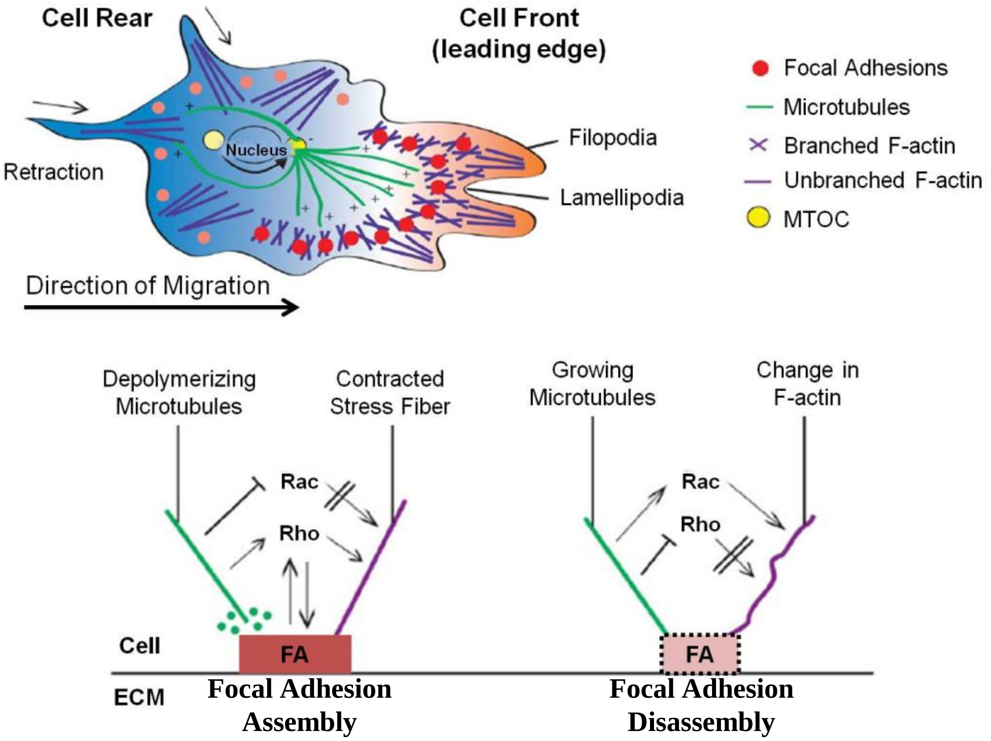

Figure 11: Migrating cells. (Top) A cartoon representation shows the complex coordination between actin and microtubules in an actively migrating cell. Arrows indicate the direction of cell movement. At the leading edge of the cell, actin and microtubules (originating from the Microtubule Organizing Center-MTOC) work together to form focal adhesions to provide areas of traction for forward movement. Stable microtubules also promote actin polymerization and formation of protrusive structures, like filopodia or lamellipodia. At the rear of the cell, these cytoskeletal elements work together to contract and remove cell-substrate contacts. (Bottom) Stabilized microtubules can affect the turnover of focal adhesions (FA) though interactions with Rac and Rho A. Disassembly of focal adhesions at the rear of the cell requires growing microtubules, while microtubule depolymerization promotes stress fiber formation and more stable adhesions. Figure modified from: (5)

a vital role in cellular motility, a crosstalk between actin filaments and the microtubule network help coordinate the major cytoskeletal changes necessary for migration (Figure 11) (5). Most specifically, it has been shown that stable microtubules help regulate actin effector proteins at the leading edge of a migrating cell and at the cell rear to promote motility (5).

At the leading edge, stabilized microtubules help activate Rac, a protein that promotes actin polymerization and growth (95). Rac activation by stable microtubules aids in the formation of actin-based protrusive structures like lamellipodia (96). At the rear of the cell, stable microtubules can also activate Rho A (97). Rho A helps regulate the formation of stress fibers, promotes contractility through contraction of nonmuscle myosin II, and stimulates disassembly of F-actin (5). Microtubule polymerization can also promote turnover of focal adhesions (stable cell-substrate contacts) to enhance forward movement (Figure 11-bottom) (5, 96).

Studies investigating acetylation of α-tubulin in the process of migration have been performed in various cell types. Acetylated α-tubulin has been shown to orient towards the wounded-edge in migrating fibroblasts (98). Overexpression of a non-acetylatable α-tubulin mutant inhibits proper migration and branching of cortical neurons (99, 100). Acetylation of α-tubulin has also been found to play an important role in regulating cell motility and uptake of external substances in keratinocyte response to skin damage (101). It is suggested that acetylated microtubules enhance trafficking of cytoskeletal regulatory proteins and organelles to the leading edge, and thus, could further promote migration (98, 102).

Although studies have been carried out in different cell types, we have very little information regarding the role of acetylated α-tubulin in migration of cancer cells. A single recent study silencing αTAT1 in the highly metastatic MDA-MB-231 breast cancer cell line found that cells depleted of αTAT1 (with decreased acetylated α-tubulin) migrated at the same velocity as control cells, but moved in less linear paths (103). This group also found that silencing αTAT1 significantly inhibited migration towards an epidermal growth factor (EGF) gradient (103). They suggested αTAT1 enhances acetylated microtubules that orient toward the leading edge of a migrating breast tumor cell to promote migration and chemotaxis (movement toward a chemical gradient) (103). More studies must be carried out to further confirm the role for acetylated α-tubulin and the tubulin acetyltransferase in migration.

On the contrary, certain studies have suggested increasing acetylated α-tubulin actually inhibits cellular migration, since knockdown or inhibition of HDAC6 decreases migratory capability in certain cell types (80, 84, 104, 105). However, it is important to note that HDAC6 also targets a number of actin-binding cytoplasmic substrates like cortactin (106), formin homology proteins, mDia1, and mDia2 (107). Acetylation of α-tubulin in the cytoplasm will increase when HDAC6 is inhibited. However, actin-polymerizing and depolymerizing proteins are also affected by a decrease in HDAC6 activity (90). Recent focus on HDAC6 and its role in cellular migration have suggested the effects on actin-binding proteins are the major cause of migration defects when HDAC6 is inhibited (108).

Acetylated α-tubulin and Invasion

There is also very little known about the role of stabilized microtubules in the process of cellular invasion. Interestingly, microtubules have been found within actin-based invasive structures called invadopodia (66). These cellular protrusions have been shown to promote matrix degradation and invasion though a basement membrane (BM) (9, 109). Intact microtubules are not necessary for initial invadopodia formation, but are necessary for invadopodia growth and elongation (Figure 12) (9, 110). It is significant to note that only stable microtubules were found within invadopodia shafts, while tyrosinated tubulin (dynamic microtubules) was restricted to the base (9). Studies have

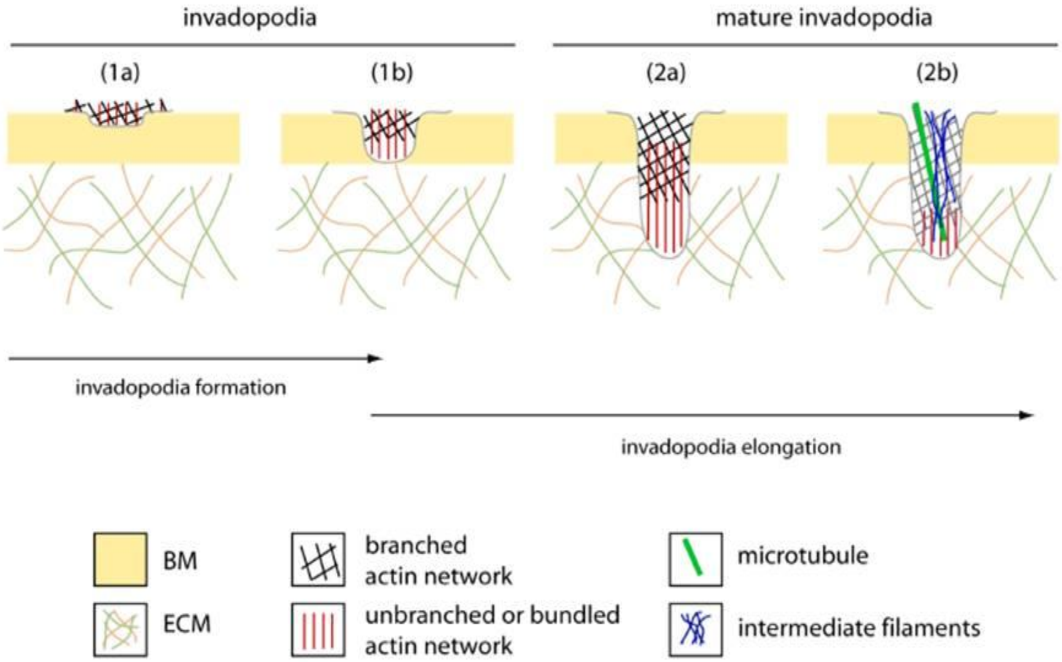

Figure 12: Microtubules and invadopodia. Invadopodia are actin-based structures formed to enhance cancer cell invasion. In early stages of invasion (1a-1b), invadopodia form and begin to degrade the basement membrane (BM). This requires a coordination of branched and unbranched actin networks as well as proteins involved in BM degradation. Invadopodia elongation or maturation requires similar actin networks; however, this is also where stabilized microtubules play an important role (2a-2b). Stable microtubules, as well as intermediate filaments, penetrate mature invadopodia to promote elongation of the structures and invasion into the surrounding extracellular matrix (ECM). Figure source: (9)

also shown that paclitaxel, a tubulin stabilizing drug, increases the number of invadopodia formed and increases gelatin degradation in head and neck small cell carcinoma (HNSCC) as well as ovarian cancer cells (111). Similar to the hypothesized role in migration, it is thought that stable microtubules provide the tracks for cargo necessary for invadopodia formation, like matrix metalloproteinases (MMPs), that aid in degradation of the basement membrane (109). Interestingly, MMP-9 expression and function is associated with increased distant metastasis risk in breast cancer patients (112), while exocytosis of this metalloproteinase has been shown to be a microtubule-dependent process that significantly affects cancer cell invasion (113).

Analogous to what is known about acetylated α-tubulin in migration, a single study has investigated breast cancer cell invasion using the metastatic MDA-MB-231 cell line (114). This group knocked down αTAT1 and decreased endogenous acetylated α-tubulin to barely detectable levels (114). When an invasion assay using a 3D collagen I matrix was carried out, αTAT1 silencing significantly inhibited invasion of MDA-MB-231 cells by 40-50% (114).

Since we have limited information as to how acetylated α-tubulin could affect metastatic progression, additional studies were carried out to further elucidate the role of acetylated α-tubulin in both processes of migration and invasion (Aim 2).

SPECIFIC AIMS AND HYPOTHESES

Rationale:

Circulating and disseminated tumor cells are believed to be the root of metastatic disease by seeding distant and recurrent metastases, the leading cause of breast cancer-related death. Current research efforts largely target tumor growth but overlook the contributions of detachment and dissemination on the metastatic process.

Microtentacles (McTNs) aid in cell-cell and cell-substrate reattachment after suspension (6, 7). These structures can be enhanced by actin depolymerization but are dependent upon microtubule stability (7, 36, 60, 61). Microtubule-associated proteins like tau (59) and the post-translational modification of α-tubulin detyrosination (6) have been shown to be enriched in and enhance the formation of McTNs. Previous studies have shown a positive correlation between McTN frequency and reattachment capability to an endothelial layer or trapping in the lungs of mice (36, 59), modeling one of the early steps in CTC retention in distant tissues (59, 63).

Despite growing understanding of the role of microtubule stability in metastasis, one α-tubulin PTM has gone largely understudied. Acetylation occurs on lysine 40 (K40) of α-tubulin by the recently identified α-tubulin acetyltransferase 1 (αTAT1) (87, 88) and is a well known marker of stable microtubules (4). New studies suggest that α-tubulin acetylation can actually increase microtubule stabilization by enhancing protofilament bundling (79, 115). Detyrosination is the only α-tubulin PTM that has been previously reported to be enriched in McTNs; however, this modification does not directly correlate with increased McTN occurrence (6). Because of this, we must examine if α-tubulin acetylation could also promote McTN formation and function. This will help us determine if acetylation of α-tubulin could be a possible target for reducing McTNs and suspended cell reattachment.

Others have shown that αTAT1 overexpression can enhance migration and invasion of a metastatic breast tumor cell line (103, 114), but very little is known about the role of acetylated α-tubulin in these metastatic processes. It is also unclear if α-tubulin acetylation could affect prognosis or overall survival of breast cancer patients. To date, there has only been one translational study investigating acetylated α-tubulin in cancer patients. This group found high acetylated α-tubulin is associated with poor patient prognosis and increased nodal metastases in squamous cell carcinoma of the head and neck (116). Along with enhancing McTNs and reattachment, tau and α-tubulin detyrosination have also been associated with poor prognosis and metastasis in breast cancer patients (72, 117). However, it is unclear if acetylation of α-tubulin could play a similar role in patient prognosis or metastatic risk.

Based on the aforementioned studies, the role of α-tubulin acetylation in a number of steps in the metastatic cascade will be further examined and the potential translational significance of this modification will be elucidated.

Overall Hypothesis: With these proposed studies, we will test the hypothesis that α-tubulin acetylation gives a metastatic advantage to breast tumor cells. We set out to test this hypothesis with the following Specific Aims:

SPECIFIC AIM 1: To define the role of α-tubulin acetylation in McTN formation and function in suspended breast tumor cells

We will determine if α-tubulin acetylation correlates with increased McTN frequency and localizes to McTNs, since previous studies have only investigated detyrosination of α-tubulin in McTNs presented on metastatic breast tumor cells (6, 7, 36, 37, 62, 73). We will then utilize genetic means to inhibit or enhance α-tubulin acetylation and examine the effects on McTN formation and function.

Hypothesis: α-tubulin acetylation will increase McTNs and reattachment

Strategy:

1.1: To evaluate McTN frequency in cell lines of increasing acetylation of α-tubulin and characterize the localization of acetylated α-tubulin within McTNs

1.2: To decrease acetylation through mutation of the acetylation site on α-tubulin in metastatic/highly acetylated cell lines and determine the effects on McTNs

1.3: To increase acetylation by overexpressing αTAT1 (tubulin acetyltransferase) in non-metastatic, low acetylated cell lines and determine the effects on McTNs

1.4: To assay reattachment efficiency of K40R and αTAT1 expressing cells to confirm McTN functionality

SPECIFIC AIM 2: To examine the effects of α-tubulin acetylation changes on proliferation, migration, and invasion

As compared to a more elucidated role in neuronal cells, the role of α-tubulin acetylation in cancer is largely unknown (4). We will utilize the genetic modifications outlined in Specific Aim 1 to investigate a number of steps in the metastatic cascade.

Proliferation, migration, and invasion are all necessary for proper metastatic dissemination and outgrowth, but it is currently unclear if the specific modification of acetylation of α-tubulin could affect these processes.

Hypothesis: Acetylated α-tubulin will promote proliferation and enhance migration and invasion

Strategy:

2.1: To investigate the effects of K40R stable expression and αTAT1 transient expression on cellular proliferation

2.2: To determine if changes in α-tubulin acetylation status affect migration in both metastatic and non-metastatic cell lines

2.3: To determine the effects of reducing α-tubulin acetylation on invasion of metastatic breast tumor cell lines

SPECIFIC AIM 3: To examine α-tubulin acetylation in breast cancer patient samples

This patient-centered aim will use immunohistochemistry of primary and matched metastatic breast tumors to determine if there are changes occurring from patient primary tumor to metastasis that could give these cells a selective advantage during the metastatic process. We will also utilize a large-scale protein array of primary breast tumors to help elucidate the translational significance of this modification for breast cancer patients, since we only know this modification correlates with patient prognosis in head and neck cancers (116).

Hypothesis: Acetylated α-tubulin will be elevated in patient metastases, as compared to the primary tumor, and associated with more aggressive breast cancer subtypes

Strategy:

3.1: To probe matched primary and metastasis patient samples for changes in acetylated α-tubulin

3.2: To utilize the Reverse Phase Protein Array to investigate acetylated α-tubulin in patient prognosis/subtype

CHAPTER 2: MATERIALS AND METHODS

Cell Culture

MCF-7, BT-20, BT-549 and Hs578T cells were obtained from American Type Culture Collection (Manassas, VA). MDA-MB-231 cells were kindly provided by Dr. X. Zhan (University of Maryland, School of Medicine). Since they were not directly purchased from American Type Culture Collection, MDA-MB-453 and MDA-MB-231 cells were authenticated by Bio-Synthesis Inc. on 23 May 2013 (Lewisville, TX). Cells were maintained at 37ºC, 5% CO_2 in Dulbecco's Modified Eagles Medium (DMEM) (CellGro), except BT-20 maintained in Eagle's Minimum Essential Medium (EMEM) (CellGro), supplemented with 10% FBS and 1% penicillin-streptomycin. Stable cell lines were also maintained in 1% Geneticin.

Plasmids and Transfections

EGFP-Tubulin.K40R (plasmid 30488, Tso-Pang Yao), EGFP-Tubulin wt (plasmid 30487, Tso-Pang Yao), and pEF5B-FRT-GFP-αTAT1 (plasmid 27099, Maxence Nachury) were obtained from Addgene. AcGFP1-C1 was obtained from Clontech.

Transient transfections were carried out with Fermentas ExGen 500 *in vivo* transfection reagent according to manufacturer's protocol (Thermo Scientific). Experiments were carried out at least 24h after transfection. Since each plasmid was tagged with GFP (green fluorescent protein), visual confirmation of transfection efficiency was always completed. Stable cell lines were selected 3 days post-transfection in 1% Geneticin. Stable pooled clones were verified after multiple passages in antibiotic selection, visually by GFP expression, and by immunoblot, as described below.

Immunoblot

Cells were plated 24h prior to immunoblot and harvested at 70-80% confluency in ice-cold radioimmunoprecipitation assay lysis buffer (RIPA) (0.5M Tris-HCl- pH 7.4, 1.5M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10mM EDTA) with 1mM phenylmethylsulfonyl fluoride (PMSF) and 1% protease inhibitor cocktail. Cell lysates were then spun at 4ºC at 14,000 RPM for 10min. Protein concentrations were determined by the Bio-Rad DC colorimetric assay (Bio-Rad). 20µg protein was separated by SDS-PAGE on 4-12% NuPage MES Bis-Tris gels (Life Technologies). Membranes were blocked with 5% milk in TBST for 1h at room temperature before an overnight incubation at 4ºC with the following primary antibodies: Acetyl-α-tubulin (Lys40) (D20G3) XP Rabbit mAb (1:1000 Cell Signaling), Anti-detyrosinated alpha tubulin antibody (1:1000 Abcam), Monoclonal Anti-α-tubulin Clone DM1A (1:5000 Sigma Aldrich), or GFP (1:5000 Santa Cruz) in 5% milk in TBST. Secondary antibodies conjugated to horseradish peroxidase were used at 1:5000 and incubated for 1h at room temperature.

Visualization was carried out with ECL+ Chemiluminescent Detection Kit (GE Healthcare) while imaging was carried out using an EpiChemi3 Imaging System (UVP) attached to a CCD camera (Hamamatsu). Densitometry was calculated utilizing 3 independent immunoblots per antibody using ImageJ software (National Institutes of Health).

Attached and Suspended Immunofluorescence

Cells were suspended for 30min with normal media in ultra low-attach plates (Corning) then spun down onto poly-L-lysine coated coverslips to preserve suspended cellular structures. Cells were then fixed with 3.7% formaldehyde in PBS, washed, permeabilized in a 0.25% Triton-X 100 solution in PBS, blocked in a 5% BSA/NP40/PBS, and then incubated overnight at 4ºC in 2.5%BSA/NP40/PBS with primary antibody. Secondary antibody was added 1:500 in PBS with Hoechst (DNA stain) at 1:1000. Images were acquired using an Olympus FV1000 confocal microscope (Olympus) and analyzed using ImageJ.

Live Cell Imaging and McTN scoring

Cells to be scored for McTNs were plated 24h prior to counting at 50-75% confluency. In order to visualize protrusions, cells were stained with CellMask Orange plasma membrane stain (Life Technologies) then suspended for 30min in phenol red-free media in 24-well ultra low-attach plates (Corning). After 30 minutes of suspension, cells were blindly scored for McTNs using an Olympus CKX41 inverted fluorescent microscope. Cells were considered positive for McTNs if they presented 2 or more protrusions that were longer than the radius of the cell. Each independent experiment counted at least 100 cells/well in triplicate, and this was repeated at least 3 times. The percentage of McTN positive cells per total cells counted is represented in each graph as %McTNs. Images were taken using the MicroSuite Five software (Olympus). Transiently transfected cells were confirmed to be GFP-positive (green= transfection-positive) prior to counting.

