2014

# A Novel Link Between Abl Family Kinases and NM23-H1 During Metastatic Progression

Leann S. Fiore
*University of Kentucky*, leann.fiore@uky.edu

STUDENT AGREEMENT:

I represent that my thesis or dissertation and abstract are my original work. Proper attribution has been given to all outside sources. I understand that I am solely responsible for obtaining any needed copyright permissions. I have obtained needed written permission statement(s) from the owner(s) of each third-party copyrighted matter to be included in my work, allowing electronic distribution (if such use is not permitted by the fair use doctrine) which will be submitted to UKnowledge as Additional File.

I hereby grant to The University of Kentucky and its agents the irrevocable, non-exclusive, and royalty-free license to archive and make accessible my work in whole or in part in all forms of media, now or hereafter known. I agree that the document mentioned above may be made available immediately for worldwide access unless an embargo applies.

I retain all other ownership rights to the copyright of my work. I also retain the right to use in future works (such as articles or books) all or part of my work. I understand that I am free to register the copyright to my work.

REVIEW, APPROVAL AND ACCEPTANCE

The document mentioned above has been reviewed and accepted by the student's advisor, on behalf of the advisory committee, and by the Director of Graduate Studies (DGS), on behalf of the program; we verify that this is the final, approved version of the student's thesis including all changes required by the advisory committee. The undersigned agree to abide by the statements above.

Leann S. Fiore, Student

Dr. Rina Plattner, Major Professor

Dr. Robert W. Hadley, Director of Graduate Studies

# A NOVEL LINK BETWEEN ABL FAMILY KINASES AND NM23-H1 DURING METASTATIC PROGRESSION

---

DISSERTATION

---

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By
Leann Sara Fiore

Lexington, KY

Director: Dr. Rina Plattner, Associate Professor

Lexington, KY

2014

ABSTRACT OF DISSERTATION


A NOVEL LINK BETWEEN ABL FAMILY KINASES AND NM23-H1 DURING METASTATIC PROGRESSION

Cancer patient mortality is caused by the ability of tumor cells to invade the extracellular matrix and metastasize. Our lab was the first to identify the role of Abl family of non-receptor tyrosine kinases (c-Abl and Arg) in the progression of solid tumor cancers. In our previous studies, we showed that high c-Abl/Arg activity promotes proliferation, invasion, and metastasis in melanoma and breast cancer cells lines. Here, we demonstrate that our previous findings are clinically relevant by showing increased c-Abl/Arg kinase activity in primary melanoma tumor tissue in comparison to low activity as compared to benign nevi. Additionally, in breast cancer tissue, we found aggressive tumor subtypes (triple-negative and high-grade breast cancer) had increased c-Abl/Arg activity as compared to less aggressive subtypes. To define the mechanism by which c-Abl and Arg promote melanoma and breast cancer metastasis, we searched for novel pathways by which c-Abl and Arg promote invasion, a key step in metastasis. Significantly, we found that c-Abl and Arg decrease the expression of non-metastatic protein, NM23-H1, a metastasis suppressor that is lost during metastatic progression. We demonstrate that NM23-H1 is localized and degraded within the lysosome via proteases, cathepsins L and B. Moreover, we show that c-Abl and Arg upregulate cathepsin mRNA levels and activate the cathepsins, which in-turn degrade NM23-H1. We demonstrate that this pathway is functionally significant as c-Abl and Arg require the downregulation of NM23-H1 to promote invasion in melanoma and breast cancer cell lines. We show that the pathway is clinically significant as c-Abl/Arg activity is inversely correlated with NM23-H1 expression in mouse lung metastases, as well as in human primary melanoma and primary breast cancer tissue. In summary, we are the first to demonstrate novel crosstalk between oncogenic and metastasis suppressor signaling pathways, and provide evidence that pharmacological inhibition of Abl family kinases in melanoma and breast cancer patients may prevent metastatic progression by stabilizing a metastasis suppressor.