Cell Reattachment Assay

Real-time cell reattachment was measured using the xCELLigence RTCA DP device (Acea Biosciences, Inc.). 20,000 cells/well were added to electrode-containing microtiter plates (E-plate 16) for analysis of re-attachment. Reattachment rates were quantitatively recorded as Cell Index- a change in electrical impedance of the current flowing through the well electrodes. Electrical impedance was recorded every 1-5min for 2h. Raw data was exported to Microsoft Excel. Three independent experimental runs were carried out per cell-line and graphs shown are representative of a single experimental run +/- standard deviation of 3 wells.

Cell Proliferation Assay

The number of viable cells in proliferation was determined by the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega). Cells were plated at 10,000 cells/well in triplicate/time point in a 96-well plate in full-serum media. To monitor proliferation, 20µl of warmed Cell Titer was added at t=0 and every 24h thereafter for 120h. 2h after adding Cell Titer, absorbance was read at 490nm using a Bio-Tek Synergy HT Multimode Microplate Reader and analyzed with KC4 Software (Bio-Tek). Viability in serum-free conditions was monitored over the same time period, except t=0 was marked by the washout of full-serum media and the addition of serum-free media. Three independent experiments were carried out per cell line and serum conditions; each was plated in triplicate. The average absorbance reading/time point/cell line and condition was determined for each independent experiment. This was then divided by the average absorbance at time 0 to determine the percentage change in proliferation rates, over time.

Using time 0 allowed us to correct for any differences in initial cell plating. This was carried out 3 separate times to form average proliferation curves +/- standard deviation from the mean.

Real-time Migration Assay

Real-time migration was measured using the xCELLigence device, as previously described. 40,000 cells/well of MDA-MB-231/MCF-7 and 20,000 cells/well of BT-549 were added to each CIM-plate 16. CIM-plates are similar to a Boyden chamber, containing an upper and a lower chamber separated by a polyethylene terephthalate (PET) membrane with an 8 µm pore size. The membrane contains gold electrodes on the underside of the upper chamber to detect movement of cells from the upper to lower chamber. The chemoattractant used was 5% FBS. Electrical impedance was recorded every 15min for 24h. Raw data was exported to Microsoft Excel. Three independent runs were carried out per cell line and experimental condition. Graphs shown are representative of a single experimental run +/- standard deviation of 3 wells. The underside of the CIM-plate upper chamber was stained with Cell Stain (Millipore) to visualize migrated cells at 24h with a Nikon SMZ1500 stereomicroscope attached to a Nikon digital camera DXM1200 using ACT-1 Software Version 2.62 (Nikon).

Invasion Assay

Real-time analysis of invasion was carried out using the xCELLigence device. CIM-16 plates utilized for migration assays (above) were coated with Standard BD Matrigel (BD Biosciences), a solubilized basement membrane preparation extracted from

Engelbreth-Holm-Swarm (EHS) mouse sarcoma. The major component of matrigel is laminin, but matrigel also contains additional ECM proteins like collagen IV and growth factors. Coating was accomplished by diluting the matrigel at 1:10 in cold serum-free media for an approximate concentration of 8-10µg/ml. 50µl of diluted matrigel was added to each upper chamber and then 30µl were removed, leaving 20µl to coat the upper chamber of the well. These plates were then placed at 37ºC for 4h to allow for matrigel polymerization. After incubation, the lower chamber was filled with serum-containing (experimental) or serum-free (control) media and the upper chamber was filled with serum-free media. This was allowed to reach equilibrium at 37ºC for 1h. Cells were then trypsinized, quenched with serum-containing media, and washed once with serum-free media. Cells were counted and 100µl of 6×10^5 cells/ml of MDA-MB-231 and 4×10^5 cells/ml of BT-549 cells stably expressing the K40R α-tubulin mutant or wild-type α-tubulin were added to each well in triplicate in serum-free media. The plates were inserted into the real-time cell analyzer and incubated at 37ºC 5% CO_2 for 48h. Cell Index readings were taken every 15 minutes for 48h. Three independent runs were carried out per cell line. Raw data was exported to Microsoft Excel. Graphs shown are representative of a single experimental run +/- standard deviation of 3 wells.

Reverse-Phase Protein Array (RPPA)

412 breast cancer patient primary tumors from The Cancer Genome Atlas (TCGA) Breast Invasive Carcinoma RPPA Set 041011-0035 were assayed for acetylated α-tubulin intensity as previously described (118). Briefly, proteins were extracted from snap-frozen tissue and printed on nitrocellulose-coated slides. These slides were probed

with anti-acetylated tubulin and a signal was obtained and visualized by DAB colorimetric reaction. Scanned slides were analyzed and quantified using Microvigene software (VigeneTech Inc.) to determine acetylated tubulin intensity. Dilution curves were fitted with a logistic model developed by MD Anderson Cancer Center and concentrations were normalized to correct for loading. Read-out of loading-corrected intensity was calculated as “Normalized Linear Value”. Data from 10 patients was excluded because molecular subtype was not determined. Molecular subtyping was carried out utilizing the PAM50 assay (20). 402 patients were matched with clinical information (current as of June 2014) presented by the TCGA Research Network (TCGA Data Portal: https://tcga-data.nci.nih.gov/tcga/). Median tubulin acetylation intensity across this patient set was determined. Acetylation intensity was classified as high vs. low if it was above or below the median acetylation intensity, respectively, for 402 patients.

Immunohistochemistry and Scoring of Matched Patient Samples

Breast cancer and matched metastatic carcinoma of lymph node tissue arrays BR1005a, BR10010a, BR1001 were obtained from US BioMax, Inc (Rockville, MD). Patient ages and histologies accompanied the arrays. Samples from 144 patients were stained by the University of Maryland Greenebaum Cancer Center (UMGCC) Pathology Biorepository and Research Core (K. Tuttle) using the anti-acetylated tubulin antibody (Cell Signaling). Blind scoring of samples was carried out by Dr. Olga Ioffe, head clinical pathologist for breast and gynecologic cancers at UMGCC. Samples were scored on a scale of 0-3 to represent staining intensity of each tumor sample. Images were scanned using the Aperio System and captured using the ImageScope Viewer (www.aperio.com). After receiving scoring results, they were matched with each patient. It was then determined if the score changed from primary tumor to matched lymph node metastasis. The sample was considered "Low" if the score was 0-1 and "High" if it was a score of 2-3. The following categories were utilized to classify the changes from primary to metastasis: Low to Low, Low to High, High to Low, or High to High. Statistical analysis is explained under "Statistics".

Statistics

Statistical significance of densitometry analysis and McTN scoring was measured by t-test (Microsoft Excel). Matched primary and metastatic patient sample biostatistics were analyzed by Dr. Olga Goloubeva, director of the Biostatistics Facility at UMGCC. The McNemar’s test was used to test the equality of binary acetylation rates from two populations (primary tumor and metastases) with the data that are paired and dependent, since tumor and metastases rates are obtained from the same patients. Welch two-sample t-test was calculated with R statistical software (http://www.R-project.org) to determine significance of basal-like acetylation intensity vs. non-basal subtypes.

CHAPTER 3: SPECIFIC AIM 1

Define the role of α-tubulin acetylation in McTN formation and function in suspended breast tumor cells

Introduction

Breast tumor cells produce dynamic microtubule-based membrane protrusions, termed microtentacles (McTNs), upon detachment (6, 7, 60). These protrusions can encircle adjacent cells promoting cell-cell aggregation as well as reattachment of tumor cells to an extracellular matrix, endothelial monolayer, and retention in the lungs of mice (59, 61, 62). McTNs are dependent upon microtubule stability (60, 61). Inhibition of McTNs by microtubule-destabilizing drugs, like nocodazole, significantly reduces cell-cell and cell-substrate reattachment efficiency of suspended breast tumor cells (6). Conversely, microtubule stability conferred by overexpression of the MAP tau as well as the PTM of α-tubulin detyrosination have been shown to enhance reattachment in both *in vitro* and *in vivo* models of metastasis (6, 36, 59, 62).

Acetylation of α-tubulin is very similar to detyrosination in that it is an α-tubulin PTM associated with stabilized microtubules in the cell cytoplasm (4). Importantly, it has been found that detyrosinated α-tubulin promotes McTN formation through increasing microtubule stability while inhibiting detyrosination decreases McTNs (6, 36, 73). However, previous studies could not establish a correlation between the invasiveness of breast cancer and detyrosination of α-tubulin (62). Given previous data showing that stable microtubules are associated with increased CTC reattachment *in vivo* (56), preliminary studies were carried out on a panel of breast tumor cell lines to determine if acetylation of α-tubulin could potentially play a role in metastatic breast cancer.

Initial Results: Metastatic breast tumor cell lines have high α-tubulin acetylation

A panel of breast cancer cell lines was chosen to represent a range of *in vivo* metastatic potential. Acetylation of α-tubulin was investigated in non-metastatic breast cancer cell lines: MCF-7, MDA-MB-453, and BT-20 as well as in metastatic breast cancer cell lines: MDA-MB-231, BT-549, and Hs578T (119). Immunoblot results indicate acetylation of α-tubulin is significantly associated with the metastatic cell lines, whereas detyrosination is not (Figure 13-top panel). A small percentage of total α-tubulin is acetylated in the non-metastatic lines while α-tubulin is highly acetylated in the

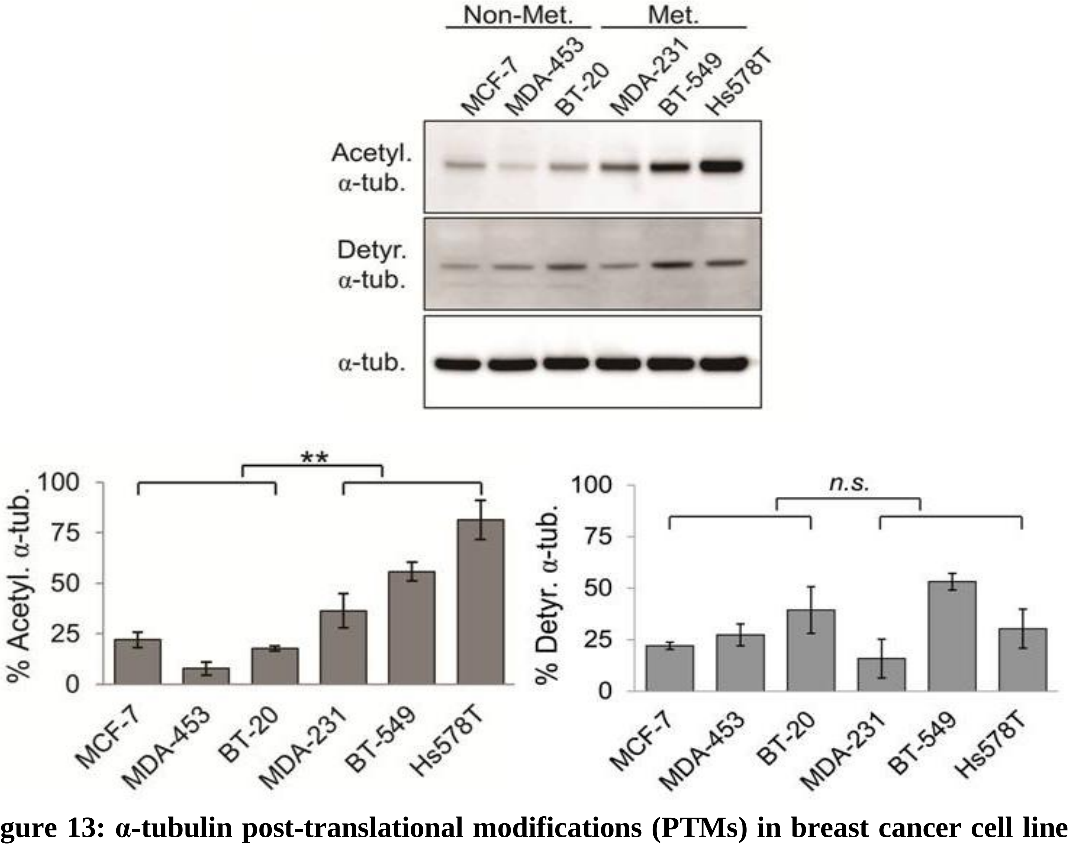

Figure 13: α-tubulin post-translational modifications (PTMs) in breast cancer cell lines. (Top) Lysates from non-metastatic (Non-Met) and metastatic (Met) breast cancer cell lines were subjected to immunoblot analysis for α-tubulin PTMs. The non-metastatic cell lysates were run separately from the metastatic cell lysates. Acetylation is associated with more metastatic cell lines. (Bottom) Densitometric analysis of acetylated or detyrosinated α-tubulin, as compared to total α-tubulin. $^{**}p<0.01$ for acetylation between Non-Met and Met. breast cancer cell lines. There is a non-significant (n.s) difference in detyrosination. Error bars indicate +/- standard deviation, n=3. *Boggs et al. 2014 Cancer Research: Under Review*

metastatic cell lines. Densitometry of three independent immunoblots showed there is over a three-fold difference between the average acetylation in non-metastatic cell lines compared to metastatic cell lines (Figure 13-bottom panel). However, there is no significant difference in detyrosination across the cell panel. This was not surprising since previous studies could not establish a correlative trend between cancer invasiveness and detyrosination of α-tubulin (62).

Immunofluorescence was then performed on attached cells to visualize differences in localization and structure of acetylated α-tubulin, as compared to the α-tubulin network. The non-metastatic cell lines MCF-7 and MDA-MB-453 exhibit low basal α-tubulin acetylation, with only BT-20 showing minimal acetylation (Figure 14-left columns). In contrast, the highly metastatic MDA-MB-231, BT-549, and Hs578T cell

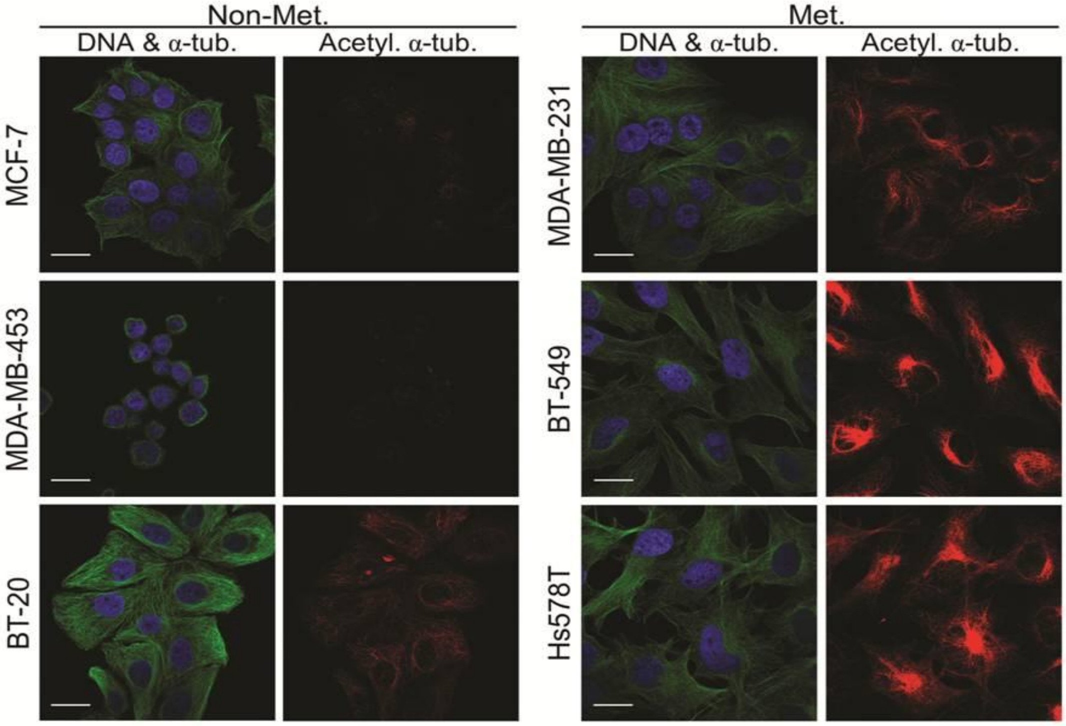

Figure 14: Acetylated α-tubulin immunofluorescence of metastatic (Met.) and non-metastatic (Non-Met.) breast cancer cell lines. Immunofluorescence of DNA (blue), α-tubulin (green), and acetylated α-tubulin (red) shows acetylation increases in more metastatic breast tumor cell lines. Scale bar=20 µm. *Boggs et al. 2014 Cancer Research: Under Review*

lines display robust acetylation of α-tubulin with bundling or increased density of acetylated microtubules radiating from the perinuclear region (Figure 14-right columns). Immunofluorescence not only confirmed the immunoblot results, but also enabled us to visualize strong bundling of the acetylated microtubule network in the cytoplasm of metastatic breast cancer cell lines. The aforementioned studies showing acetylated α-tubulin is increased in metastatic breast cancer cells, as compared to non-metastatic cells, was the foundation for further investigation into the role of this modification in McTN formation and function.

Aim 1.1: Acetylation of α-tubulin correlates with increased McTN frequency and is enriched in McTNs

We wanted to first determine if acetylation is maintained under suspended conditions before investigating its potential role in McTN formation. The cell line panel utilized for initial studies was detached for 30min under ultra-low attachment conditions, lysed, subjected to immunoblot, and compared to their attached counterparts (Figure 15).

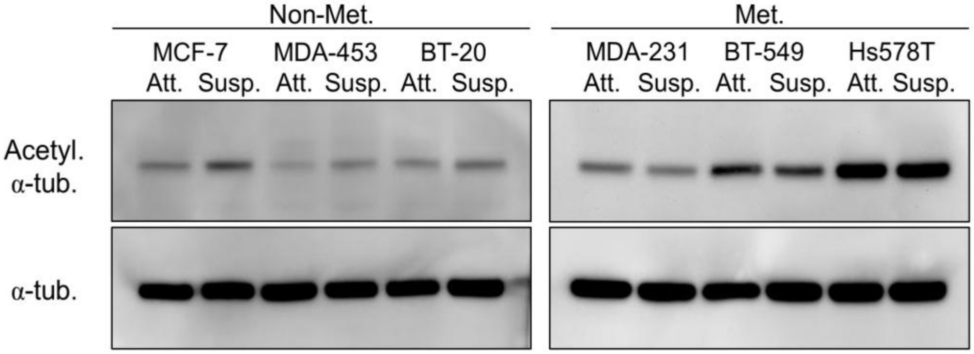

Figure 15: Acetylated α-tubulin and suspension. Non-metastatic and metastatic breast cancer cells were lysed after 24h attachment (Att.) or 30min suspension (Susp.) under low-attachment conditions and subjected to immunoblot for α-tubulin acetylation. Acetylation is maintained and possibly elevated in non-metastatic (Non-Met.) cell lines and remains elevated in metastatic (Met.) cell lines upon suspension. Non-Met. and Met. lysates were run on separate gels. *Boggs et al. 2014 Cancer Research: Under Review*

It was found that even though the non-metastatic cell lines have low levels of acetylated α-tubulin under attached conditions, slight increases in acetylation were observed when comparing attached lysates to suspended lysates (Figure 15). Importantly, acetylation of α-tubulin remains elevated in the metastatic cell lines under suspended conditions and could be investigated in suspended-cell protrusions.

The role of α-tubulin acetylation in McTN formation and function has not been previously investigated in metastatic breast tumor cell lines. Given the increased α-tubulin acetylation detected in more metastatic cell lines (Figures 13-14) that remains elevated under suspended conditions (Figure 15), blinded quantitation of McTN frequencies was carried out on this panel of breast cancer cell lines. We found metastatic cell lines, which have higher acetylation of α-tubulin, have significantly more McTNs than the non-metastatic lines (**p<0.01) (Figure 16). Suspended cell images of non-

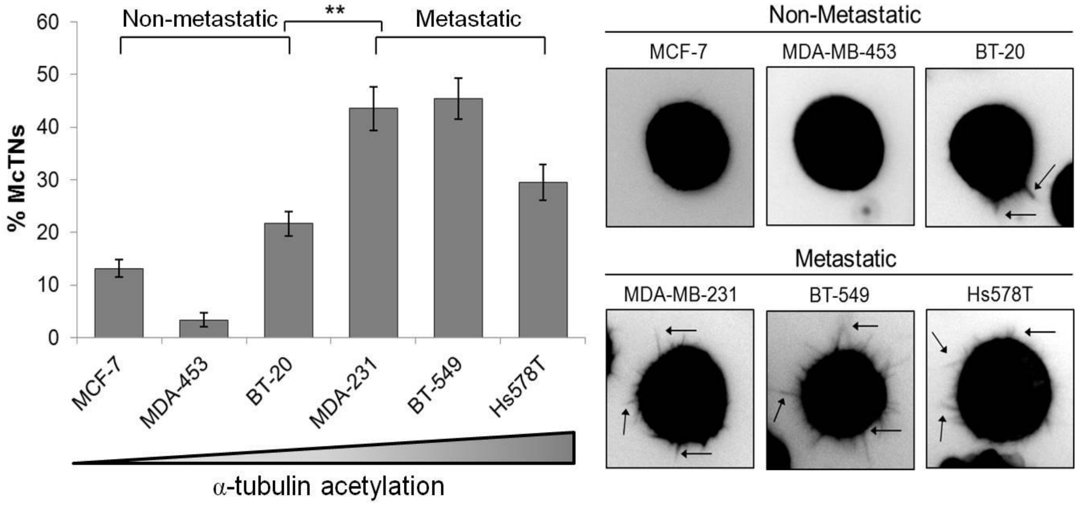

Figure 16: Acetylation and microtentacle (McTN) frequency. (Left) McTN counts were carried out. Data is represented as the mean +/- standard deviation of n=3 experiments with at least 100 cells scored blindly/experiment. Cells with higher acetylation also have significantly higher McTNs. **p<0.01 between non-metastatic and metastatic McTN counts. %McTNs indicates the percentage of McTN positive cells. (Left) Representative suspended-cell images highlighting McTN protrusions (arrows) in more metastatic/acetylated cell lines, as compared to less acetylated/ non-metastatic cell lines. Modified from: *Boggs et al. 2014 Cancer Research: Under Review*

metastatic and metastatic cell lines are also presented in Figure 16. Metastatic breast tumor cells present more numerous McTNs upon suspension than non-metastatic cells.

Detyrosination is the only α-tubulin PTM to date that has been reported to be enriched in McTNs; however, detyrosination does not directly correlate with increased McTN occurrence or *in vivo* invasiveness (6). Given the increased acetylation correlated with higher McTN frequency in metastatic lines, we examined if α-tubulin acetylation is a component of McTNs. Immunofluorescence of suspended metastatic cell lines with high McTN frequency and α-tubulin acetylation was carried out. Briefly, this was done by suspending MDA-MB-231 and BT-549 cells under low-attachment conditions for 30min then gently spinning the cells down onto poly-L-lysine coated coverslips to preserve any protrusive structures in suspension. We found acetylated α-tubulin extends along the lengths of McTNs in these highly metastatic breast tumor cell lines (Figure 17, arrows), highlighting for the first time that this PTM is a constituent of McTNs.

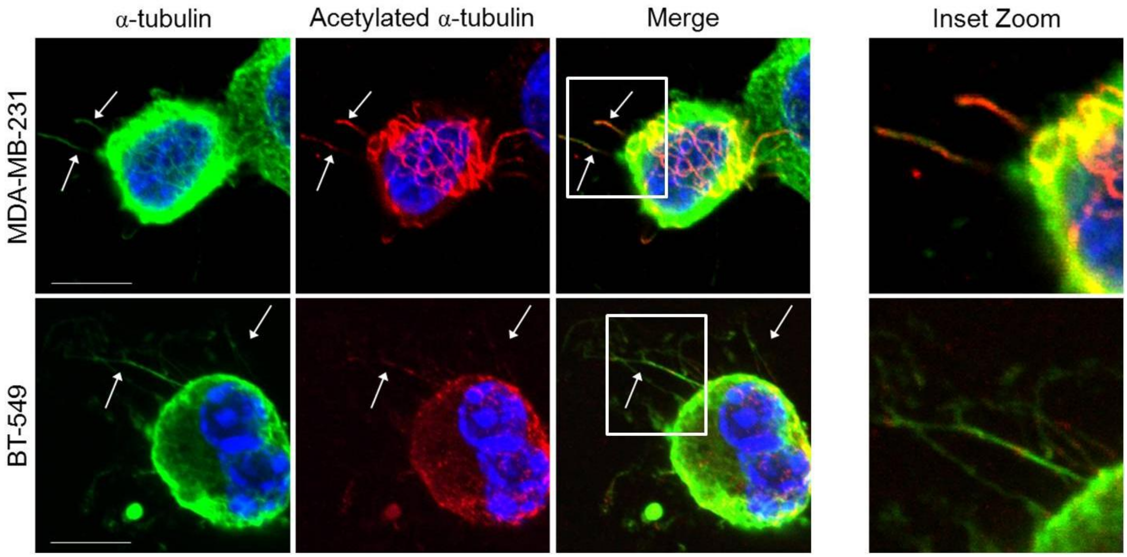

Figure 17: Suspended cell immunofluorescence for acetylated α-tubulin. Immunofluorescence of suspended metastatic breast tumor cell lines indicates acetylated α-tubulin (red) localizes along α-tubulin (green)-based protrusions in metastatic cells. DNA is stained in blue. Arrows indicate McTNs. Scale bar=10 µm. Modified from: *Boggs et al. 2014 Cancer Research: Under Review*

Aim 1.2: K40R α-tubulin mutation decreases endogenous acetylation and McTN frequency

We previously observed metastatic cell lines have increased α-tubulin acetylation that is maintained upon suspension and localizes to McTNs. These studies propelled further investigation into the mechanistic role of α-tubulin acetylation in McTN formation. We first used site directed mutagenesis to determine if decreasing α-tubulin acetylation in highly acetylated and metastatic cell lines could affect McTNs. Acetylation occurs on lysine 40 of α-tubulin (K40), a highly conserved site shown to affect microtubule stability (79, 115). Previous research has shown the lysine 40 to arginine α-tubulin point mutation (K40R) is acetylation-resistant but can still incorporate into the microtubule polymer (120). MDA-MB-231 and BT-549 cells were selected to investigate the effects of the K40R mutation given their high endogenous α-tubulin acetylation and McTN frequency. Immunofluorescence was carried out on transient transfections with either K40R α-tubulin-GFP or a wild-type control α-tubulin-GFP (Figure 18) to compare

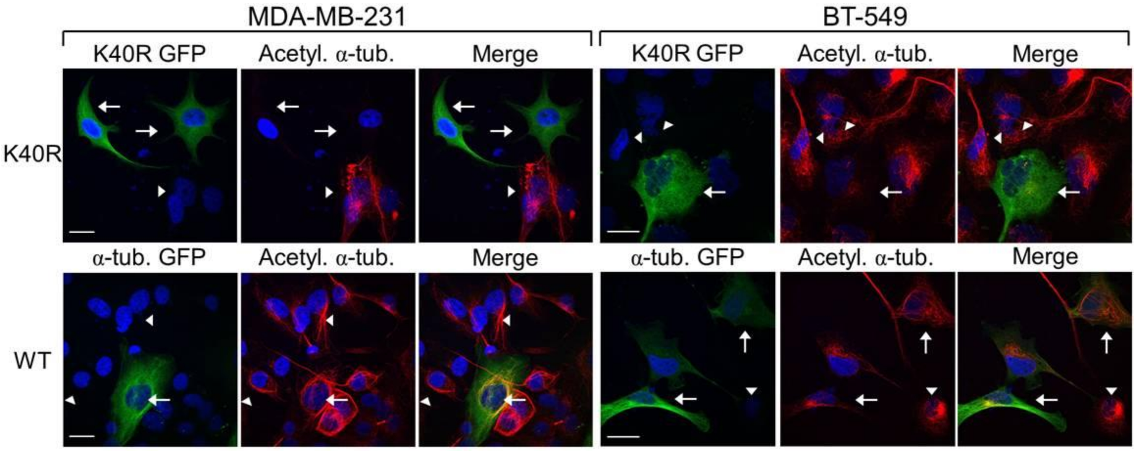

Figure 18: K40R α-tubulin mutant decreases acetylation. MDA-MB-231 and BT-549 cells were transiently transfected with GFP-labeled (green) K40R α-tubulin (arrows, top row) or GFP (green) α-tubulin control (arrows, bottom row) and subjected to immunofluorescence for acetylated α-tubulin (red). The K40R mutant dramatically decreased α-tubulin acetylation, while the wild-type (WT) control did not. DNA is in blue. Arrowheads indicate non-transfected cells. Scale bar=20 µm. *Boggs et al. 2014 Cancer Research: Under Review*

the effects of this mutant on acetylation of the α-tubulin network. MDA-MB-231 and BT-549 cells expressing the K40R α-tubulin-GFP showed major disruption of acetylated α-tubulin filaments after 24h (Figure 18, top row, arrows), compared to adjacent untransfected cells (Figure 18, top row, arrowheads) or transfection of the α-tubulin-GFP control (Figure 18, bottom row). Stable cell lines expressing K40R α-tubulin GFP or the wild-type α-tubulin GFP control were then created in MDA-MB-231 and BT-549 cells and pooled clones were utilized in subsequent experiments. It is important to mention stable cell lines expressing the K40R α-tubulin mutant could not be created in the most highly acetylated cell line, Hs578T.

Under attached and suspended conditions, MDA-MB-231 and BT-549 cells stably expressing the K40R α-tubulin-GFP mutant show decreased endogenous acetylation of α-tubulin, as compared to α-tubulin-GFP control cells (Figure 19). Since K40R expression decreased endogenous acetylation and significantly reduced acetylated microtubules, the impact of reducing α-tubulin acetylation on McTN formation in cell lines with high McTN frequency was investigated.

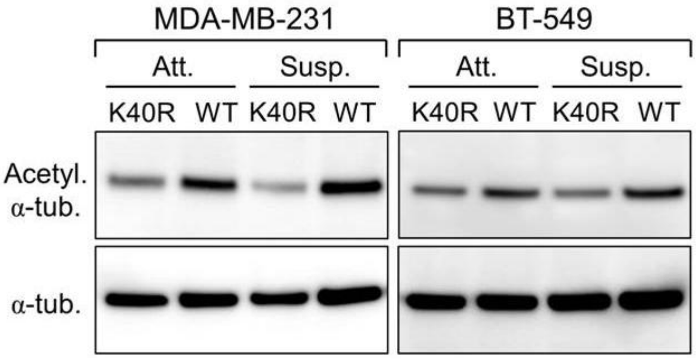

Figure 19: K40R stable expression decreases acetylation of α-tubulin. Pooled clones were lysed of both attached (Att.) and suspended (Susp.) cells expressing the K40R α-tubulin mutant or wild-type (WT) α-tubulin control and subjected to immunoblot. K40R stable expression decreased acetylation of α-tubulin, as compared to the wild-type control.
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MDA-MB-231 and BT-549 cells expressing the control wild-type α-tubulin exhibit numerous long McTNs when suspended (Figure 20A, arrows). However, when these metastatic cells stably express the non-acetylatable K40R α-tubulin mutant, the McTN protrusions are significantly reduced (Figure 20A, B). McTN frequency was reduced by more than 45% in MDA-MB-231 and 62% in BT-549 K40R expressing cells, as compared to controls (Figure 20B).

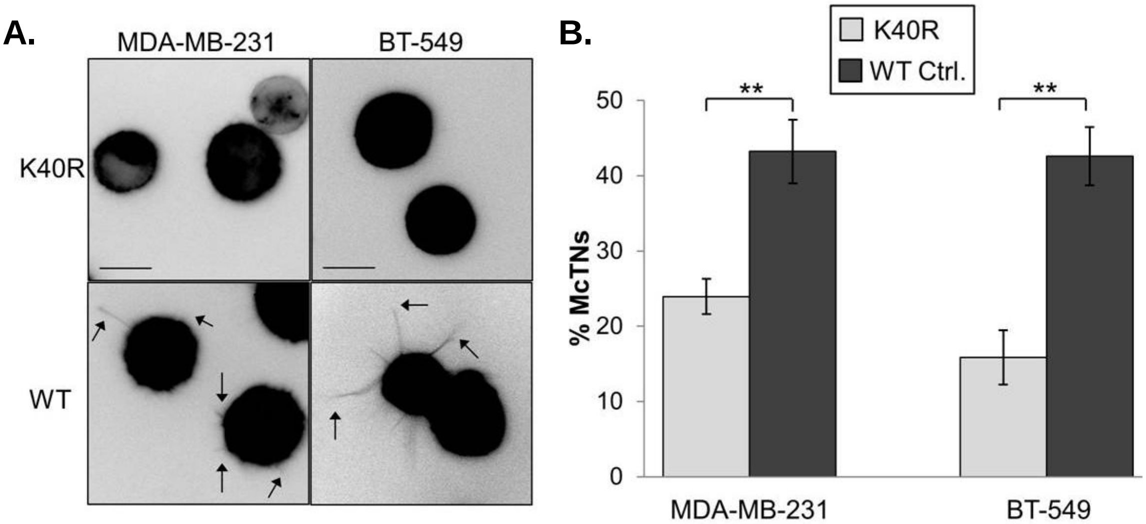

Figure 20: K40R α-tubulin mutant significantly decreases McTNs. (A) Representative McTN images of MDA-MB-231 and BT-549 stable cell lines suspended for 30min under low-attachment conditions. Arrows indicate McTNs that are normally present under wild-type conditions (bottom panels). However, once the K40R mutant is introduced, McTNs are dramatically reduced (top panels) Scale bar=10 µm. (B) McTN counts were carried out on the suspended stable cell lines. Error bars indicate +/- standard deviation of n=3 in triplicate. **$p<0.01$. % McTNs indicates the percentage of McTN positive cells per population of cells counted. The K40R mutant significantly decreases McTN frequency in both metastatic breast tumor cell lines. *Boggs et al. 2014 Cancer Research: Under Review*

Additional McTN counts were carried out on transiently transfected MDA-MB-231 and BT-549 cells to ensure the process of creating the stable cell lines did not adversely affect McTNs. Similar to what was seen in the stable lines, both metastatic cell lines significantly decreased McTN frequency even when transiently expressing the K40R mutant (Figure 21).

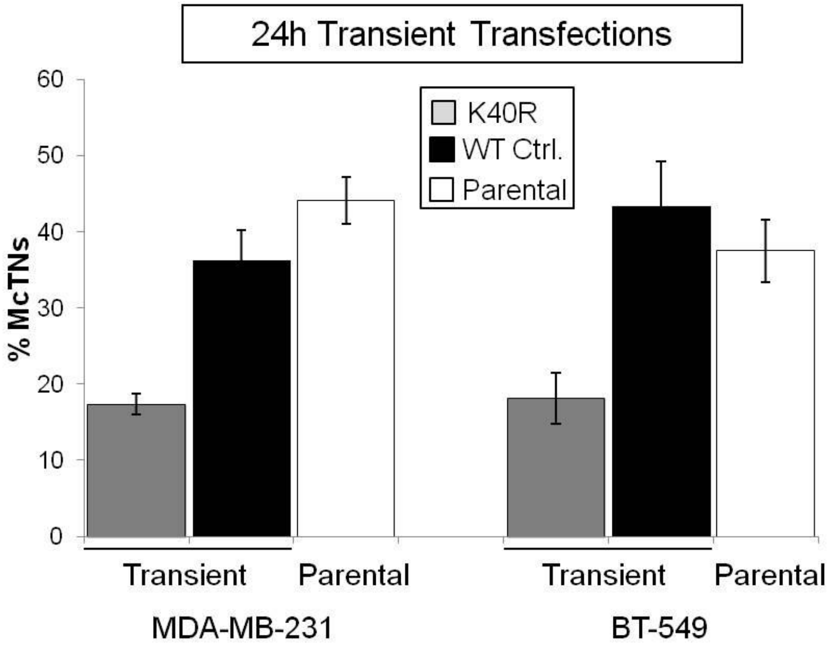

Figure 21: K40R α-tubulin decreases McTN frequency under transient transfection conditions. McTN counts were carried out on MDA-MB-231 and BT-549 cells transiently transfected with the K40R α-tubulin mutant or the wild-type α-tubulin GFP control 24h post-transfection and compared to parental control cells. GFP expression was confirmed prior to scoring transfected cells. Error bars indicate +/- standard deviation of n=3 counts carried out in triplicate. %McTNs indicates the percentage of McTN positive cells per population of cells counted. Creation of the stable cell lines did not affect trends seen in McTN frequency (compare to Figure 20). Modified from: *Boggs et al. 2014 Cancer Research: Under Review*

Aim 1.3: Overexpression of αTAT1 increases tubulin acetylation and enhances McTN frequency

Since reducing acetylation of α-tubulin decreased McTN formation and frequency in highly acetylated and metastatic breast tumor cells, we reversed this molecular mechanism to determine if increasing acetylation in a non-metastatic cell line would promote McTNs. The α-tubulin acetyltransferase 1 (αTAT1) was recently demonstrated to specifically acetylate α-tubulin on lysine 40 in mammalian cells (87, 88). MCF-7 cells were selected to overexpress αTAT1 because they have low endogenous acetylation (Figures 13-14) and low McTN frequency (Figure 16).

Transient overexpression of αTAT1-GFP (87) in MCF-7 cells caused robust acetylation of α-tubulin throughout the cytoplasm of transfected cells (Figure 22, top row, arrows) while the GFP control (Figure 22, bottom row, arrows) and non-transfected cells (arrowheads) were unaffected. Overexpression of αTAT1 or the GFP control did not affect the overall α-tubulin network (Figure 23). Transfection in Figures 22-23 was scaled down to visualize transfected cells (green) adjacent to non-transfected cells in the same field of view.

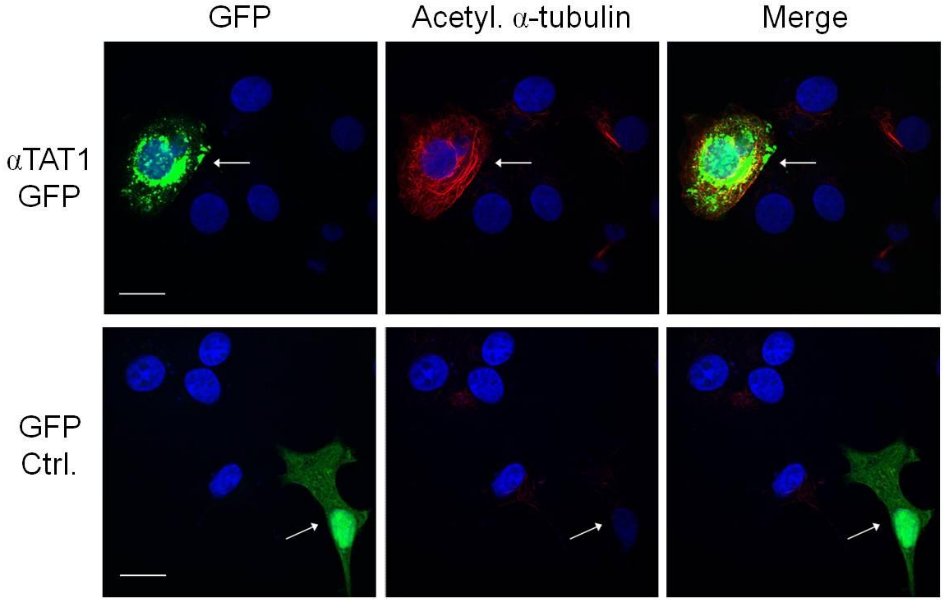

Figure 22: αTAT1 significantly increases α-tubulin acetylation. MCF-7 cells were transiently transfected with αTAT1-GFP or GFP control (green) and subjected to immunofluorescence for acetylated α-tubulin (red). DNA is shown in blue. Transfected cells are indicated by arrows; non-transfected cells are indicated by arrowheads. Scale bar=20 μm. Only αTAT1 transfected cells show increases in acetylation of α-tubulin, while the GFP control transfected cells are unaffected. Transfection efficiency was scaled down to visualize transfected cells adjacent to non-transfected cells in the same field of view. *Boggs et al. 2014 Cancer Research: Under Review*

Concurrently, immunoblot shows that αTAT1 overexpression greatly increased endogenous acetylation of α-tubulin in both attached and suspended MCF-7 cells, as compared to the GFP-control (Figure 24A).