KEYWORDS: c-Abl, Arg, NM23-H1, melanoma, breast cancer

Leann Fiore
Student's Signature

5/29/14
Date

# A NOVEL LINK BETWEEN ABL FAMILY KINASES AND NM23-H1 DURING METASTATIC PROGRESSION

By

Leann Sara Fiore

Rina Plattner, Ph.D
Director of Dissertation

Robert Hadley, Ph.D
Director of Graduate Studies

5/29/14
Date

Dedicated to William and Nancy Fiore

ACKNOWLEDGMENTS

I would like to thank my mentor, Dr. Rina Plattner, for providing me with the opportunity to train in her lab. Furthermore, I thank her for her guidance, always making time for me, and providing me with an excellent education and understanding of the scientific technique and critical thinking. I would like to thank my committee members Dr. David Kaetzel, Dr. Rolf Craven, and Dr. John D'Orazio for the direction and encouragement they have provided throughout my graduate career. I would also like to thank my outside examiner, Dr. Don Gash, for taking the time to meet and discuss my work. I would like to thank former members of the lab for their insight in lab meetings, help with experiments, moral support and comic relief: Dr. Jonathan Sims, Dr. Sourik Ganguly, Holly Bennett, Woodrow Friend, Aruna Visavadiya, and Dr. Divyamani Srinivasan. I would also like to thank Dr. Aditi Jain, Dr. Rakshamani Tripathi, and Stacy Hinchey for moral support and useful discussions for this dissertation. I appreciate the support and friendships of the faculty, students, and support staff of the Department of Molecular and Biomedical Pharmacology. I would also like to thank members of Dr. David Kaetzel's lab, Dr. John D'Orazio's lab, and Dr. Christian Paumi's lab for providing resources for experiments.

I am extremely grateful for the strong friendships I have formed in graduate school: Kathleen "Kigga" Schoch, Mary Catherine "MC" and Dexter Reneer, Heather Buechel, Gavin "Gavie Bear" Ellis, Alejandra Catalina "Cata" Velez-Ortega, Holly "Didi" Bennett, Cassandra "Cassie" Reiling, Matthew "Cakes" Thacker, Margo Ubele, Maxwell Merkel and Catie Reneer. I would like to thank my mentors at Oklahoma State University for the foundation and encouragement to continue a career in science. Finally, I would like to thank William, Nancy, and Philip Fiore, for without them I would not have had the support, confidence or resources to pursue a graduate career.

# Table of Contents

# List of Tables

# List of Figures

# Chapter 1: Introduction

## Melanoma

*Risk Factors, Molecular Mechanisms, and Subtyping*

Melanoma accounts for only 4% of all skin cancers; however, it is responsible for 80% of skin cancer deaths [1]. The risk factors for melanoma include family history, presence of atypical nevi, skin pigmentation patterns, and ultraviolet light exposure [1, 2]. Nevi (common moles) form from abnormal proliferation of melanocytes in the epidermis [1]. Further aberrant proliferation within a nevus or, more commonly, a new location of melanocytes forms a lesion that presents histologically with asymmetry, irregular coloration, and increasing size [1]. Progression to the radial growth phase is characterized by melanocyte invasion into the epidermal skin layer [1], followed by progression to the vertical growth phase marked by dermal skin layer invasion allowing for metastatic spread to other organs (Figure 1.1) [1].

Initiation of aberrant proliferation of normal melanocytes is most commonly, albeit not exclusively, through activation of the mitogen-activated protein kinase (MAPK) pathway via two mutually exclusive activators: the mutated form of the serine/threonine protein kinase BRAF or the small GTPase NRAS (Figure 1.1) [1, 3]. Mutated BRAF (V600E) or NRAS (Q61K/R) alone in a nevus cannot stimulate progression to a malignant phenotype [3]. In fact, forced expression of these oncogenes results in senescence within the nevi, thus an additional “hit” is required for melanoma progression [4]. Additional mutations promoting melanoma include activation of the PI3K-AKT pathway due to the loss of the Phosphatase and Tensin Homolog (PTEN) protein and activation of the WNT/β-catenin pathway promoting survival, cell proliferation, spreading and migration. Other mutations include hypermethylation, deletion, or mutation of cyclin-dependent kinase inhibitor 2A (CDKN2A), amplification of the Cyclin D1 (CCND1) locus [5, 6], or activation of the receptor tyrosine kinase, cKIT [1]. It is apparent that melanoma pathogenesis yields a complex signaling profile, therefore several melanoma subtypes have been identified.

Melanoma cases can be grouped into subtypes based on lesion location and sun-exposure. Minimal sun-exposed acral melanomas (palms of hands and soles of feet), sun protected mucosal melanomas, and chronic sun-exposed (face) melanomas have the largest number of genomic instabilities, which cause mutations within the