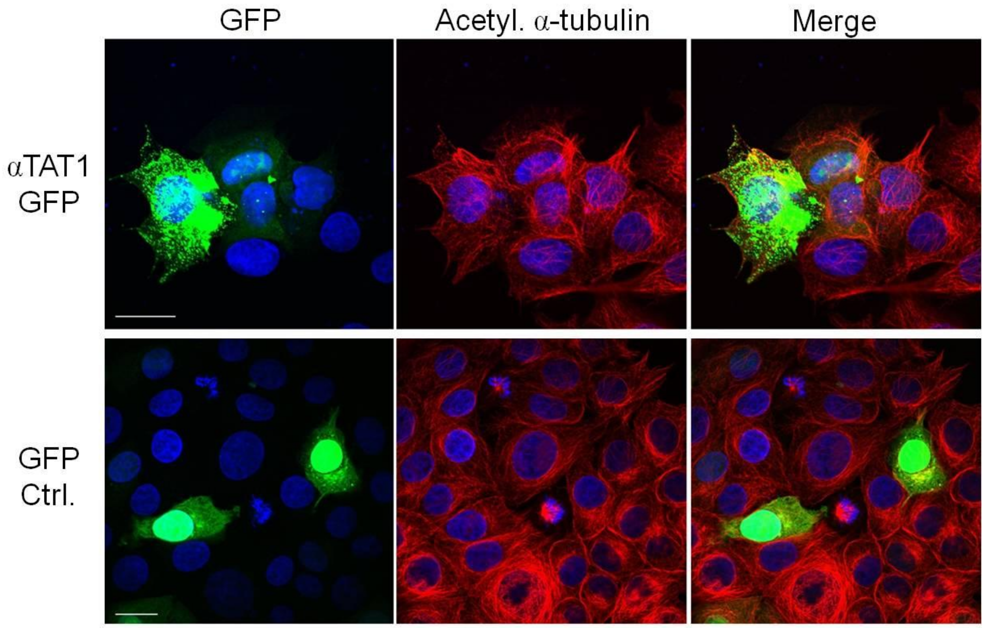

Figure 23: αTAT1-GFP or GFP control transfection does not affect the α-tubulin network. MCF-7 cells were transiently transfected with αTAT1-GFP or the GFP control (green). 24h post-transfection, immunofluorescence was carried out for α-tubulin (red) and DNA (blue) and shows αTAT1 does not affect the overall α-tubulin network. Scale bar=20µm. *Boggs et al. 2014 Cancer Research: Under Review*

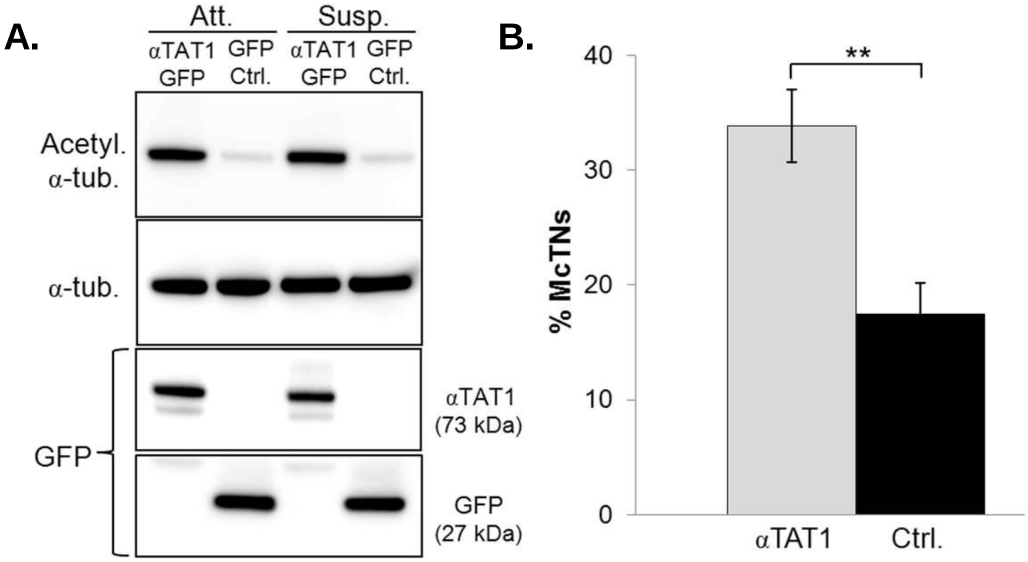

Figure 24: αTAT1 significantly increases α-tubulin acetylation and McTNs. (A) Immunoblot of cells lysed after 24h transfection under attached or 30min suspended conditions shows αTAT1 overexpression greatly increases acetylation of α-tubulin in MCF-7 cells. (B) McTN counts of MCF-7 cells transiently transfected with αTAT1-GFP or GFP control shows a significant increase in McTNs in cells overexpressing αTAT1. %McTNs indicates the percentage of McTN positive cells. Data represents n=3 in triplicate +/- standard deviation. **p<0.01. *Boggs et al. 2014 Cancer Research: Under Review*

αTAT1-GFP or the GFP control MCF-7 cells were then suspended to determine the effects of increased acetylation on McTN formation and function. We found that overexpression of αTAT1 significantly increased the percentage of McTN positive cells by approximately two-fold over control cells (Figure 24B).

Because of the significant difference in McTN frequency, we then examined if αTAT1-induced acetylation of α-tubulin localized along the lengths of McTNs. Suspended cell immunofluorescence revealed that acetylated α-tubulin extended within McTNs in the αTAT1 overexpressing cells (Figure 25, top row, arrows) but not in the GFP-transfected control (Figure 25, bottom row).

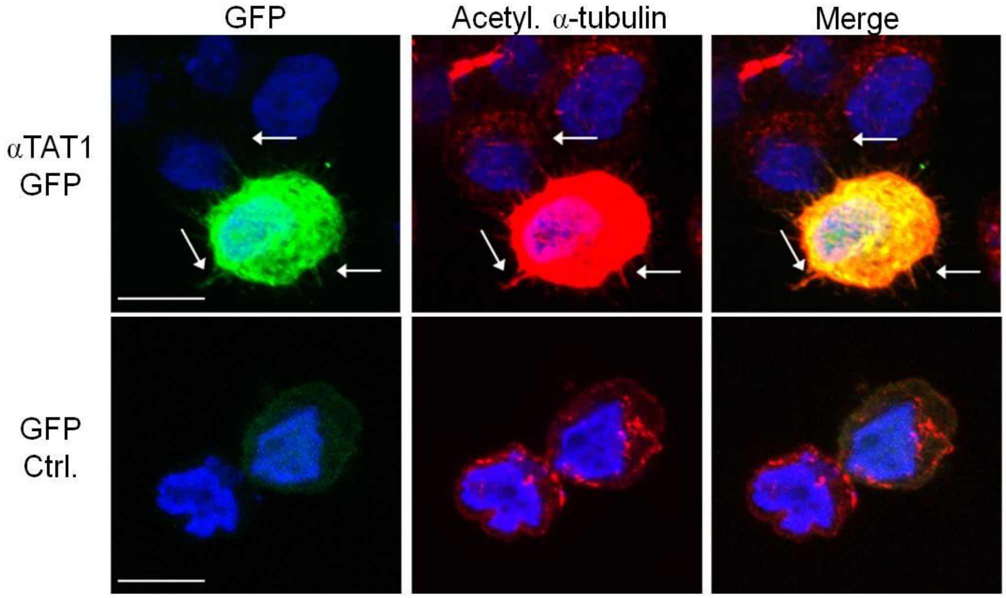

Figure 25: Acetylated α-tubulin localizes to McTNs in cells overexpressing αTAT1. Suspended MCF-7 cells overexpressing αTAT1-GFP (green) not only increased acetylated α-tubulin (red), but localized this PTM to McTN protrusions upon suspension. GFP control overexpression (green) did not affect acetylation or promote acetylated α-tubulin protrusions upon suspension. DNA is represented in blue. Arrows indicate McTNs. Scale bar=10 µm. Transfection efficiency was scaled-down to visualize transfected (green) cells adjacent to non-transfected cells in the same field of view. *Boggs et al. 2014 Cancer Research: Under Review*

Aim 1.4: Altering acetylation of α-tubulin influences the reattachment of suspended breast tumor cells

McTN function is evaluated by the ability of detached cells to reattach in order to model one of the early steps in CTC retention in distant tissues (7). To assess how the reduction in acetylation affects reattachment of metastatic cell lines, MDA-MB-231 and BT-549 cells expressing the K40R stable mutation were analyzed (Figure 26A, B).

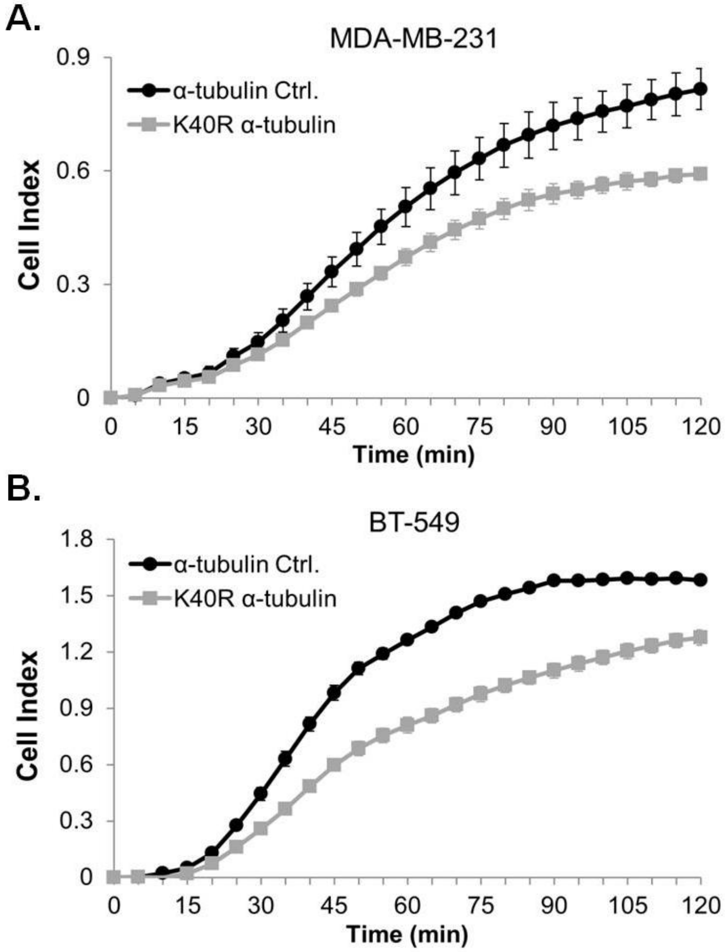

Figure 26: α-tubulin acetylation significantly affects reattachment rates of suspended breast tumor cells. Real-time cell reattachment was analyzed. Each cell line was plated in triplicate and error bars indicate +/- standard deviation of those 3 wells. Cell Index represents the change in electrical impedance over time. Graphs shown are representative of n=3 independent runs/cell line. (A-B) Representative graphs of reattachment for stable pooled clones expressing the mutant K40R α-tubulin or the α-tubulin wild-type control. Cells expressing the K40R mutant attached at a decreased rate. *Boggs et al. Cancer Research: Under Review*

The K40R mutant α-tubulin expressing cells reattached significantly less over 2h, as compared to controls (Figure 26A, B). It is interesting to note that BT-549 cells had a greater difference in McTN frequency between those stably expressing the K40R mutant and the α-tubulin control (Figure 20B). This could explain the larger difference in reattachment (Figure 26B), as compared to the significant but less drastic reduction in reattachment in the MDA-MB-231 cells (Figure 26A).

In complementary experiments, increasing acetylation by overexpressing αTAT1 in low acetylated and non-metastatic MCF-7 cells revealed that increased acetylation significantly increases reattachment, compared to the GFP control (Figure 27). This elevation in attachment efficiency parallels the increased McTN counts in Figure 24B, not only confirming McTN function but demonstrating for the first time the importance of α-tubulin acetylation in suspended cell reattachment.

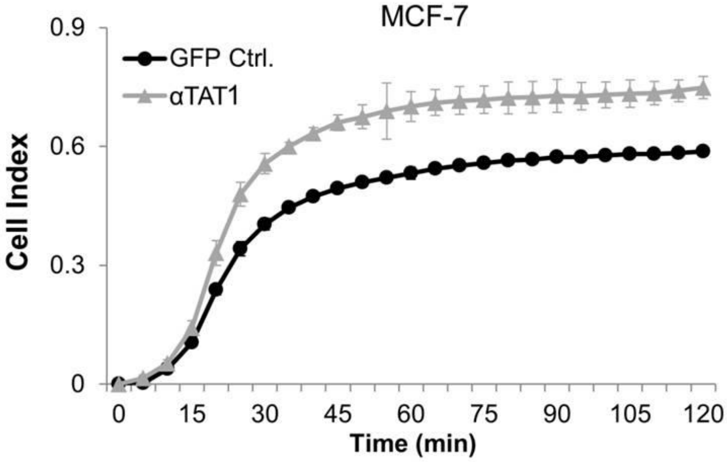

Figure 27: αTAT1 overexpression increases reattachment rates of suspended breast tumor cells. Real-time cell reattachment was analyzed using the xCELLigence system, as in Figure 27. Graphs shown are representative of n=3 independent runs/cell line. MCF-7 cells were transiently transfected with αTAT1 or GFP control 24h prior to analysis. Overexpression of αTAT1 in MCF-7 cells significantly increased reattachment over 2h compared to control. *Boggs et al. 2014 Cancer Research: Under Review*

Summary and Discussion

Aim 1 focused on the specific role of α-tubulin acetylation at lysine 40 in the formation of suspended cell protrusions and tumor cell reattachment. Prior to these studies, the only α-tubulin PTM thought to localize to McTNs in suspended metastatic breast tumor cells was detyrosination. We found that metastatic breast tumor cell lines have high acetylation of α-tubulin that is maintained when these cells are suspended. It was also determined that acetylated α-tubulin is enriched in McTN protrusions in metastatic breast tumor cells.

Specifically reducing acetylation of α-tubulin with the K40R α-tubulin mutant significantly decreased McTN frequency in metastatic breast tumor cells and inhibited reattachment upon suspension. This suggests suspended cell reattachment to a substrate may be dependent upon proper α-tubulin acetylation. Elevating acetylation via αTAT1 overexpression also increased McTNs in a less aggressive MCF-7 breast cancer cell line and enhanced reattachment efficiency. The current results define a novel mechanism where acetylation of lysine 40 of α-tubulin promotes microtentacle generation and tumor cell reattachment that could be selective advantages during metastasis.

These studies support the accepted model of McTN formation in which tubulin stabilization promotes outward growth of microtubules that are capable of overcoming the inward force of the contractile actin cortex (see Figure 7) (7). In this case, instead of detyrosination (6, 36, 37, 73), we have also found that acetylation could also promote McTN formation. Because of these studies, acetylation should also be considered a target in future studies aimed at reducing McTN frequency and CTC reattachment.

The reattachment of suspended and circulating tumor cells is one of many steps in the metastatic cascade that could potentially be targeted to prevent disseminated disease. Aim 2 examines additional steps of the metastatic cascade that could be affected by alterations in α-tubulin acetylation.

CHAPTER 4: SPECIFIC AIM 2

Examine the effects of α-tubulin acetylation changes on proliferation, migration, and invasion

Introduction

Studies have suggested high HDAC6 levels (increased deacetylase= lower α-tubulin acetylation) are associated with good prognosis and increased survival in breast cancer patients (83, 84). However, the mechanisms behind this correlation are still unclear. Since McTN formation/reattachment (Aim 1) is only one step in the metastatic cascade that could promote metastatic progression, we next investigated the role of acetylated α-tubulin in additional metastatic processes dependent upon cytoskeletal coordination.

Migration and invasion are both dependent upon cytoskeletal coordination and necessary for disseminated cells at a number of steps in the metastatic cascade, like intravasation and extravasation (121, 122). Migration of cells toward a specific chemical gradient is considered chemotaxis. Tumor cells and tumor-associated cells (like inflammatory and stromal cells) are often directed towards a chemoattractant, like chemokines or growth factors (121). It is believed that downstream signaling of chemoattractants can promote changes in cytoskeletal dynamics to direct cellular motility (121). It was recently shown that acetylated α-tubulin orients toward the leading edge of a migrating cell (103), but it was unclear if this modification is necessary for proper migration. The studies carried out in Aim 2 further investigate the effects of altering acetylation of α-tubulin in chemotaxis of breast cancer cell lines to determine if this modification is necessary or sufficient for migration.

Exogenous αTAT1 overexpression has also been shown to promote invasion of the metastatic MDA-MB-231 cell line through an ECM (103, 114). These studies only investigated one cell line and were unable to determine if acetylation of α-tubulin was necessary for invasion or if αTAT1 was promoting invasion by interacting with other proteins. Utilizing the K40R α-tubulin mutant to knock down acetylation in metastatic breast tumor cell lines, we were also able to investigate the role of α-tubulin acetylation in invasion.

Aim 2.1: K40R stable expression and αTAT1 transient expression does not significantly affect cellular proliferation

Since normal cell division requires stable microtubules at the mitotic spindle (123), we first sought to determine if K40R stable expression or αTAT1 transient expression affects cell proliferation. These experiments were carried out for 120h in normal serum-containing media as well as under serum-free conditions. This was done to monitor normal cellular proliferation as well as determine viability under serum-free conditions. We found that K40R α-tubulin stable expression did not significantly affect proliferation in MDA-MB-231 or BT-549 cells under normal serum (solid lines) and serum-free conditions (dashed lines), as compared to cells stably expressing the wild-type α-tubulin control (Figure 28).

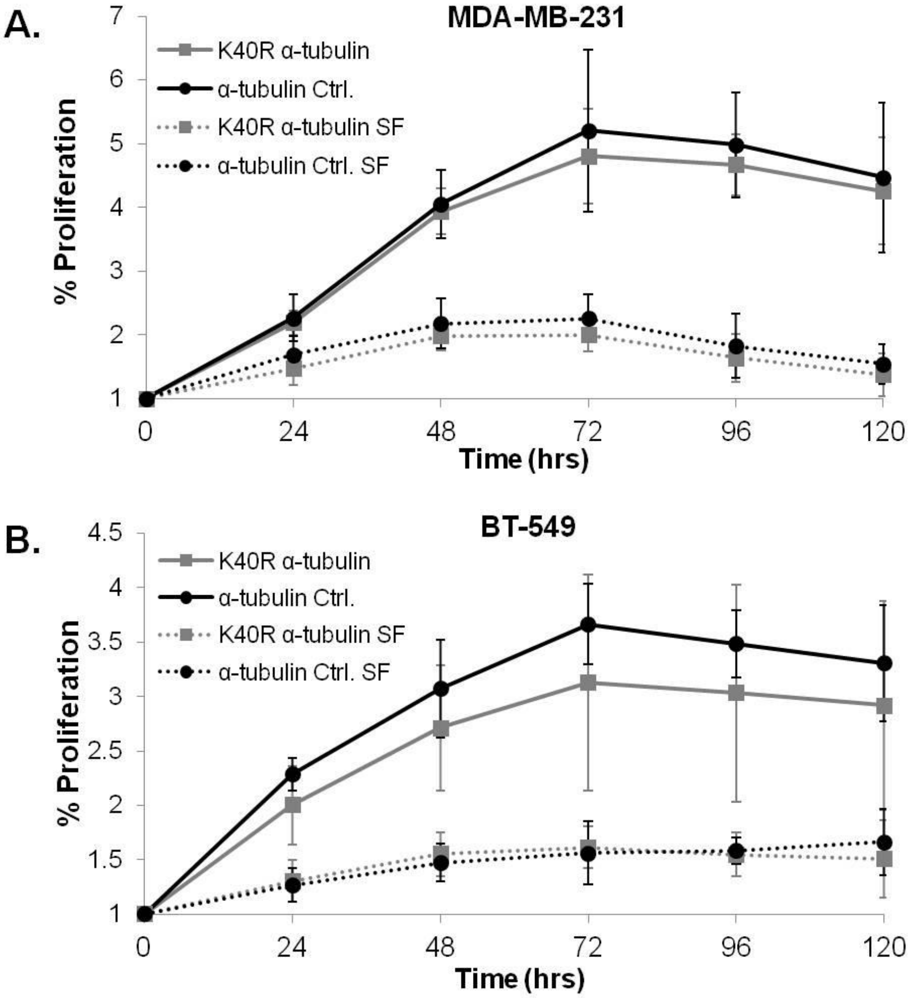

Figure 28: K40R α-tubulin does not affect proliferation in MDA-MB-231 or BT-549 cells. (A-B) Proliferation (solid lines) was monitored over a period of 120h in cells stably expressing the K40R α-tubulin mutant or the wild-type α-tubulin control. Viability in serum-free (SF) conditions was also monitored in these cell lines (dashed lines). Error bars indicate +/- standard deviation of the average absorbance of triplicate wells, n=3.
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We also found that αTAT1 transient overexpression did not affect proliferation or serum-free viability in MCF-7 cells, as compared to cells overexpressing the GFP control (Figure 29). It is important to note that transient overexpression is still detectable in these cells via GFP expression and immunoblot for at least 96h.