**Figure 1.1 Biological and Molecular Events in the Initiation and Progression of Melanoma**

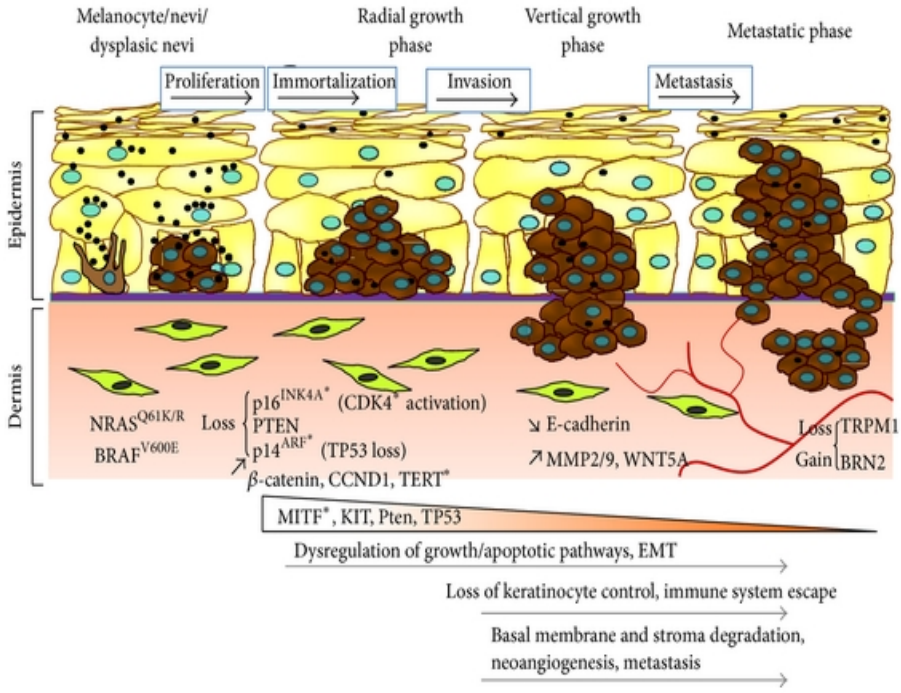


**Figure 1.1 Biological and Molecular Events in the Initiation and Progression of Melanoma.** A benign nevus (common mole) or a new location of melanocytes can undergo uncontrolled growth. This is most commonly due to a BRAF or NRAS mutations activating the mitogen-activated protein kinase (MAPK) pathway. Radial growth phase (RGP) lesions often have loss of PTEN, CCND1 amplification, cKIT mutation and are marked by melanocyte invasion into the epidermal layer. Invasion into the dermis marks the vertical growth phase (VGP). Figure adapted from: Bertolotto, C. (2013). Scientifica (Cairo).

CDKN2A/ CCND1 locus paired with amplification of cKIT (Figure1.2) [2, 7]. In contrast, intermittent sun-exposed melanomas (trunk, arms, legs) carry a high incidence of BRAF or NRAS activating mutations [7]. During chronic sun-exposure, UV radiation leads to Melanocortin 1 Receptor (MC1R) activation causing the subsequent activation of Microphthalmia Transcription Factor (MITF) which promotes a UV-protective, darkened skin pigment [7].

The term “melanoma” blankets several disease subtypes with unique complex signaling pathways. Due to the mutually exclusive nature of these pathways, several therapeutic regimens have been designed to combat the disease.

*Current Therapeutics*

With the discovery that 50% of melanoma patients have the activating BRAF mutation, clinical trials were conducted using vemurafenib, a BRAF inhibitor, and trametinib, a MEK inhibitor to target the downstream MAPK pathway [8]. Treatment with vemurafenib increased median patient survival from 5.7 months to 13.2 months, and trametinib gave a median progression free survival at 4.8 months compared to 1.5 months on a chemotherapy arm [8]. Although these inhibitors showed great promise, some tumors harbor intrinsic resistance or acquire resistance over time [8] due to the upregulation of compensatory survival pathways such as: reactivation of MEK via NRAS or CRAF upregulation, ERK mutations, Cyclin D1 amplification, PI3K/AKT signaling, PTEN mutations, PDGFβ overexpression, or IGF1R upregulation [7, 9, 10]. Due to the reactivation of MEK signaling in resistant tumors, clinical trials testing combination therapies of BRAF and MEK inhibitors are showing promise by inhibiting tumor progression and thus increasing survival [7]. However, as previously mentioned, only 50% of patients have the activating BRAF mutation, therefore other targetable therapies are necessary for the treatment of melanoma patients.

Tumors without mutant BRAF commonly have mutated NRAS. Unfortunately, treatment with this mutation has proven to be difficult as NRAS mutations upregulate other molecular pathways [8]. Mutated NRAS activates the MAPK pathway via the serine/threonine kinase CRAF. Sorafenib, an inhibitor of CRAF as well as BRAF, cKIT, and PDGFR, has shown promise by inducing apoptosis in melanoma cell lines [8]. Therefore, clinically targeting CRAF could prove to be beneficial in patients with NRAS mutations or those that have become resistant to BRAF inhibition [9]. Acral and mucosal

**Figure 1.2 Genetic Mutations in Melanoma Subtypes**

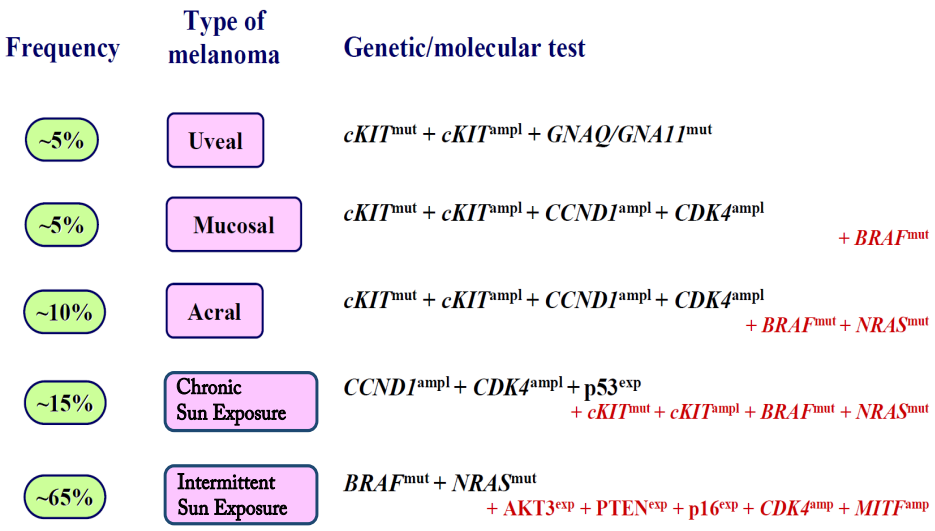


**Figure 1.2 Genetic mutations in melanoma subtypes.** Melanoma cases can be grouped into subtypes based on lesion location and sun exposure. Minimal sun-exposed acral melanomas (palms of hands and soles of feet), sun- protected mucosal melanomas, and chronic sun-exposed (face) melanomas have the largest number of genomic instabilities affecting the CDKN2A/ CCND1 locus paired with amplification of cKIT. In contrast, intermittent sun-exposed melanomas (trunk, arms, legs) carry a high incidence of activating mutations of BRAF and NRAS. Figure adapted from: Palmieri, G., et al. (2013). Melanoma - From Early Detection to Treatment, InTech.

melanomas harbor cKIT activating mutations, which are exclusive from, albeit more rare, than BRAF and NRAS mutations [7, 9]. A clinical trial using the multitarget inhibitor , imatinib mesylate (Gleevec, STI571; targets cKIT, c-Abl/Arg, and platelet derived growth factor (PDGFR)) in patients not selected for cKIT mutations resulted in a minimal response rate [11]. The response rate observed in phase II trials selecting for patients with cKIT activating mutations showed more promise [12, 13]; however, patients treated with this inhibitor often progress due to resistance induced by secondary cKIT mutations [3].

It is clear that melanoma classification is critical for choosing the proper signaling pathway to target therapeutically. However, even with proper subtyping, patient treatment options are partially successful at best, and the 5-year survival rate for patients with metastatic melanoma remains at 14% [14]. This statistic boasts the need for additional identification of compensatory mechanisms upregulated with drug resistance to provide a more well-rounded treatment option to melanoma patients.

## Breast Cancer

*Molecular Mechanisms, Progression and Clinical Grade*

Similar to melanoma, metastatic, aggressive breast cancer is also difficult to treat. With breast cancer being the most common type of cancer among women [15], several diagnostic techniques are used to identify treatment options patients. Non-invasive breast cancer, ductal carcinoma *in situ* (DCIS), can develop invasive properties after acquiring genetic and epigenetic alterations [16]. Using immunohistological techniques, invasive carcinoma has been subtyped into estrogen receptor (ER)/progesterone receptor (PR) positive (60% of breast cancers), human epidermal growth factor 2 (HER2) positive (20% of breast cancers), or triple-negative (lacking ER/PR expression and HER2 amplification, 20% of all breast cancers) [15]. The combinations of the presence/absence of these markers give five possible molecular classifications (Figure 1.3a). $ER^+/PR^+$ breast cancers can be subtyped into Luminal A, lacking HER2 expression, or Luminal B having HER2 enrichment. $ER^-/PR^-$ cancers can be HER2+ or triple-negative, lacking all three receptors. Triple-negative breast cancers can be further divided into basal-like or claudin-low breast cancers, although not all basal-like cancers are triple-negative. Both basal-like and claudin-low classifications are aggressive with basal-like being highly proliferative and claudin-low exhibiting markers of epithelial to mesenchymal transition (EMT) [16, 17]. Finally, tumors classified as normal