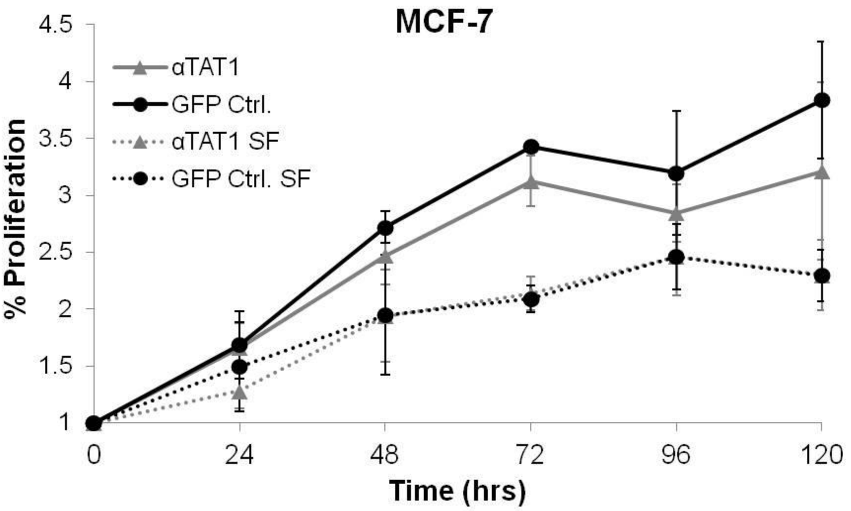


Figure 29: αTAT1 transient overexpression does not significantly affect proliferation. Proliferation was monitored over a period of 120h in cells transiently expressing αTAT1 or the GFP control. Viability in serum-free (SF) conditions was also monitored over 120h in these cell lines (dashed lines). Error bars indicate +/- standard deviation of the average absorbance of triplicate wells, n=3. *Boggs et al. 2014 Cancer Research: Under Review*

Since there is no significant difference in proliferation among each of these experimental groups, the subsequent migration and invasion assays could be carried out without the confounding factor of differences in proliferation.

Aim 2.2: Decreasing α-tubulin acetylation with the K40R mutant inhibits migration

MDA-MB-231 and BT-549 cell lines stably expressing the K40R α-tubulin mutant or the α-tubulin control were subjected to a real-time migration assay in which fetal bovine serum (FBS) was used as a chemoattractant. This assay utilized CIM-16 plates (Figure 30) to quantify cellular movement towards the chemoattractant gradient. CIM-16 plates are similar to a Boyden chamber, except movement from the upper (serum free) chamber to the lower (serum-containing) chamber can be monitored continuously and quantified. Each well contains a polyethylene terephthalate (PET) membrane of 8 µm pore size in which the underside is covered in gold electrodes. The electrodes sense when a cell has contacted the lower chamber and this is read out as a change in electrical impedance (Cell Index). The higher the Cell Index, the more cells are migrating from the upper to lower chambers. The migration experiments were carried out on MDA-MB-231 and BT-549 cells stably expressing the K40R or α-tubulin control as well as MCF-7 cells transiently expressing αTAT1 or the GFP control. Negative controls were also included in each experiment in which serum-free media was added to the lower chamber. These cells should not migrate because there is no chemoattractant to promote movement.

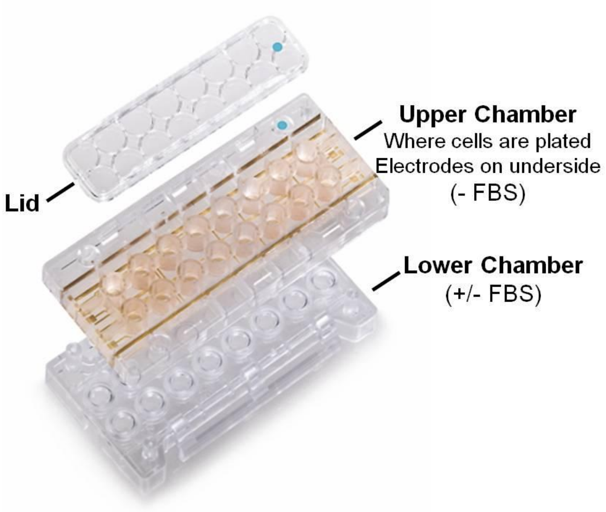


Figure 30: Image of CIM plate 16 utilized for the migration assay. In order to monitor chemotaxis, the upper chamber is filled with serum-free media and the lower chamber either contains full serum (experimental) or serum-free (negative control) media. Cells are plated into the upper chamber and allowed to migrate towards the serum-containing media in the lower chamber. Electrodes on the underside of the upper chamber detect cell movement from the upper to lower chamber. Image modified from Acea Biosciences: http://www.aceabio.com/

MDA-MB-231 and BT-549 stable cell lines expressing the K40R α-tubulin mutant migrated at a significantly reduced rate, as compared to cells expressing the α-tubulin control (Figure 31A-D). As expected, the serum-free negative controls did not migrate towards the lower chamber (Figure 31A-D). These results suggest acetylation of α-tubulin may be necessary for migration of more motile and metastatic breast tumor cells.

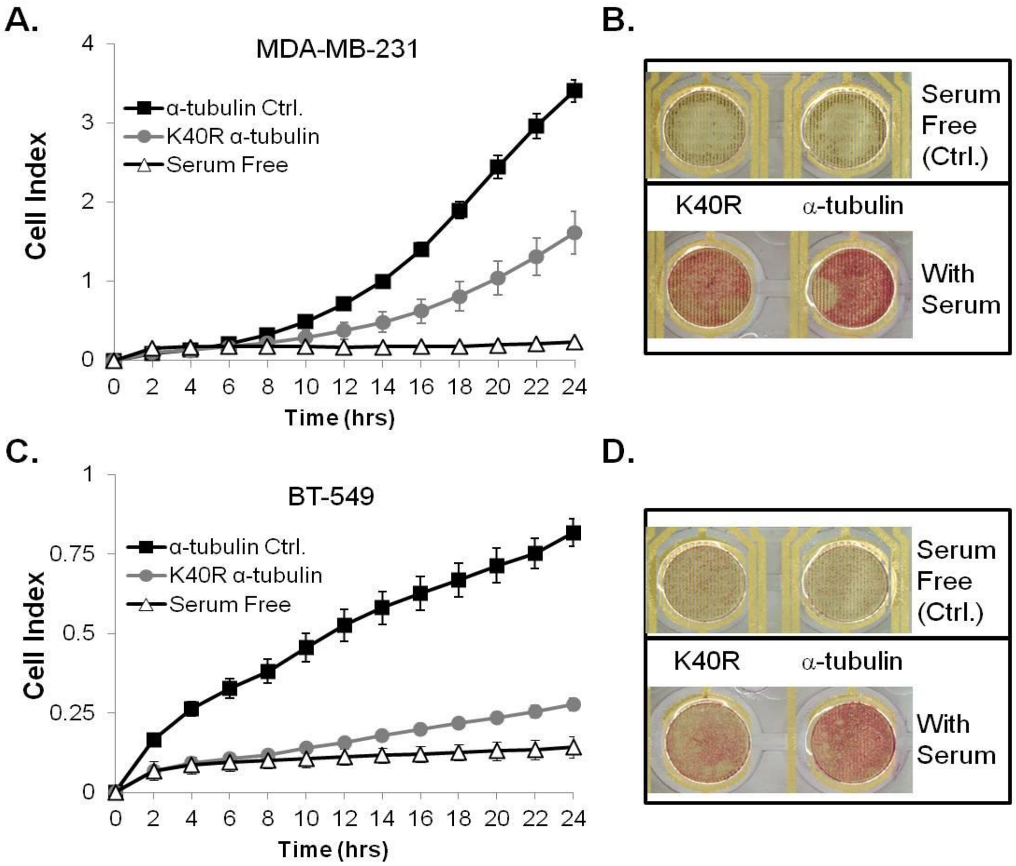


Figure 31: Migration of breast tumor cells is affected by reducing acetylated α-tubulin. Each cell line was plated in triplicate into serum-free media (upper chamber) and allowed to migrate towards the lower chamber containing 5% FBS for 24h. The serum-free control represents control cells plated in duplicate into serum-free media (upper chamber), where the lower chamber also contained serum-free media. Error bars indicate +/- standard deviation of the 3 experimental or 2 control wells/run. Graphs shown are representative of n=3 independent runs/cell line. Cell Index is a measure of movement from the upper to lower chamber. (A, C) Representative graphs of MDA-MB-231 or BT-549 stable pooled clones expressing the K40R α-tubulin mutant or the α-tubulin control. (B, D) The underside of the upper chamber was stained at 24h and representative images are shown. Modified from: *Boggs et al. 2014 Cancer Research: Under Review*

MCF-7 cells transiently overexpressing αTAT1-GFP or the GFP control showed no significant difference in chemotaxis over 24h, while the serum-free controls did not migrate (Figure 32A, B). This may suggest transient increases in acetylated tubulin are not sufficient to promote chemotaxis in a non-metastatic cell line.

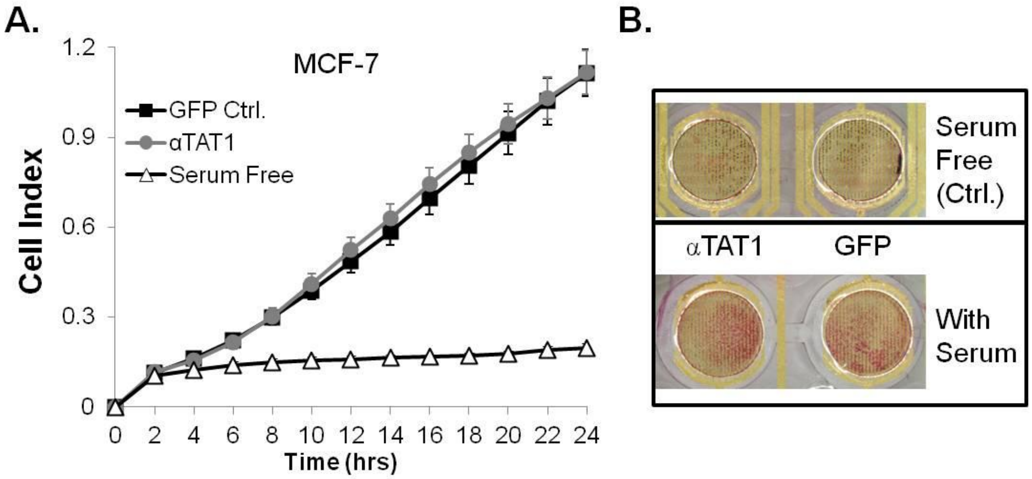


Figure 32: Transient overexpression of αTAT1 is not sufficient to enhance migration. (A) Real time migration was monitored as in Figure 31. MCF-7 cells were transiently transfected with either αTAT1 or GFP control 24h prior to experimentation. Graphs shown are representative of n=3 independent runs/cell line. Cell Index quantifies the change in electrical impedance measuring movement from the upper to lower chamber.

(B) The underside of the upper chamber was stained at 24h and representative images are shown to the right of the graph. Modified from: *Boggs et al. 2014 Cancer Research: Under Review*

Aim 2.3: Changes in α-tubulin acetylation status do not significantly affect invasion of metastatic breast cancer cell lines

Similar to migration, invasion of cancer cells requires a complex coordination of cytoskeletal elements (66). Although actin-based structures have been shown to promote invasion, stable microtubules could also play an important role in mediating this process (9). We found decreasing acetylated α-tubulin through the K40R mutant significantly decreased migration in metastatic MDA-MB-231 and BT-549 cells (Figure 31). Since MDA-MB-231 and BT-549 cell lines are also invasive *in vitro*, we utilized the stable K40R mutant and α-tubulin control to determine if decreasing acetylated α-tubulin can affect invasion. MCF-7 cells overexpressing αTAT1 were not utilized for this experiment since the parental cells are normally noninvasive.

In order to investigate invasion, we first performed a number of traditional invasion assays using Boyden chambers coated with ECM proteins. These experiments yielded inconsistent results. One of the biggest problems we encountered using these end-point assays was quantifying invasion. In order to better understand invasion in real-time, CIM-16 plates utilized for migration assays were coated with a thin layer of matrigel, a basement membrane matrix extracted from mouse sarcoma cells. Serum-containing media was added to the underside of the wells to act as a chemoattractant, while the cells were plated in serum-free media over the matrigel layer in the upper chamber. This promotes the invasion of cancer cells through the basement membrane matrix towards an increasing serum gradient.

When we carried out this real-time assay of cell invasion, it was difficult to determine if there were any significant differences in invasion between cells stably expressing the K40R α-tubulin mutant or the wild-type α-tubulin control (Figure 33A, B). Each time this experiment was carried out (n=3 for each set of stable cell lines), no significant trends could be obtained from the data. In fact, each subsequent run had an

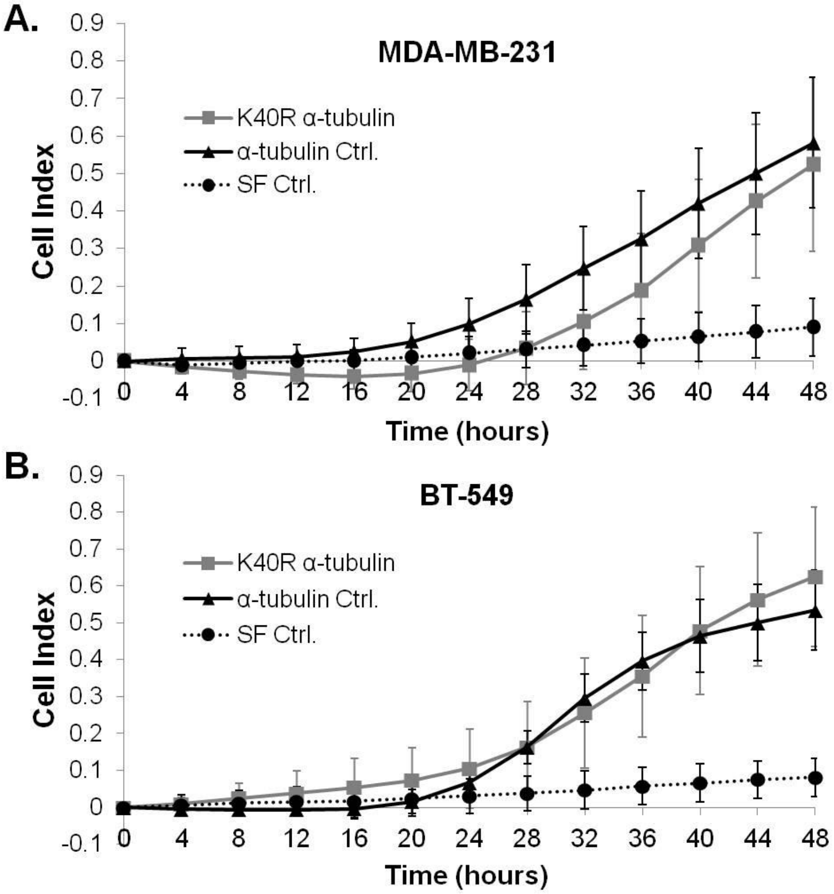


Figure 33: Decreasing acetylation of α-tubulin through stable K40R expression is not rate-limiting in invasion through a matrigel layer. MDA-MB-231 and BT-549 cells stably expressing the K40R mutant or wild-type α-tubulin control were subjected to a real-time invasion assay. This utilized the same protocol as the migration assays, except a thin layer of matrigel was coated on the upper chamber and Cell Index was read for 48h. SF = serum free. (A-B) Results suggest there may not be a significant difference in invasion between cells stably expressing K40R or the α-tubulin control. However, error bars indicate a wide range of recordings within the same experimental group per independent run. These studies will have to be optimized to better understand the impact of the K40R mutation on invasion of metastatic breast tumor cell lines. Figure Credit: *Amanda Boggs- Unpublished*

even bigger discrepancy between experimental groups, resulting in very large error bars. Because of this, we cannot conclude that there are any significant differences in invasion through a matrigel layer between cells stably expressing the K40R α-tubulin mutant or the wild-type α-tubulin control.

Summary and Discussion

Before carrying out assays investigating migration and invasion, we had to ensure the genetic alterations (K40R or αTAT1) affecting acetylation of α-tubulin did not affect cellular proliferation. If this was the case, results from migration and invasion assays could be skewed in favor of cells with higher proliferation rates. We found that stable expression of K40R α-tubulin in MDA-MB-231 and BT-549 cells as well as transient overexpression of αTAT1 in MCF-7 cells does not significantly affect cellular proliferation or viability under serum-free conditions. This enabled us to carry out longer-term assays (24-48h) without the confounding factor of significant differences in proliferation.

We then found reducing acetylation of α-tubulin through stable expression of the K40R mutant in metastatic breast cancer cells significantly inhibits migration towards a chemoattractant. However, transient αTAT1 overexpression in non-metastatic MCF-7 cells did not promote migration, as compared to control cells. This supports a conclusion that α-tubulin acetylation is required for proper migration in metastatic cells but is not sufficient to enhance migration in a non-metastatic cell line.

Unfortunately, the results of the invasion assay utilizing K40R stable expression in the metastatic cell lines will require further studies. There are many reasons this assay did not show any differences in invasion between the two experimental groups, even though significant differences were seen in chemotaxis studies. The process of invasion requires a complex coordination of not only cytoskeletal elements, but a number of other factors that promote degradation of BM and ECM proteins (1).

Also, previously mentioned studies in MDA-MB-231 cells overexpressing αTAT1 utilized type I collagen for invasion assays (103, 114). Matrigel was used in our studies and is composed mainly of laminin and collagen IV, proteins found in the basement membrane surrounding breast epithelial cells (124). Type I collagen is a stromal ECM protein, but has been shown to promote invasion in breast cancer models (125, 126). In ovarian cancer cells, it has been found that MMPs are not required for invasion through a layer of matrigel, but are required for invasion through collagen I (124). It has also been shown in breast cancer cells that certain MMPs are activated by collagen I but not by laminin (127). It is suggested that stable microtubules, most specifically acetylated α-tubulin, could promote invasion by trafficking MMPs along invadopodia to promote matrix degradation (9, 109). Future studies using collagen I instead of matrigel will help determine if acetylated α-tubulin promotes invasion of breast cancer cells in an MMP-dependent manner.

CHAPTER 5: SPECIFIC AIM 3

Examine α-tubulin acetylation in breast cancer patient samples

Introduction

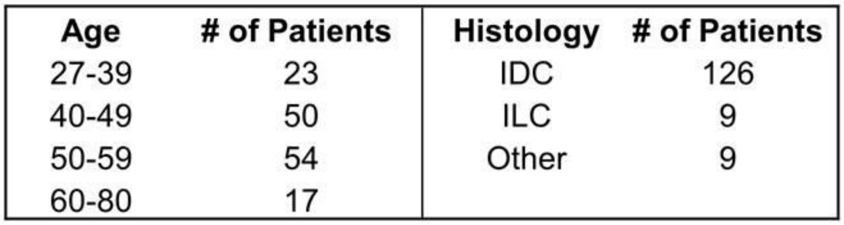
The *in vitro* studies in Aims 1-2 suggested α-tubulin acetylation may promote metastatic progression in breast cancer by enhancing McTNs, reattachment, and migration. The goal of this Specific Aim (Aim 3) is to elucidate the translational significance of α-tubulin acetylation for breast cancer patients. Matched primary and metastatic breast tumors were stained and examined for changes in α-tubulin acetylation intensity from the primary tumor to the matched metastatic tumor. Since tumors are highly heterogeneous, changes in protein expression or modifications could indicate a selective advantage for disseminated cells in metastatic progression.

We also investigated the possibility that acetylation could be associated with more aggressive breast cancer subtypes in a much larger patient cohort. As previously noted, breast cancers can be molecularly classified by one of four distinct subtypes: luminal A, luminal B, HER2+, and basal-like (128, 129). These subtypes have been associated with significantly different patient outcomes and can influence therapeutic strategies or encourage subsequent screening for distant metastases (23, 130). Basal-like breast cancers have poor patient prognosis with high metastasis rates, while the luminal A subtype is associated with a better response to therapy and higher patient survival rates (27, 131).

We utilized "The Cancer Genome Atlas" (TCGA) data-sets of primary tumor samples from over 400 patients and probed these tumors for acetylated α-tubulin using the Reverse Phase Protein Array (RPPA). This allowed us to make correlations between acetylated α-tubulin intensity and breast cancer patient subtype/prognosis because TCGA data sets contain important clinical information regarding the patient, their tumor, and metastatic events following treatment. Studying tumors associated with TCGA provides a major advantage to researchers in that the clinical information for each tumor core is readily available and this information is constantly being updated to reflect major medical changes for each enrolled patient (metastatic disease, new cancer diagnosis, death, etc.).

Aim 3.1: Acetylation of α-tubulin increases from primary tumor to lymph node metastases in breast cancer patients

To extend the *in vitro* findings that tubulin acetylation promotes McTN generation, tumor cell reattachment, and affects migration, tubulin acetylation was examined in patient tumor samples. Tumor microarrays of primary lesions and matched lymphatic metastases of 144 breast cancer patients were examined to determine if changes in tubulin acetylation status from patient primary to metastasis could reflect a selective advantage for primary tumor cells with increased tubulin acetylation during the metastatic process. Patient age and pathologic diagnosis can be viewed in Table 1.



Age	# of Patients	Histology	# of Patients
27-39	23	IDC	126
40-49	50	ILC	9
50-59	54	Other	9
60-80	17		

Table 1: Patient ages and pathological diagnosis for 144 matched patient samples. A range of ages and histologies were investigated to determine if there were changes in acetylated α-tubulin from the primary tumor to the matched lymph node metastasis. IDC=invasive ductal carcinoma, ILC=invasive lobular carcinoma. *Boggs et al. 2014 Cancer Research: Under Review*

Immunohistochemistry was carried out for acetylated α-tubulin and each primary and matched metastatic tumor sample was blindly scored for acetylated α-tubulin intensity by a board-certified pathologist. Figure 34 shows matched tumor core samples probed for acetylated α-tubulin at 8x and 20x magnification. Each horizontal set of tumors represents matched primary and metastatic samples from a single patient.

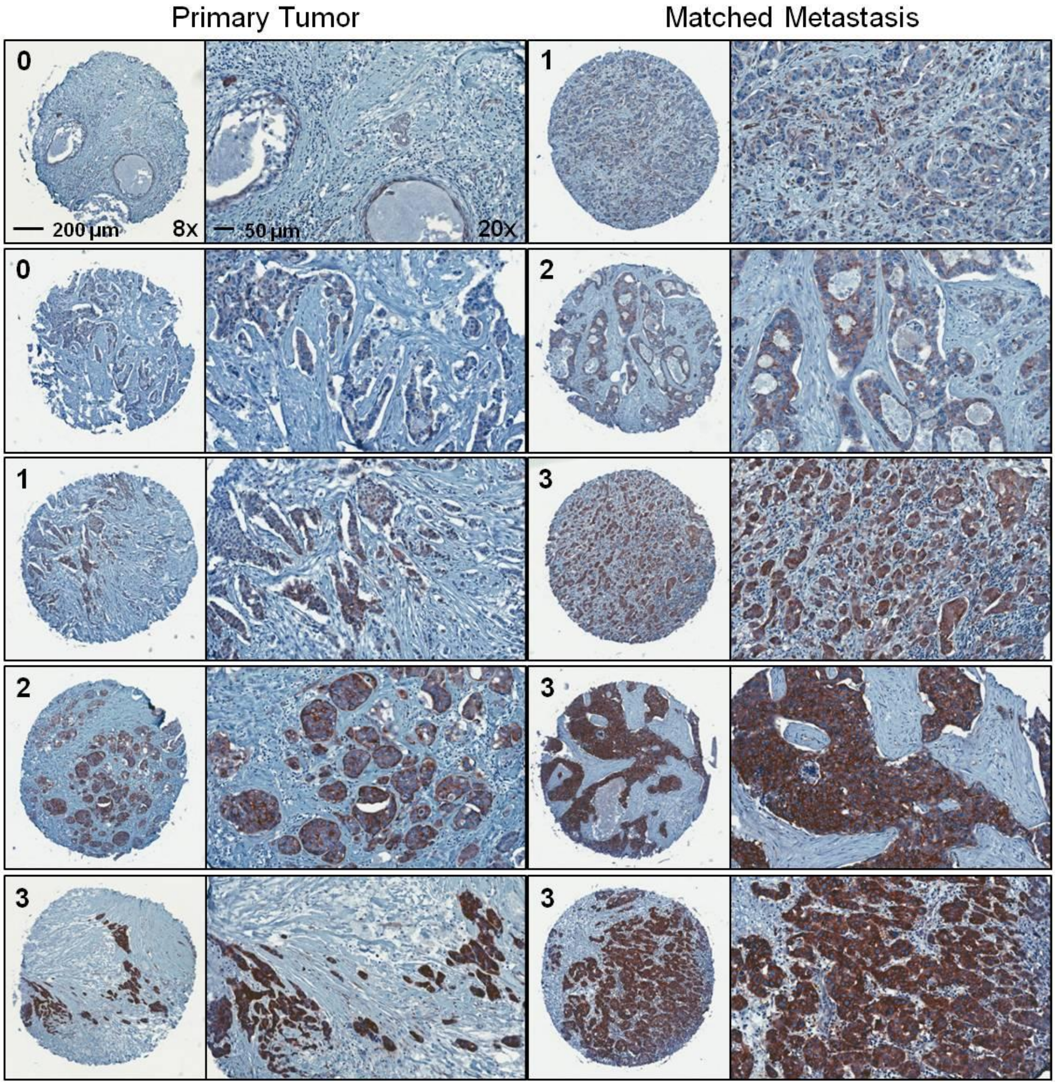
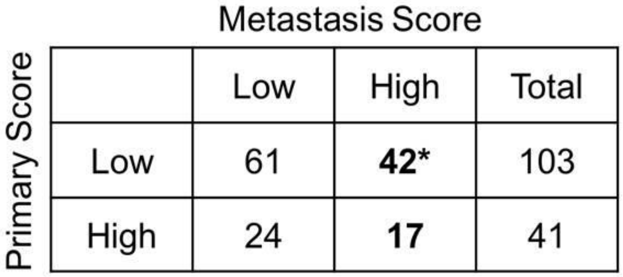


Figure 34: Acetylated α-tubulin is detected in patient primary and matched metastatic tumors. Representative images of matched patient primary invasive ductal carcinomas and lymph node metastases stained for acetylated α-tubulin. Horizontal pairings represent the primary tumor and the matched lymphatic metastasis from the same patient. 8x magnification image contains the tumor score (scale bar=200 µm) and is compared to 20x of the same sample (scale bar=50 µm). *Boggs et al. 2014 Cancer Research: Under Review*

The tumor score of 0-1 was considered "Low" for α-tubulin acetylation, while the score of 2-3 was considered "High" α-tubulin acetylation intensity within the breast primary tumor or metastasis. The two-sided exact McNemar's test for paired data revealed that based on the 144 samples, there is a statistically significant increase (*p=0.03) in acetylated α-tubulin score from the primary to matched metastasis in almost 30% of patients (42 total) (Table 2). 17 patients also maintained high acetylation in their metastasis, as compared to the primary tumor. Only 17% of patients decreased in acetylation intensity, as compared to the 41% of patients that increased or remained strongly positive from primary to matched lymph node metastasis (59 total).



Metastasis Score

Primary Score	Low	High	Total
Low	61	**42***	103
High	24	**17**	41

Table 2: Acetylated α-tubulin is increased from patient primary to matched metastatic tumors. Tumor scores for primary and matched lymph node metastases were compared for 144 patients. Tumor score of 0-1 is "Low"; 2-3 is "High" α-tubulin acetylation. McNemar’s test was carried out and the two-sided exact test revealed that these rates were statistically different: *p-value=0.03. *Boggs et al. 2014 Cancer Research: Under Review*

Aim 3.2: High α-tubulin acetylation is associated with the aggressive basal-like breast cancer subtype

We next examined a large primary tumor sample set associated with The Cancer Genome Atlas (TCGA). TCGA is a cancer genomics program supported by the National Cancer Institute and the National Human Genome Research Institute of the National Institutes of Health (132). This program provides access to patient clinical information to correspond with genomic and proteomic analysis of individual tumors (TCGA Data Portal: https://tcga-data.nci.nih.gov/tcga/). A TCGA breast tumor sample set was probed for acetylated α-tubulin intensity using the Reverse Phase Protein Array (RPPA) (Figure 35) (133) to determine if acetylated α-tubulin correlates with patient subtype.

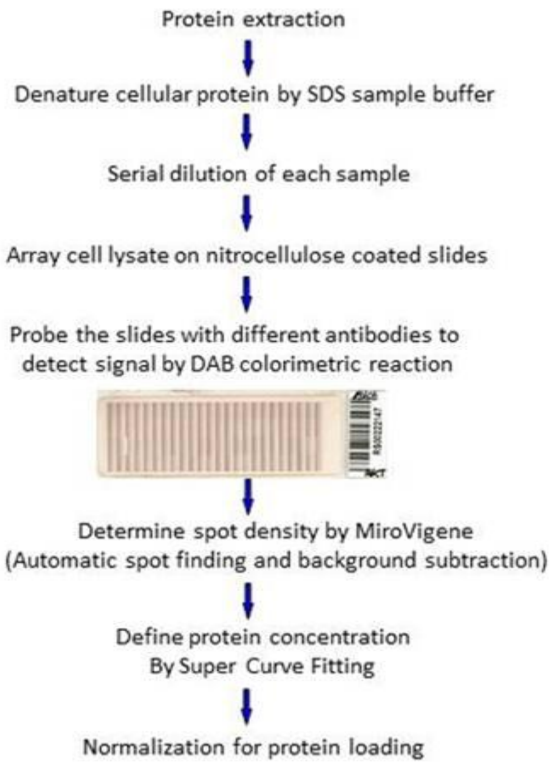


Figure 35: Reverse Phase Protein Array (RPPA) Process. RPPA utilizes a multi-step process to extract proteins from patient tumor samples for analysis.
Image source: MD Anderson Cancer Center- http://www.mdanderson.org

A large patient cohort (n=402) representing a wide range of ages and histologies, including the most commonly diagnosed histologies, IDC and ILC, was investigated (Table 3).

Age	# of Patients	Histology	# of Patients
26-39	36	IDC	348
40-49	74	ILC	30
50-59	103	Medullary	1
60-69	118	Mixed	7
70-79	46	Mucinous	2
80+	25	Other	14

Total: 402 patients

Table 3: Patient age at diagnosis and tumor histological information. The ages and histologies of patient tumors examined are shown (n=402). IDC= Invasive Ductal Carcinoma, ILC= Invasive Lobular Carcinoma. *Boggs et al. 2014 Cancer Research: Under Review*

When patients were separated by breast cancer subtype, over 72% (60/83) of patients diagnosed with basal-like breast cancer had high acetylated α-tubulin, as compared to median acetylation intensity of all 402 patients (Figure 36A). The non-basal subtypes combined did not show a strong association with acetylation; 56% had low

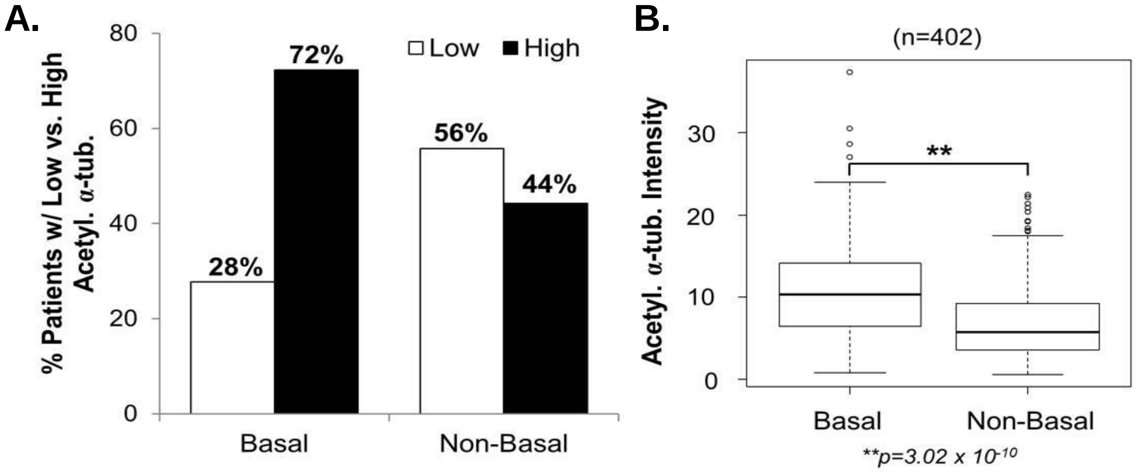


Figure 36: Basal-like breast cancers have high acetylation of α-tubulin. (A) Acetylated α-tubulin intensity of basal-like vs. non-basal cancers. Acetylation was considered "Low" if it was below and "High" if it was above the median acetylation of n=402 patients. (B) Raw intensity for acetylated α-tubulin in basal-like (n=83) and non-basal breast cancers (n=319) are compared. **p<0.001. There is over a 1.5-fold difference between acetylation intensity in basal-like and non-basal cancers. *Boggs et al. 2014 Cancer Research: Under Review*

while 44% had high α-tubulin acetylation (Figure 36A). Comparing basal-like to all non-basal breast cancers reveals significantly increased acetylated α-tubulin in the basal-like breast cancers, with over 1.5-fold higher acetylation than non-basal cancers (Figure 36B).

When separated out by subtype, basal-like tumors had an average acetylated α-tubulin intensity around 1.5x higher than the luminal subtypes and over 2x higher than HER2 subtype tumors (Figure 37). HER2+ patients averaged the lowest acetylation of α-tubulin but had considerably fewer patients than the basal or luminal groups. It is important to note the "Normal" subtype was given as classification for 5 of the patient tumors in the sample set of 402 patients. The normal subtype classification was given to breast tumors that presented molecular markers reminiscent of the normal breast (128). More recently, the normal subtype is not included in breast cancer subtyping. Despite these changes, we did not want to disregard the patients categorized under this subtype and chose to present their clinical and molecular information under the classification system assigned to them by TCGA.

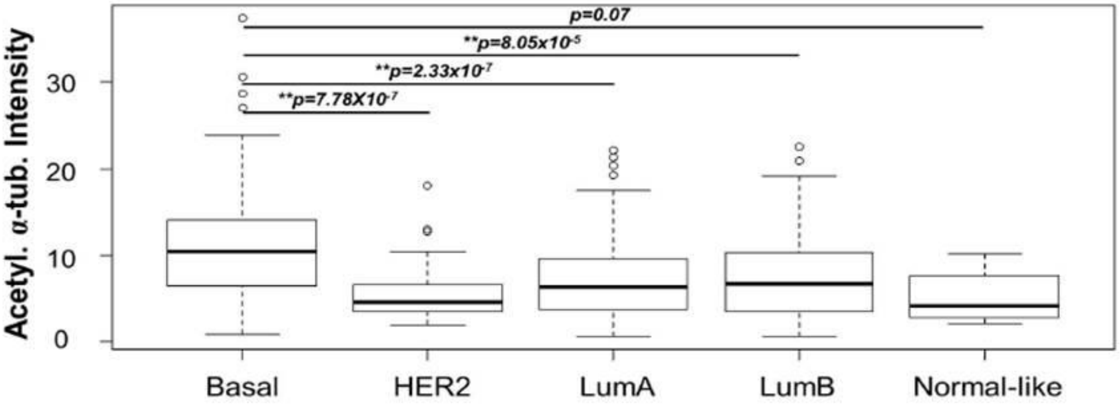


Figure 37: Acetylated α-tubulin intensity separated by tumor subtype. Box plots represent the average acetylation intensity as well as the range of intensities seen across each specific subtype. Basal-like subtype tumors have higher average acetylated α-tubulin intensity than the remainder of the subtypes. **p<0.001 between basal-like (n=83) and HER2 (n=48), luminal A (n=169) and luminal B (n=97). p=0.07 between basal and normal-like subtypes because of the small number of patients (n=5) in the normal-like subtype. *Boggs et al. 2014 Cancer Research: Under Review*

Thus far, 14 of the 402 patients screened in this sample set formed metastases after initial treatment. Trends for α-tubulin acetylation in metastases arising from the non-basal subtypes followed what was seen for overall tubulin acetylation across the population of 402 patients (compare Figures 37 and 38). Interestingly, all of the basal-like breast cancer patients that metastasized after treatment (n=5) had high α-tubulin acetylation in their primary tumor (Figure 38).

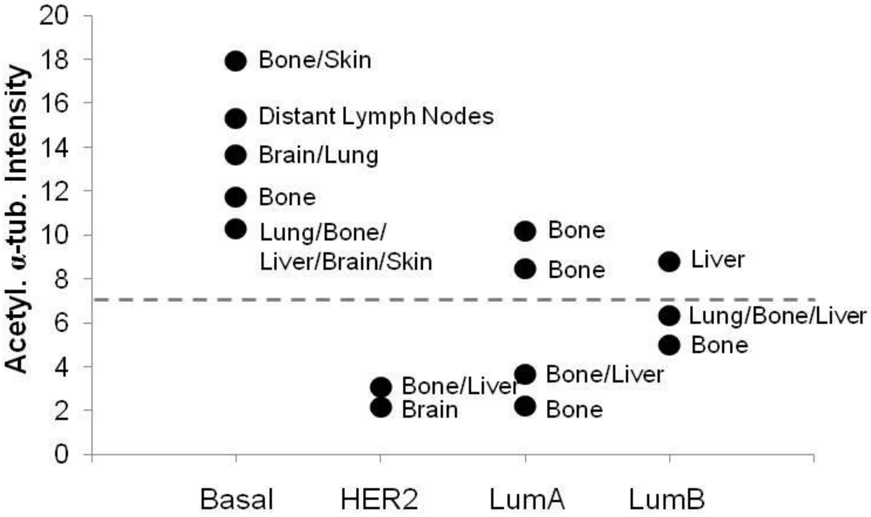


Figure 38: Basal-like breast tumors that metastasized had high acetylated tubulin intensity in the primary tumor. Each dot represents a patient that formed a distant metastasis subsequent to treatment and matches it to the intensity of acetylated α-tubulin in their primary tumor. The site of metastatic outgrowth is indicated. The dotted line represents the median acetylated tubulin intensity across the cohort of 402 patients. *Boggs et al. 2014 Cancer Research: Under Review*

Summary and Discussion

Our current results show α-tubulin acetylation is increased from the primary tumor to the matched lymph node metastasis in 30% of patients (42/144) while high acetylation was detected in 41% of screened breast cancer metastases (59/144). This could indicate that high α-tubulin acetylation gives some sort of advantage to disseminated cells in the metastatic process. The studies carried out in Aims 1-2 suggest this could be due to acetylated α-tubulin enhancing pro-metastatic phenotypes like increased McTN formation and reattachment as well as migration.

We also report for the first time that α-tubulin acetylation is associated with the aggressive basal-like breast cancer subtype in patients. We found that over 72% of basal-like tumors had high α-tubulin acetylation. The average acetylated tubulin intensity for basal tumors was approximately 1.5x higher than the luminal subtypes and over 2x the average for HER2+ tumors. Also, all basal-like primary tumors that metastasized to distant sites had high α-tubulin acetylation. Future studies will investigate an even larger breast cancer patient cohort, since our collaborators at MD Anderson have analyzed approximately 187 proteins and protein modifications in over 740 breast cancer patients. Once the RPPA data concerning acetylation of α-tubulin has been collected for more patients, more substantial analyses can be made on the basis of breast cancer subtype and prognosis.

CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

McTNs are microtubule-based membrane protrusions formed upon suspension that have been found to play a role in CTC aggregation and reattachment to an endothelial monolayer (7, 36, 60-62). Previous McTN-based studies have focused on the specific α-tubulin PTM of detyrosination, since it was first to be found localized to McTNs in suspended tumor cells (6). High detyrosination of α-tubulin is also associated with poor patient prognosis in breast cancer (72). Although a number of studies have investigated the role of detyrosination in McTN formation and function, a second PTM associated with microtubule stability, α-tubulin acetylation, has been largely overlooked.

Through these studies, we have elucidated a novel role for acetylated α-tubulin in McTN formation and function. We found metastatic breast tumor cell lines have high acetylation of α-tubulin that is enriched in McTN protrusions upon suspension. Modifying the acetylation site on α-tubulin through the K40R point mutation significantly decreases McTN frequency in metastatic cell lines while increasing acetylation via αTAT1 overexpression in a less aggressive cell line increases McTNs. Manipulation of acetylated α-tubulin though mutation and enzymatic regulation also shows suspended cell reattachment is dependent upon this modification. Stable McTNs have been associated previously with enhanced reattachment to endothelial monolayers (36) and CTC lung trapping in a murine metastasis model (59). Here we find the specific PTM of α-tubulin acetylation enhances McTN formation to promote suspended cell reattachment, one key and potentially targetable step in the metastatic cascade.

To validate our findings *in vivo*, future studies could be carried out to investigate the significance of altering α-tubulin acetylation in a mouse model of metastasis. Stable luciferase-expressing metastatic breast tumor cells with the K40R α-tubulin mutant or wild-type α-tubulin control would be injected into the tail vein of mice. Since the lungs are the first capillary bed to be encountered by these circulating human breast tumor cells, trapping and retention in the lung could be monitored by measuring bioluminescence over time. Similar to what has been previously done in the lab (Figure 39) (59, 63), this will help determine if decreasing acetylation of α-tubulin can inhibit lung-trapping in an *in vivo* metastasis model. This may further suggest this modification could be an ideal target in preventing CTC reattachment.