**Figure 1.3 Invasive Ductal Carcinoma Classifications and Grade**

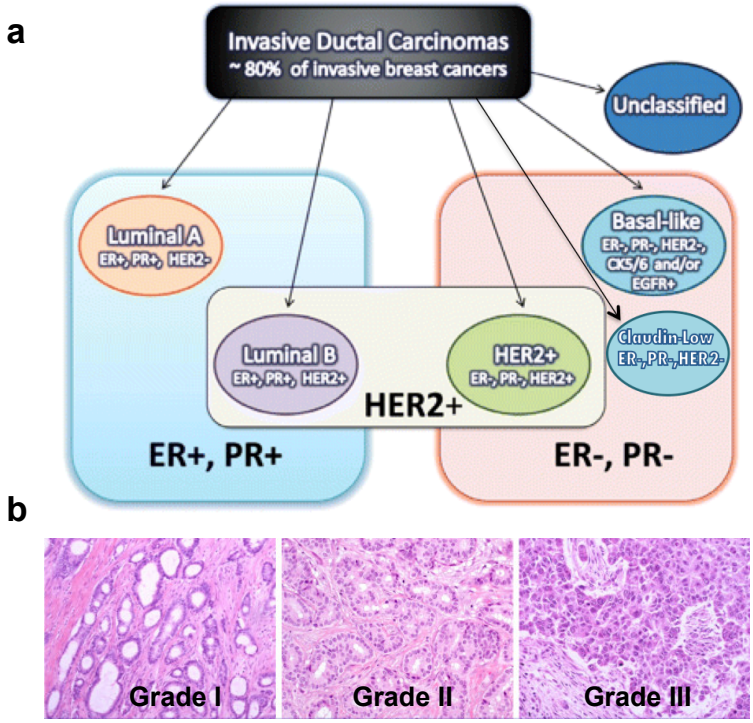


**Figure 1.3 Invasive ductal carcinoma classifications and grade. a)** Invasive carcinoma is been subtyped into estrogen receptor (ER)/progesterone receptor (PR) positive (60% of breast cancers), human epidermal growth factor 2 (HER2) positive (20% of breast cancers), or triple-negative (lacking ER/PR expression and HER2 amplification, 20% of all breast cancers). The combinations of the presence/absence of these markers give five possible molecular classifications. $ER^+/PR^+$ breast cancers can be subtyped into Luminal A, lacking HER2 expression, or Luminal B having HER2 enrichment. $ER^-/PR^-$ cancers can be HER2+ or triple negative, lacking HER2. Triple-negative breast cancers can be further divided into basal-like or claudin-low breast cancers. Both basal-like and claudin-low classifications are aggressive with basal-like being highly proliferative and claudin-low exhibiting markers of epithelial to mesenchymal transition (EMT). Adapted from Sandhu et al. (2010) LabMedicine, 41, 364-372. **b)** Grade I is a well differentiated tumor with normal breast tissue homology, mild nuclear pleomorphism, and low mitotic count. Grade II is moderately differentiated and grade III is poorly differentiated with high pleomorphism, frequent mitosis, and lack of tubule formation. Figure adapted from Rakha et al. *Breast Cancer Research* 2010, 12:207 .

breast-like resemble normal breast tissue and can be characterized molecularly due to high expression of non-epithelial markers, however a distinct molecular profile has yet to be defined [18]. Normal breast-like tumors have a good prognosis [18].

Histological tumor grade is a clinical prognostic tool used to evaluate tumor morphological features such as tubule/gland formation, nuclear pleomorphism, and mitotic count [19]. Grade I is a well differentiated tumor with normal breast tissue homology, mild nuclear pleomorphism, and low mitotic count. Grade II is moderately differentiated and Grade III is poorly differentiated with high pleomorphism, frequent mitosis, and no tubule formation (Figure 1.3b) [19]. Increasing grade has been linked with decreased survival and it has been suggested that tumor grade predicts tumor behavior accurately, even more so than tumor size [19, 20]. In addition to grade, TNM tumor staging is used clinically to assess progression. The "T" (0-4) describes tumor size, the "N" (0-3) indicates lymph node spreading, and "M" (0 or 1) indicates metastatic spread [21]. One of five stages can be assigned to a tumor: Stage 0 (non-invasive), Stage I (invasive, less than 2cm), Stage II (spread to lymph nodes), Stage III (spread to lymph nodes near the breast bone), Stage IV (metastatic spread to distant organs) [22].

Current treatments for patients with early stage breast cancer yield positive outcomes; however, 33% of patients with early stage breast cancer will develop metastases. Metastatic breast cancer remains incurable with a five-year survival rate of only 23% [15] indicating the need for further investigation of the molecular mechanisms causing metastasis and decreased mortality in breast cancer patients.

*Current Therapeutics*

As previously mentioned, immunohistological examination of breast tumors allows for targeted therapy for the patient. $ER^+/PR^+$ tumor patients have a favorable prognosis (80-85% 5 year survival rate) [16] due to the availability of targeted treatment using an estrogen receptor antagonist such as Tamoxifen or estrogen lowering aromatase inhibitors such as Letrozole. Similarly, some patients with $HER2^+$ tumors also may have a favorable outcome due to targetable therapy against the HER2 receptor using the HER2 monoclonal antibody, Trastuzumab, or inhibition of HER2 downstream signaling via Lapatinib [15]. Unfortunately, many of these $HER2^+$ tumors (70%) show drug resistance against Trastuzumab, therefore other therapeutic options are currently in clinical trials such as Trastuzumab-DMI, a combination of the monoclonal antibody with a fungal toxin for targeted drug delivery of Trastuzumab to HER2 overexpressing cells [15].