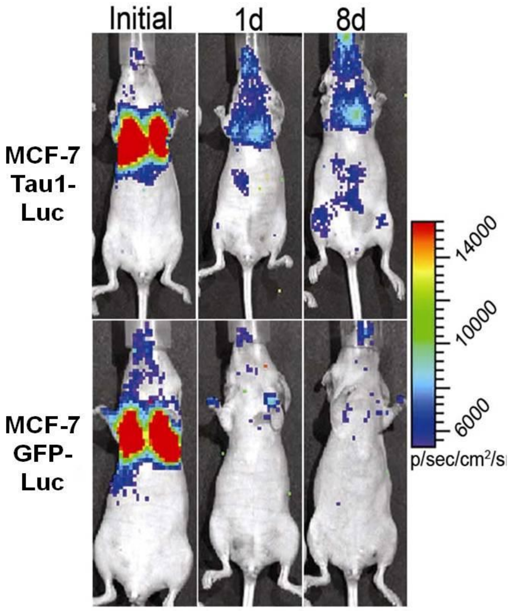


Figure 39: Murine metastasis model of CTC lung trapping. Previous studies in the lab have injected stable luciferase-expressing cell lines (Luc) into the tail vein of mice. Bioluminescence imaging is able to monitor trapping of these cells in the lungs over time.
These studies found that cells with high McTN frequency (MCF-7-Tau-Luc) retained in the lung longer, as compared to controls (MCF-7-GFP-Luc). After 8 days, there was still significant detectable signal in the lungs in the tau-expressing cells, whereas the control cells were mostly cleared. Experiment performed by M. Matrone and K. Thompson.
Figure modified from: (7)

Migration is another necessary step in cancer dissemination. However, the role of stabilized microtubules in migration is greatly understudied, as compared to the more established role of actin. It has been shown that a reduction in acetylated α-tubulin impairs migration in neuronal cell lines (99, 100), but little is known about how it affects cancer cell motility. We find that chemotaxis is significantly reduced with overexpression of the K40R α-tubulin mutant in metastatic breast tumor cell lines. This suggests acetylation may be necessary for proper chemotaxis in more invasive breast cancer cells. We did not find a significant difference in migration of non-metastatic MCF-7 cells transiently overexpressing αTAT1. Transient increases in α-tubulin acetylation were not sufficient to promote migration in a non-metastatic breast cancer cell line. Future studies could determine if increases in acetylation can further enhance migratory capabilities by investigating αTAT1 overexpression in metastatic breast tumor cell lines.

Our results indicate αTAT1 overexpression increases McTNs and reattachment efficiency while others have utilized the metastatic MDA-MB-231 breast cancer cell line and found that silencing αTAT1 inhibits proper migration (103) as well as invasion through a collagen matrix (114). Very recently, it has also been found that α-tubulin acetylation promoted by αTAT1 activity (and not inhibition of tubulin deacetylases) is an induced stress response that promotes cell survival through induction of autophagy (134). Together, these studies suggest that αTAT1 can enhance a number of metastatic properties like cell survival, migration, reattachment, and invasion, and could potentially serve as a therapeutic target in metastasis prevention. However, there are no known chemical inhibitors of αTAT1 for current investigative use. Until inhibitors are developed, insight can be gained from current tools that could affect α-tubulin acetylation. αTAT1 is thought to be the major tubulin acetyltransferase *in vivo*, but histone acetyltransferases (HATs) like Gcn5 (86) have also been shown to acetylate α-tubulin. Interestingly, overexpression of Gcn5 is linked to progression of acute lymphoblastic leukemia (ALL) and enhanced lung cancer growth and is currently under investigation as a potential chemotherapeutic target (135, 136). HAT inhibitors have also been shown to have potent anti-tumor activity against triple-negative breast cancer xenografts *in vivo* (137). These targeted therapies are mainly aimed at inhibiting histone acetyltransferase activity but should be further investigated to determine if HAT inhibition could also reduce α-tubulin acetylation at clinically tolerable levels. This dual mechanism of action may prove to be more efficacious in certain cancers, specifically those with high acetylation of α-tubulin.

Future studies could knock down αTAT1 in metastatic breast tumor cell lines using siRNA to investigate the effects on McTN formation, reattachment, and migration. Three groups have knocked down αTAT1 utilizing commercially available siRNA pools and found this significantly decreases endogenous α-tubulin acetylation (103, 114, 134). We obtained the published pool of four siRNAs against αTAT1 (along with scrambled controls) and began preliminary experiments in both MDA-MB-231 and BT-549 cells. One hurdle to overcome is that we, along with other groups (103, 114, 134), have not had success identifying any commercially available antibodies against αTAT1. Instead of being able to immunoblot for αTAT1 to evaluate knockdown efficiency, the only available readout has been a decrease in acetylation of α-tubulin. An alternative strategy for determining knockdown efficiency could utilize RT-PCR to monitor αTAT1 mRNA changes due to siRNA.

HDAC inhibitors are currently approved by the FDA for the treatment of hematologic malignancies and are being investigated in a number of different cancers, including triple-negative breast cancer, through ongoing clinical trials (138, 139). Although the exact mechanism of action of these compounds is yet to be elucidated, the anti-cancer properties of HDAC inhibitors are thought to come from histone deacetylase activity and regulation of cell fate (140). However, our results suggest caution when utilizing therapeutic treatments like HDAC inhibitors that would not only block tumor growth, but could also raise acetylated α-tubulin as an off-target effect. HDAC6 is a major tubulin deacetylase that acts to reduce α-tubulin acetylation in the cytoplasm (80). Small-molecule HDAC inhibitors can bind and inhibit the activity of a number of HDACs in both the nucleus and cytoplasm (140), and the consequences of these off-target effects have not yet been determined. Paramount to the current studies, inhibition of HDAC6 would result in an increase in α-tubulin acetylation and very little is known about how these therapies could influence CTC metastasis. Since clinical cancer imaging requires a foci of at least 10^7 cells (141), current interpretations of drug effects are limited to large changes in tumor size rather than specific effects on metastatic dissemination. The ability of elevated α-tubulin acetylation to increase McTNs, promote tumor cell reattachment and migration emphasizes the importance of understanding how this modification influences metastasis so therapies aimed at tumor growth do not inadvertently elevate metastatic risk.

In order to study the translational significance of α-tubulin acetylation, we investigated matched primary tumor and metastatic lymph node samples. These patient studies indicated α-tubulin acetylation is increased from the primary tumor to the matched lymph node metastasis in 30% of patients (42/144) while high acetylation was detected in 41% of screened breast cancer metastases (59/144). The dependence upon endogenous acetylation for reattachment and migration of metastatic breast tumor cell lines and the increase of α-tubulin acetylation in metastatic tumors support a model in which tubulin acetylation confers a selective advantage for disseminated tumors during the metastatic process. This model is further supported by recent evidence that high tubulin acetylation is associated with lymph node metastasis and poor patient prognosis in head and neck squamous cell carcinoma (116).

A large patient cohort (n=402) associated with The Cancer Genome Atlas (TCGA) was also investigated to determine if acetylated α-tubulin intensity correlates with breast cancer patient prognostic factors. The clinical correlations did reveal a significant association between the basal-like breast cancer subtype and high α-tubulin acetylation. Basal-like breast cancers are negative for estrogen receptor (ER) or progesterone receptor (PR)-responsive genes and genes associated with HER2 amplification (128). This aggressive subtype is correlated with increased risk of metastatic spread and overall poor patient prognosis (23, 27). Although there is consensus for what characteristics basal-like tumors lack (ER/PR/HER2), there are very few positive markers that can help define the basal-like subtype (27). This also hampers development of targeted therapies to enhance survival rates of patients with basal-like breast tumors. Our studies found that over 72% of basal-like tumors had high α-tubulin acetylation and the average acetylated tubulin intensity for basal tumors was approximately 1.5x higher than the luminal subtypes. This is very interesting, since it has been found that HDAC6 (tubulin deacetylase that decreases α-tubulin acetylation) is an estrogen-regulated gene (84). Luminal subtypes are positive for ER and tend to have high expression of ER-regulated genes (11, 23). It has also been shown that HDAC6 and ERα co-localize at the plasma membrane (142), although the resulting phenotypic changes are unknown.

We also found that all basal-like primary tumors that metastasized to distant sites had high α-tubulin acetylation (n=5). It is difficult to make conclusions with such a small sample set of patients across all subtypes that formed metastatic disease (n=14). While having very few patients progress metastatically is obviously a desirable clinical outcome, having a limited number of patients with distant metastases that were also therapeutically treated differently creates challenges for determining the significance of experimental findings. With many breast cancer metastases occurring 10-15+ years after diagnosis, most specifically in luminal cancers (143), follow-up on the rest of the patient cohort within the next few decades will help identify possible correlations between primary tumor characteristics and the potential for metastatic spread. Mouse models of metastatic basal-like breast cancer could also be utilized in the short-term.

Despite the need for treatments to prevent metastatic dissemination, there are a number of challenges in developing such therapies. A major challenge in targeting metastasis is that tumor cells can leave the primary site and begin metastatic progression before cancer is clinically detected (144). Although CTCs can enter the bloodstream before diagnosis, the early steps of the metastatic cascade are still reasonable targets for therapeutic intervention. Tumor cells have been shown in animal models to reenter the circulation and seed other metastatic sites as well as self-seed the primary tumor during cancer progression (29, 145). Surgery to remove a primary tumor alone seeds millions of cells into the patient's bloodstream (146) and CTCs can be detected in a patient's blood for many years after primary tumor resection (48). Anti-metastatic therapies may be most effective prior to surgery on the primary tumor or in patients at high risk of disseminated disease (29). A key advantage of targeting disseminated and circulating cells is that they are more accessible for chemotherapeutics in the bloodstream (29). Ideally, targeted therapies against dissemination, reattachment, or invasion could be combined with existing cancer treatments to control or prevent metastatic disease (29, 121).

The current results define a novel mechanism where acetylation of lysine 40 of α-tubulin promotes microtentacle generation, tumor cell reattachment, and chemotaxis that are selective advantages during metastasis. Figure 40 summarizes each of the steps in metastatic progression that are impacted *in vitro* by alterations in acetylated α-tubulin.

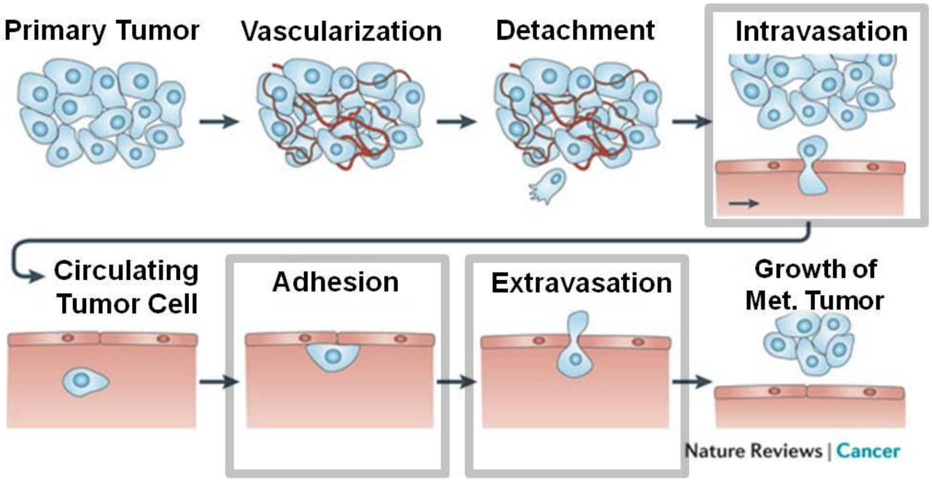


Figure 40: Acetylated α-tubulin as a therapeutic target in metastatic progression. A simplified version of the metastatic cascade begins with vascularization of the primary tumor that leads to tumor cells leaving the breast. Intravasation and extravasation of disseminated tumor cells are influenced by migration, while adhesion of circulating tumor cells is a microtubule-dependent process facilitated by McTNs. Acetylation of α-tubulin has been shown to affect both McTN formation/ reattachment of suspended tumor cells as well as migration (chemotaxis). Each box represents a step in the metastatic cascade that could be affected by alterations in acetylated α-tubulin and opportunities for development of targeted therapies to prevent outgrowth of a metastatic (Met.) tumor. Figure modified from: (10)

In patients, acetylated α-tubulin is also particularly enriched in basal-like breast cancers and increased in a significant number of lymph node metastases. The resulting opportunity for α-tubulin acetylation to serve as both a diagnostic and therapeutic target for metastatic breast cancer will be an important avenue of ongoing investigation.

REFERENCES

1. Reymond N, d'Agua BB, Ridley AJ. Crossing the endothelial barrier during metastasis. Nat Rev Cancer. 2013;13:858-70.

2. Polyak K, Metzger Filho O. SnapShot: breast cancer. Cancer Cell. 2012;22:562-e1.

3. Cheeseman IM, Desai A. Molecular architecture of the kinetochore-microtubule interface. Nat Rev Mol Cell Biol. 2008;9:33-46.

4. Janke C, Bulinski JC. Post-translational regulation of the microtubule cytoskeleton: mechanisms and functions. Nat Rev Mol Cell Biol. 2011;12:773-86.

5. Akhshi TK, Wernike D, Piekny A. Microtubules and actin crosstalk in cell migration and division. Cytoskeleton (Hoboken). 2014;71:1-23.

6. Whipple RA, Cheung AM, Martin SS. Detyrosinated microtubule protrusions in suspended mammary epithelial cells promote reattachment. Exp Cell Res. 2007;313:1326-36.

7. Matrone MA, Whipple RA, Balzer EM, Martin SS. Microtentacles tip the balance of cytoskeletal forces in circulating tumor cells. Cancer Res. 2010;70:7737-41.

8. Yu M, Stott S, Toner M, Maheswaran S, Haber DA. Circulating tumor cells: approaches to isolation and characterization. J Cell Biol. 2011;192:373-82.

9. Schoumacher M, Goldman RD, Louvard D, Vignjevic DM. Actin, microtubules, and vimentin intermediate filaments cooperate for elongation of invadopodia. J Cell Biol. 2010;189:541-56.

10. Wirtz D, Konstantopoulos K, Searson PC. The physics of cancer: the role of physical interactions and mechanical forces in metastasis. Nat Rev Cancer. 2011;11:512-22.

11. ACS. American Cancer Society Cancer Facts & Figures. Atlanta, GA: American Cancer Society; 2013.

12. Hutchinson L. Breast cancer: challenges, controversies, breakthroughs. Nat Rev Clin Oncol. 2010;7:669-70.

13. Bertos NR, Park M. Breast cancer - one term, many entities? J Clin Invest. 2011;121:3789-96.

14. Yerushalmi R, Hayes MM, Gelmon KA. Breast carcinoma--rare types: review of the literature. Ann Oncol. 2009;20:1763-70.

15. Rakha EA, Reis-Filho JS, Baehner F, Dabbs DJ, Decker T, Eusebi V, et al. Breast cancer prognostic classification in the molecular era: the role of histological grade. Breast Cancer Res. 2010;12:207.

16. Engstrom MJ, Opdahl S, Hagen AI, Romundstad PR, Akslen LA, Haugen OA, et al. Molecular subtypes, histopathological grade and survival in a historic cohort of breast cancer patients. Breast Cancer Res Treat. 2013;140:463-73.

17. Ignatiadis M, Sotiriou C. Understanding the molecular basis of histologic grade. Pathobiology. 2008;75:104-11.

18. Webster LR, Lee SF, Ringland C, Morey AL, Hanby AM, Morgan G, et al. Poor-prognosis estrogen receptor-positive breast cancer identified by histopathologic subclassification. Clin Cancer Res. 2008;14:6625-33.

19. Haibe-Kains B, Desmedt C, Loi S, Culhane AC, Bontempi G, Quackenbush J, et al. A three-gene model to robustly identify breast cancer molecular subtypes. J Natl Cancer Inst. 2012;104:311-25.

20. Parker JS, Mullins M, Cheang MC, Leung S, Voduc D, Vickery T, et al. Supervised risk predictor of breast cancer based on intrinsic subtypes. J Clin Oncol. 2009;27:1160-7.

21. Prat A, Parker JS, Fan C, Perou CM. PAM50 assay and the three-gene model for identifying the major and clinically relevant molecular subtypes of breast cancer. Breast Cancer Res Treat. 2012;135:301-6.

22. Tran B, Bedard PL. Luminal-B breast cancer and novel therapeutic targets. Breast Cancer Res. 2011;13:221.

23. Kennecke H, Yerushalmi R, Woods R, Cheang MC, Voduc D, Speers CH, et al. Metastatic behavior of breast cancer subtypes. J Clin Oncol. 2010;28:3271-7.

24. Chien AJ, Rugo HS. Emerging treatment options for the management of brain metastases in patients with HER2-positive metastatic breast cancer. Breast Cancer Res Treat. 2013;137:1-12.

25. Ahn ER, Vogel CL. Dual HER2-targeted approaches in HER2-positive breast cancer. Breast Cancer Res Treat. 2012;131:371-83.

26. Prat A, Adamo B, Cheang MC, Anders CK, Carey LA, Perou CM. Molecular characterization of basal-like and non-basal-like triple-negative breast cancer. Oncologist. 2013;18:123-33.

27. Valentin MD, da Silva SD, Privat M, Alaoui-Jamali M, Bignon YJ. Molecular insights on basal-like breast cancer. Breast Cancer Res Treat. 2012;134:21-30.

28. Howlader N, Noone AM, Krapcho M, Neyman N, Aminou R, Waldron W, et al. SEER Cancer Statistics Review, 1975-2008, National Cancer Institute. 2011; based on November 2010 SEER data submission, posted to the SEER web site, 1.]. Available from: http://seer.cancer.gov/csr/1975_2008/

29. Labelle M, Hynes RO. The initial hours of metastasis: the importance of cooperative host-tumor cell interactions during hematogenous dissemination. Cancer Discov. 2012;2:1091-9.

30. Weigelt B, Peterse JL, van 't Veer LJ. Breast cancer metastasis: markers and models. Nat Rev Cancer. 2005;5:591-602.

31. Nguyen DX, Bos PD, Massague J. Metastasis: from dissemination to organ-specific colonization. Nat Rev Cancer. 2009;9:274-84.

32. Mego M, Mani SA, Cristofanilli M. Molecular mechanisms of metastasis in breast cancer--clinical applications. Nat Rev Clin Oncol. 2010;7:693-701.

33. Wan L, Pantel K, Kang Y. Tumor metastasis: moving new biological insights into the clinic. Nat Med. 2013;19:1450-64.

34. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Invest. 2009;119:1420-8.

35. Bonnomet A, Brysse A, Tachsidis A, Waltham M, Thompson EW, Polette M, et al. Epithelial-to-mesenchymal transitions and circulating tumor cells. J Mammary Gland Biol Neoplasia. 2010;15:261-73.

36. Whipple RA, Matrone MA, Cho EH, Balzer EM, Vitolo MI, Yoon JR, et al. Epithelial-to-mesenchymal transition promotes tubulin detyrosination and microtentacles that enhance endothelial engagement. Cancer Res. 2010;70:8127-37.

37. Charpentier MS, Whipple RA, Vitolo MI, Boggs AE, Slovic J, Thompson KN, et al. Curcumin targets breast cancer stem-like cells with microtentacles that persist in mammospheres and promote reattachment. Cancer Res. 2014;74:1250-60.

38. Charpentier M, Martin S. Interplay of Stem Cell Characteristics, EMT, and Microtentacles in Circulating Breast Tumor Cells. Cancers (Basel). 2013;5:1545-65.

39. Chambers AF, Groom AC, MacDonald IC. Dissemination and growth of cancer cells in metastatic sites. Nat Rev Cancer. 2002;2:563-72.

40. Balkwill FR. The chemokine system and cancer. J Pathol. 2012;226:148-57.

41. Teicher BA, Fricker SP. CXCL12 (SDF-1)/CXCR4 pathway in cancer. Clin Cancer Res. 2010;16:2927-31.

42. Luker KE, Lewin SA, Mihalko LA, Schmidt BT, Winkler JS, Coggins NL, et al. Scavenging of CXCL12 by CXCR7 promotes tumor growth and metastasis of CXCR4-positive breast cancer cells. Oncogene. 2012;31:4750-8.

43. Ma X, Norsworthy K, Kundu N, Rodgers WH, Gimotty PA, Goloubeva O, et al. CXCR3 expression is associated with poor survival in breast cancer and promotes metastasis in a murine model. Mol Cancer Ther. 2009;8:490-8.

44. Zlotnik A, Burkhardt AM, Homey B. Homeostatic chemokine receptors and organ-specific metastasis. Nat Rev Immunol. 2011;11:597-606.

45. Bednarz-Knoll N, Alix-Panabieres C, Pantel K. Clinical relevance and biology of circulating tumor cells. Breast Cancer Res. 2011;13:228.

46. Baccelli I, Schneeweiss A, Riethdorf S, Stenzinger A, Schillert A, Vogel V, et al. Identification of a population of blood circulating tumor cells from breast cancer patients that initiates metastasis in a xenograft assay. Nat Biotechnol. 2013;31:539-44.

47. Cristofanilli M. Circulating tumour cells: telling the truth about metastasis. Lancet Oncol. 2014;15:365-6.

48. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med. 2004;351:781-91.

49. Cristofanilli M, Hayes DF, Budd GT, Ellis MJ, Stopeck A, Reuben JM, et al. Circulating tumor cells: a novel prognostic factor for newly diagnosed metastatic breast cancer. J Clin Oncol. 2005;23:1420-30.

50. Bidard FC, Madic J, Mariani P, Piperno-Neumann S, Rampanou A, Servois V, et al. Detection rate and prognostic value of circulating tumor cells and circulating tumor DNA in metastatic uveal melanoma. Int J Cancer. 2014;134:1207-13.

51. Liu MC, Shields PG, Warren RD, Cohen P, Wilkinson M, Ottaviano YL, et al. Circulating tumor cells: a useful predictor of treatment efficacy in metastatic breast cancer. J Clin Oncol. 2009;27:5153-9.

52. Slade MJ, Payne R, Riethdorf S, Ward B, Zaidi SA, Stebbing J, et al. Comparison of bone marrow, disseminated tumour cells and blood-circulating tumour cells in breast cancer patients after primary treatment. Br J Cancer. 2009;100:160-6.

53. Muller V, Stahmann N, Riethdorf S, Rau T, Zabel T, Goetz A, et al. Circulating tumor cells in breast cancer: correlation to bone marrow micrometastases, heterogeneous response to systemic therapy and low proliferative activity. Clin Cancer Res. 2005;11:3678-85.

54. Hekimian K, Meisezahl S, Trompelt K, Rabenstein C, Pachmann K. Epithelial cell dissemination and readhesion: analysis of factors contributing to metastasis formation in breast cancer. ISRN Oncol. 2012;2012:601810.

55. Labelle M, Begum S, Hynes RO. Direct signaling between platelets and cancer cells induces an epithelial-mesenchymal-like transition and promotes metastasis. Cancer Cell. 2011;20:576-90.

56. Korb T, Schluter K, Enns A, Spiegel HU, Senninger N, Nicolson GL, et al. Integrity of actin fibers and microtubules influences metastatic tumor cell adhesion. Exp Cell Res. 2004;299:236-47.

57. Craig DH, Owen CR, Conway WC, Walsh MF, Downey C, Basson MD. Colchicine inhibits pressure-induced tumor cell implantation within surgical wounds and enhances tumor-free survival in mice. J Clin Invest. 2008;118:3170-80.

58. Thamilselvan V, Basson MD. The role of the cytoskeleton in differentially regulating pressure-mediated effects on malignant colonocyte focal adhesion signaling and cell adhesion. Carcinogenesis. 2005;26:1687-97.

59. Matrone MA, Whipple RA, Thompson K, Cho EH, Vitolo MI, Balzer EM, et al. Metastatic breast tumors express increased tau, which promotes microtentacle formation and the reattachment of detached breast tumor cells. Oncogene. 2010;29:3217-27.

60. Balzer EM, Whipple RA, Cho EH, Matrone MA, Martin SS. Antimitotic chemotherapeutics promote adhesive responses in detached and circulating tumor cells. Breast Cancer Res Treat. 2010;121:65-78.

61. Vitolo MI, Boggs AE, Whipple RA, Yoon JR, Thompson K, Matrone MA, et al. Loss of PTEN induces microtentacles through PI3K-independent activation of cofilin. Oncogene. 2013;32:2200-10.

62. Whipple RA, Balzer EM, Cho EH, Matrone MA, Yoon JR, Martin SS. Vimentin filaments support extension of tubulin-based microtentacles in detached breast tumor cells. Cancer Res. 2008;68:5678-88.

63. Balzer EM, Whipple RA, Thompson K, Boggs AE, Slovic J, Cho EH, et al. c-Src differentially regulates the functions of microtentacles and invadopodia. Oncogene. 2010;29:6402-8.

64. Bugyi B, Carlier MF. Control of actin filament treadmilling in cell motility. Annu Rev Biophys. 2010;39:449-70.

65. Alberts B. Molecular biology of the cell. 5th ed. New York: Garland Science; 2008.

66. Nurnberg A, Kitzing T, Grosse R. Nucleating actin for invasion. Nat Rev Cancer. 2011;11:177-87.

67. Remmerbach TW, Wottawah F, Dietrich J, Lincoln B, Wittekind C, Guck J. Oral cancer diagnosis by mechanical phenotyping. Cancer Res. 2009;69:1728-32.

68. Byun S, Son S, Amodei D, Cermak N, Shaw J, Kang JH, et al. Characterizing deformability and surface friction of cancer cells. Proc Natl Acad Sci U S A. 2013;110:7580-5.

69. Kreitzer G, Liao G, Gundersen GG. Detyrosination of tubulin regulates the interaction of intermediate filaments with microtubules in vivo via a kinesin-dependent mechanism. Mol Biol Cell. 1999;10:1105-18.

70. Yoon JR, Whipple RA, Balzer EM, Cho EH, Matrone MA, Peckham M, et al. Local anesthetics inhibit kinesin motility and microtentacle protrusions in human epithelial and breast tumor cells. Breast Cancer Res Treat. 2011;129:691-701.

71. Jordan MA, Wilson L. Microtubules as a target for anticancer drugs. Nat Rev Cancer. 2004;4:253-65.

72. Mialhe A, Lafanechere L, Treilleux I, Peloux N, Dumontet C, Bremond A, et al. Tubulin detyrosination is a frequent occurrence in breast cancers of poor prognosis. Cancer Res. 2001;61:5024-7.

73. Whipple RA, Vitolo MI, Boggs AE, Charpentier MS, Thompson K, Martin SS. Parthenolide and costunolide reduce microtentacles and tumor cell attachment by selectively targeting detyrosinated tubulin independent from NF-kappaB inhibition. Breast Cancer Res. 2013;15:R83.

74. Perdiz D, Mackeh R, Pous C, Baillet A. The ins and outs of tubulin acetylation: more than just a post-translational modification? Cell Signal. 2011;23:763-71.

75. Hammond JW, Cai D, Verhey KJ. Tubulin modifications and their cellular functions. Curr Opin Cell Biol. 2008;20:71-6.

76. Wloga D, Gaertig J. Post-translational modifications of microtubules. J Cell Sci. 2010;123:3447-55.

77. Lafanechere L, Job D. The third tubulin pool. Neurochem Res. 2000;25:11-8.

78. Taschner M, Vetter M, Lorentzen E. Atomic resolution structure of human alpha-tubulin acetyltransferase bound to acetyl-CoA. Proc Natl Acad Sci U S A. 2012;109:19649-54.

79. Cueva JG, Hsin J, Huang KC, Goodman MB. Posttranslational acetylation of alpha-tubulin constrains protofilament number in native microtubules. Curr Biol. 2012;22:1066-74.

80. Hubbert C, Guardiola A, Shao R, Kawaguchi Y, Ito A, Nixon A, et al. HDAC6 is a microtubule-associated deacetylase. Nature. 2002;417:455-8.

81. North BJ, Marshall BL, Borra MT, Denu JM, Verdin E. The human Sir2 ortholog, SIRT2, is an NAD+-dependent tubulin deacetylase. Mol Cell. 2003;11:437-44.

82. Aldana-Masangkay GI, Sakamoto KM. The role of HDAC6 in cancer. J Biomed Biotechnol. 2011;2011:875824.

83. Zhang Z, Yamashita H, Toyama T, Sugiura H, Omoto Y, Ando Y, et al. HDAC6 expression is correlated with better survival in breast cancer. Clin Cancer Res. 2004;10:6962-8.

84. Saji S, Kawakami M, Hayashi S, Yoshida N, Hirose M, Horiguchi S, et al. Significance of HDAC6 regulation via estrogen signaling for cell motility and prognosis in estrogen receptor-positive breast cancer. Oncogene. 2005;24:4531-9.

85. Ohkawa N, Sugisaki S, Tokunaga E, Fujitani K, Hayasaka T, Setou M, et al. N-acetyltransferase ARD1-NAT1 regulates neuronal dendritic development. Genes Cells. 2008;13:1171-83.

86. Conacci-Sorrell M, Ngouenet C, Eisenman RN. Myc-nick: a cytoplasmic cleavage product of Myc that promotes alpha-tubulin acetylation and cell differentiation. Cell. 2010;142:480-93.

87. Shida T, Cueva JG, Xu Z, Goodman MB, Nachury MV. The major alpha-tubulin K40 acetyltransferase alphaTAT1 promotes rapid ciliogenesis and efficient mechanosensation. Proc Natl Acad Sci U S A. 2010;107:21517-22.

88. Akella JS, Wloga D, Kim J, Starostina NG, Lyons-Abbott S, Morrissette NS, et al. MEC-17 is an alpha-tubulin acetyltransferase. Nature. 2010;467:218-22.

89. Neumann B, Hilliard MA. Loss of MEC-17 leads to microtubule instability and axonal degeneration. Cell Rep. 2014;6:93-103.

90. Garnham CP, Roll-Mecak A. The chemical complexity of cellular microtubules: tubulin post-translational modification enzymes and their roles in tuning microtubule functions. Cytoskeleton (Hoboken). 2012;69:442-63.

91. Takemura R, Okabe S, Umeyama T, Kanai Y, Cowan NJ, Hirokawa N. Increased microtubule stability and alpha tubulin acetylation in cells transfected with microtubule-associated proteins MAP1B, MAP2 or tau. J Cell Sci. 1992;103 (Pt 4):953-64.

92. Cohen TJ, Friedmann D, Hwang AW, Marmorstein R, Lee VM. The microtubule-associated tau protein has intrinsic acetyltransferase activity. Nat Struct Mol Biol. 2013;20:756-62.

93. Mattila PK, Lappalainen P. Filopodia: molecular architecture and cellular functions. Nat Rev Mol Cell Biol. 2008;9:446-54.

94. Hall A. The cytoskeleton and cancer. Cancer Metastasis Rev. 2009;28:5-14.

95. Waterman-Storer CM, Worthylake RA, Liu BP, Burridge K, Salmon ED. Microtubule growth activates Rac1 to promote lamellipodial protrusion in fibroblasts. Nat Cell Biol. 1999;1:45-50.

96. Rodriguez OC, Schaefer AW, Mandato CA, Forscher P, Bement WM, Waterman-Storer CM. Conserved microtubule-actin interactions in cell movement and morphogenesis. Nat Cell Biol. 2003;5:599-609.

97. Ren XD, Kiosses WB, Schwartz MA. Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. EMBO J. 1999;18:578-85.

98. Palazzo A, Ackerman B, Gundersen GG. Cell biology: Tubulin acetylation and cell motility. Nature. 2003;421:230.

99. Creppe C, Malinouskaya L, Volvert ML, Gillard M, Close P, Malaise O, et al. Elongator controls the migration and differentiation of cortical neurons through acetylation of alpha-tubulin. Cell. 2009;136:551-64.

100. Li L, Wei D, Wang Q, Pan J, Liu R, Zhang X, et al. MEC-17 deficiency leads to reduced alpha-tubulin acetylation and impaired migration of cortical neurons. J Neurosci. 2012;32:12673-83.

101. Spallotta F, Cencioni C, Straino S, Sbardella G, Castellano S, Capogrossi MC, et al. Enhancement of lysine acetylation accelerates wound repair. Commun Integr Biol. 2013;6:e25466.

102. Ogden A, Rida PC, Aneja R. Heading off with the herd: how cancer cells might maneuver supernumerary centrosomes for directional migration. Cancer Metastasis Rev. 2013;32:269-87.

103. Montagnac G, Meas-Yedid V, Irondelle M, Castro-Castro A, Franco M, Shida T, et al. alphaTAT1 catalyses microtubule acetylation at clathrin-coated pits. Nature. 2013;502:567-70.

104. Zilberman Y, Ballestrem C, Carramusa L, Mazitschek R, Khochbin S, Bershadsky A. Regulation of microtubule dynamics by inhibition of the tubulin deacetylase HDAC6. J Cell Sci. 2009;122:3531-41.

105. Haggarty SJ, Koeller KM, Wong JC, Grozinger CM, Schreiber SL. Domain-selective small-molecule inhibitor of histone deacetylase 6 (HDAC6)-mediated tubulin deacetylation. Proc Natl Acad Sci U S A. 2003;100:4389-94.

106. Zhang X, Yuan Z, Zhang Y, Yong S, Salas-Burgos A, Koomen J, et al. HDAC6 modulates cell motility by altering the acetylation level of cortactin. Mol Cell. 2007;27:197-213.

107. Boyault C, Sadoul K, Pabion M, Khochbin S. HDAC6, at the crossroads between cytoskeleton and cell signaling by acetylation and ubiquitination. Oncogene. 2007;26:5468-76.

108. Kozyreva VK, McLaughlin SL, Livengood RH, Calkins RA, Kelley LC, Rajulapati A, et al. NEDD9 Regulates Actin Dynamics through Cortactin Deacetylation in an AURKA/HDAC6-dependent Manner. Mol Cancer Res. 2014.

109. Schoumacher M, Louvard D, Vignjevic D. Cytoskeleton networks in basement membrane transmigration. Eur J Cell Biol. 2011;90:93-9.

110. Kikuchi K, Takahashi K. WAVE2- and microtubule-dependent formation of long protrusions and invasion of cancer cells cultured on three-dimensional extracellular matrices. Cancer Sci. 2008;99:2252-9.

111. Quintavalle M, Elia L, Price JH, Heynen-Genel S, Courtneidge SA. A cell-based high-content screening assay reveals activators and inhibitors of cancer cell invasion. Sci Signal. 2011;4:ra49.

112. Vizoso FJ, Gonzalez LO, Corte MD, Rodriguez JC, Vazquez J, Lamelas ML, et al. Study of matrix metalloproteinases and their inhibitors in breast cancer. Br J Cancer. 2007;96:903-11.

113. Schnaeker EM, Ossig R, Ludwig T, Dreier R, Oberleithner H, Wilhelmi M, et al. Microtubule-dependent matrix metalloproteinase-2/matrix metalloproteinase-9 exocytosis: prerequisite in human melanoma cell invasion. Cancer Res. 2004;64:8924-31.

114. Castro-Castro A, Janke C, Montagnac G, Paul-Gilloteaux P, Chavrier P. ATAT1/MEC-17 acetyltransferase and HDAC6 deacetylase control a balance of acetylation of alpha-tubulin and cortactin and regulate MT1-MMP trafficking and breast tumor cell invasion. Eur J Cell Biol. 2012;91:950-60.

115. Topalidou I, Keller C, Kalebic N, Nguyen KC, Somhegyi H, Politi KA, et al. Genetically separable functions of the MEC-17 tubulin acetyltransferase affect microtubule organization. Curr Biol. 2012;22:1057-65.

116. Saba NF, Magliocca KR, Kim S, Muller S, Chen Z, Owonikoko TK, et al. Acetylated Tubulin (AT) as a Prognostic Marker in Squamous Cell Carcinoma of the Head and Neck. Head Neck Pathol. 2013.

117. Wang K, Deng QT, Liao N, Zhang GC, Liu YH, Xu FP, et al. Tau expression correlated with breast cancer sensitivity to taxanes-based neoadjuvant chemotherapy. Tumour Biol. 2013;34:33-8.

118. Iadevaia S, Lu Y, Morales FC, Mills GB, Ram PT. Identification of optimal drug combinations targeting cellular networks: integrating phospho-proteomics and computational network analysis. Cancer Res. 2010;70:6704-14.

119. Shibue T, Brooks MW, Inan MF, Reinhardt F, Weinberg RA. The outgrowth of micrometastases is enabled by the formation of filopodium-like protrusions. Cancer Discov. 2012;2:706-21.

120. Gao YS, Hubbert CC, Yao TP. The microtubule-associated histone deacetylase 6 (HDAC6) regulates epidermal growth factor receptor (EGFR) endocytic trafficking and degradation. J Biol Chem. 2010;285:11219-26.

121. Roussos ET, Condeelis JS, Patsialou A. Chemotaxis in cancer. Nat Rev Cancer. 2011;11:573-87.

122. Condeelis J, Singer RH, Segall JE. The great escape: when cancer cells hijack the genes for chemotaxis and motility. Annu Rev Cell Dev Biol. 2005;21:695-718.

123. Piperno G, LeDizet M, Chang XJ. Microtubules containing acetylated alpha-tubulin in mammalian cells in culture. J Cell Biol. 1987;104:289-302.

124. Sodek KL, Brown TJ, Ringuette MJ. Collagen I but not Matrigel matrices provide an MMP-dependent barrier to ovarian cancer cell penetration. BMC Cancer. 2008;8:223.

125. Provenzano PP, Eliceiri KW, Campbell JM, Inman DR, White JG, Keely PJ. Collagen reorganization at the tumor-stromal interface facilitates local invasion. BMC Med. 2006;4:38.

126. Nguyen-Ngoc KV, Cheung KJ, Brenot A, Shamir ER, Gray RS, Hines WC, et al. ECM microenvironment regulates collective migration and local dissemination in normal and malignant mammary epithelium. Proc Natl Acad Sci U S A. 2012;109:E2595-604.

127. Thompson EW, Yu M, Bueno J, Jin L, Maiti SN, Palao-Marco FL, et al. Collagen induced MMP-2 activation in human breast cancer. Breast Cancer Res Treat. 1994;31:357-70.

128. Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci U S A. 2003;100:8418-23.

129. Cancer Genome Atlas N. Comprehensive molecular portraits of human breast tumours. Nature. 2012;490:61-70.

130. Sasa M, Bando Y, Takahashi M, Hirose T, Nagao T. Screening for basal marker expression is necessary for decision of therapeutic strategy for triple-negative breast cancer. J Surg Oncol. 2008;97:30-4.

131. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A. 2001;98:10869-74.

132. International Cancer Genome C, Hudson TJ, Anderson W, Artez A, Barker AD, Bell C, et al. International network of cancer genome projects. Nature. 2010;464:993-8.

133. Tabchy A, Hennessy BT, Gonzalez-Angulo AM, Bernstam FM, Lu Y, Mills GB. Quantitative proteomic analysis in breast cancer. Drugs Today (Barc). 2011;47:169-82.

134. Mackeh R, Lorin S, Ratier A, Mejdoubi-Charef N, Baillet A, Bruneel A, et al. Reactive Oxygen Species, AMP-activated Protein Kinase, and the Transcription Cofactor p300 Regulate alpha-Tubulin Acetyltransferase-1 (alphaTAT-1/MEC-17)-dependent Microtubule Hyperacetylation during Cell Stress. J Biol Chem. 2014;289:11816-28.

135. Chen L, Wei T, Si X, Wang Q, Li Y, Leng Y, et al. Lysine acetyltransferase GCN5 potentiates the growth of non-small cell lung cancer via promotion of E2F1, cyclin D1, and cyclin E1 expression. J Biol Chem. 2013;288:14510-21.

136. Holmlund T, Lindberg MJ, Grander D, Wallberg AE. GCN5 acetylates and regulates the stability of the oncoprotein E2A-PBX1 in acute lymphoblastic leukemia. Leukemia. 2013;27:578-85.

137. Yang H, Pinello CE, Luo J, Li D, Wang Y, Zhao LY, et al. Small-molecule inhibitors of acetyltransferase p300 identified by high-throughput screening are potent anticancer agents. Mol Cancer Ther. 2013;12:610-20.

138. West AC, Johnstone RW. New and emerging HDAC inhibitors for cancer treatment. J Clin Invest. 2014;124:30-9.

139. Bayraktar S, Rocha-Lima CM. Molecularly targeted therapies for advanced or metastatic non-small-cell lung carcinoma. World J Clin Oncol. 2013;4:29-42.

140. Bantscheff M, Hopf C, Savitski MM, Dittmann A, Grandi P, Michon AM, et al. Chemoproteomics profiling of HDAC inhibitors reveals selective targeting of HDAC complexes. Nat Biotechnol. 2011;29:255-65.

141. Serres S, Soto MS, Hamilton A, McAteer MA, Carbonell WS, Robson MD, et al. Molecular MRI enables early and sensitive detection of brain metastases. Proc Natl Acad Sci U S A. 2012;109:6674-9.

142. Azuma K, Urano T, Horie-Inoue K, Hayashi S, Sakai R, Ouchi Y, et al. Association of estrogen receptor alpha and histone deacetylase 6 causes rapid deacetylation of tubulin in breast cancer cells. Cancer Res. 2009;69:2935-40.

143. Rice J. Metastasis: The rude awakening. Nature. 2012;485:S55-7.

144. Husemann Y, Geigl JB, Schubert F, Musiani P, Meyer M, Burghart E, et al. Systemic spread is an early step in breast cancer. Cancer Cell. 2008;13:58-68.

145. Kim MY, Oskarsson T, Acharyya S, Nguyen DX, Zhang XH, Norton L, et al. Tumor self-seeding by circulating cancer cells. Cell. 2009;139:1315-26.

146. Brown DC, Purushotham AD, Birnie GD, George WD. Detection of intraoperative tumor cell dissemination in patients with breast cancer by use of reverse transcription and polymerase chain reaction. Surgery. 1995;117:95-101.