Unlike $ER^+/PR^+$ and $HER2^+$ tumors, triple-negative tumors are more difficult to treat due to lack of therapeutic targets [17]. Therefore the most common treatments are cytotoxic agents including anthracyclines (topoisomerase II inhibition) such as doxorubicin or taxanes (mitotic spindle inhibition) such as paclitaxel [17]. Inhibition of poly ADP-ribose polymerase (PARP), vascular endothelial growth factor (VEGF), and PI3K/AKT are a few of the current molecular targets being investigated. The need for molecular targets for triple-negative breast cancer is urgent to provide more rational treatment options for patients [17].

## Vesicular Trafficking

*Endosome to Lysosome Maturation and Function*

Once thought to be simple vesicles utilized for protein transport, we now know that vesicular trafficking and endosomes play a role in several cancer functions such as receptor tyrosine kinase regulation, cytokinesis, and migration [23, 24]. Cargo proteins from the endoplasmic reticulum (ER) are delivered to early endosomes from the trans-golgi network (TGN) or from the plasma membrane via endocytosis (Figure1.4) [25]. Once in the early endosome, proteins are either trafficked back to the plasma membrane via recycling endosomes or are ubiquitin-tagged for degradation [25]. Ubiquitinated proteins are sorted into endosomal membrane invaginations known as intraluminal vesicles (ILVs) via a process mediated by Endosomal Sorting Complex Required for Transport (ESCRT) proteins. However, ESCRT-independent ILV sorting for non-ubiquitinated cargo exists for a subset of ILVs (melanosomes and exosomes) [26]. The ILV-filled endosome, known as a multi-vesicular body, is considered a hybrid between the early and late endosomes [23, 25]. Further endosomal maturation forms the endolysosome (a hybrid vesicle prior to full lysosome maturation) [23].

Just as the endosome has been debunked as a simplistic organelle, the lysosome can no longer be thought of as simply the trashcan at the end of the endocytic pathway. The lysosome is best known for its role in the degradation of cellular components via 50 different hydrolases (proteases, phosphatases, nucleases, etc.) [27, 28]. However, it has also been shown to have roles in cholesterol homeostasis, plasma membrane repair, cell death, and cell signaling [27]. Proteins destined for degradation can be delivered to the lysosome via protein chaperones, autophagosomes, or

**Figure 1.4 Endosomal/Lysosomal Vesicular Trafficking**

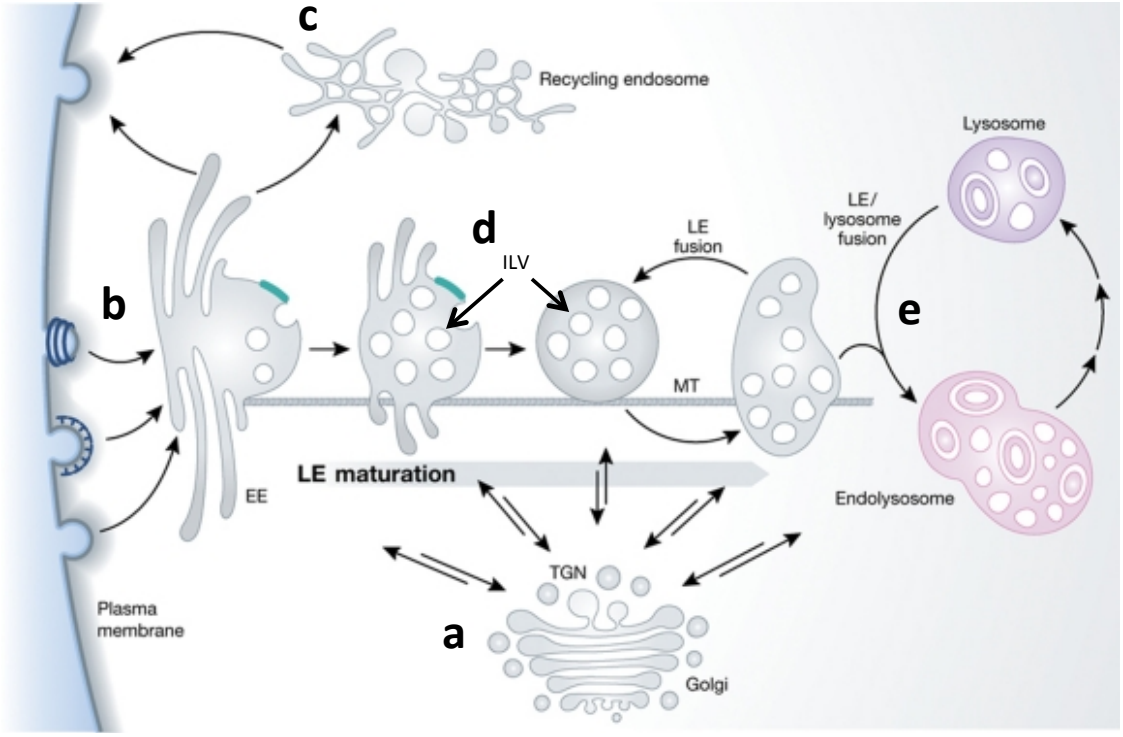


**Figure 1.4 Endosomal/Lysosomal vesicular trafficking.** **a)** Cargo proteins from the trans-golgi network (TGN) or **b)** endocytosed from the plasma membrane are delivered to the early endosomes. Once in the early endosome, proteins are either **c)** trafficked back to the plasma membrane via a recycling endosome or ubiquitin tagged for degradation. **d)** Ubiquitinated proteins are sorted into endosomal membrane invaginations known as intraluminal vesicles (ILVs). The ILV filled endosome, known as a multi-vesicular body, is considered a hybrid between the early and late endosomes. **e)** Continued endosomal maturation forms the endolysosome (a hybrid vesicle prior to full lysosome maturation) which matures into the lysosome. Figure adapted from Huotary et al. EMBO J. 2011 Aug 31;30(17):3481-500.

endosomes [29] and cleaved via cathepsin-mediated hydrolysis in a substrate-specific manner [28]. During the maturation of endosomes to lysosomes several changes occur: acquisition and loss of membrane components, intraluminal acidification, and vesicular localization from the cell periphery to the perinuclear region [23].

Dynamic vesicular membrane components are necessary for endosome maturation and also provide convenient markers for vesicular identification. Rab GTPase proteins have an integral role in vesicle docking, fusion, and motility by recruiting effector proteins via activation by a guanine exchange factor (GEF) [30]. The Rab GTPase is delivered to the vesicle membrane via a GDP dissociation inhibitor (GDI). GEFs convert the Rab-GDP state to GTP, which can then recruit and bind an effector (Figure1.5a). At the early endosome, recruitment and activation of the Rab5 GTPase, via the Rabex5 GEF promotes the recruitment of the Rab5 effector protein phosphoinositide 3-kinase (PI3K) generating phosphatidylinositol 3-phosphate (PI3P) lipid accumulation (Figure1.5b) [30]. This accumulation recruits the late endosome-specific GEF, Mon1/Ccz1. Mon1/Ccz1 then recruits the late endosome specific Rab7 GTPase [30]. Mon1/ Ccz1 has been shown to displace Rabex5, yielding a Rab5/Rab7 switch [30, 31]. Further maturation involves the acquisition of over 25 known lysosomal membrane proteins, the most abundant being LAMP1 and LAMP2 [27, 32, 33] resulting in lysosomal localization to the perinuclear region and fusion with other vesicles [33, 34].

In addition to the presence of essential membrane proteins, endosome/lysosome functionality is dependent upon intraluminal acidification [35]. As the endosome matures into a lysosome the pH decreases from ~6.8 to ~4.5 [35]. Acidification is achieved via large trans-membrane protein pumps, V-ATPases, which promote protease activation, routing of sorted cargo, and membrane trafficking [23]. Unprotonated lysosomotropic weak bases (ammonium chloride, chloroquine) act as lysosome inhibitors by binding free protons thus increasing the pH. Another inhibitor, bafilomycin A, increases pH via direct ATPase inhibition. Interestingly, bafilomycin A blocks maturation of late endosomes to lysosomes whereas the lysosomotropic weak bases do not, suggesting that in addition to pH changes, these inhibitors have other effects [23].

As early endosomes mature, they migrate from the cell periphery towards the perinuclear region [23]. Endosome movement along microtubules is regulated by kinesin and dynein motor proteins. This endosomal movement is necessary for the Rab5/Rab7 switch and cargo delivery for lysosomal degradation [23]. In addition to microtubules promoting endosomal movement, localization of the actin nucleation promoting factor,

**Figure 1.5 Endosomal Membrane Protein Recruitment**

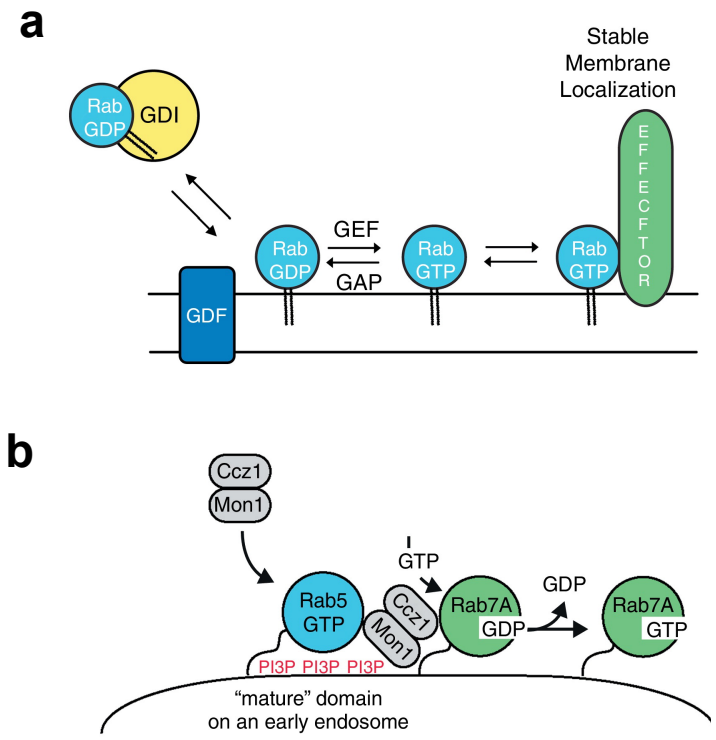


**Figure 1.5 Endosomal membrane protein recruitment.** Rab GTPase proteins have an integral role in vesicle docking, fusion, and motility by recruiting effector proteins via activation of a guanine exchange factor (GEF). **a)** The Rab GTPase is delivered to the vesicle membrane via a GDP dissociation inhibitor (GDI). GEFs convert the GDP to GTP which can then recruit and bind to an effector. **b)** Rab5 GTPase is recruited to the early endosome via the Rab5 GEF, Rabex 5. Rab5 promotes the recruitment of the Rab5 effector protein phosphoinositide 3-kinase (PI3K) generating phosphatidylinositol 3-phosphate (PI3P) lipid accumulation. This accumulation recruits Mon1/Ccz1 (a late endosome specific GEF). Mon1/Ccz1 then recruits the late endosome specific Rab7 GTPase. Mon1/ Ccz1 has been shown to displace Rabex5 and Rab5 GTPase, an action known as the Rab5/Rab7 switch. Figure adapted from: Pfeffer et al. Curr Opin Cell Biol. 2013 Aug;25(4):414-9.

WASH, to the endosomal membrane promotes endosomal trafficking and sorting via activation of the actin nucleation complex, ARP2/3 [36].

Perinuclear localization, membrane component switching, and acidification are all necessary for the delivery of proteins destined for degradation to the lysosome. Additionally, proper delivery and activation of cathepsin proteases is essential for lysosomal protein degradation and function.

*Cathepsin Activation and Function*

Within the lysosome, cathepsin proteases are primarily responsible for protein degradation. There are currently 15 cathepsins classified based on the active site residues: 11 cysteine, 2 serine and 2 aspartic proteases [37]. Most cathepsins are synthesized as preproenzymes with “pre” referring to the N-terminal signal targeting the cathepsin to the Endoplasmic Reticulum (Figure 1.6a) [37], however alternative splicing can produce cathepsins lacking the signal peptide, targeting these cathepsins to areas other than the ER such as the nucleus or mitochondria [38]. The “pro” refers to the N-terminal propeptide which assists in proper protein folding and additionally acts as an inhibitor covering the enzyme active site [37]. Cathepsin inhibition is necessary to prevent damage to cellular components prior to the cathepsin being localized to a vesicle [37]. The “pre” signaling peptide is cleaved in the ER and the glycosylated cathepsin is trafficked to the golgi apparatus where it undergoes mannose-6-phosphate (M6P) modification promoting mannose-6-phosphate receptor (M6PR) binding (Figure1.6b). Binding to the M6PR results in sorting of the cathepsins to the early endosomes and late endosomes [38]. The low pH within the early endosomes induces cathepsin release from the M6PR and the active site is exposed via protein unfolding [39]. Once localized within the late endosome, the cathepsin propeptide is cleaved due to an increase in acidity, producing an active single-chain form of the cathepsin (Figure 1.6c) [38]. Trafficking of the single-chain active cathepsin to the even more acidic lysosome induces an additional cleavage producing the double-chain active form of the cathepsin (Figure 1.6d) [38].

Cathepsins have a variety of functions in addition to lysosomal protein turnover including antigen presentation (cathepsin S), cell cycle regulation (cathepsin L), bone remodeling (cathepsin K), reproduction (cathepsin V), hormone activation (cathepsin V) and cancer progression (cathepsin L, B, D) [37, 40]. Cathepsins L and B are commonly upregulated in melanoma and breast cancer, and promote tumor progression, invasion and metastasis [37, 41]. Increased expression of these cathepsins is accompanied by

**Figure 1.6 Cathepsin Activation via Endosomal Trafficking**

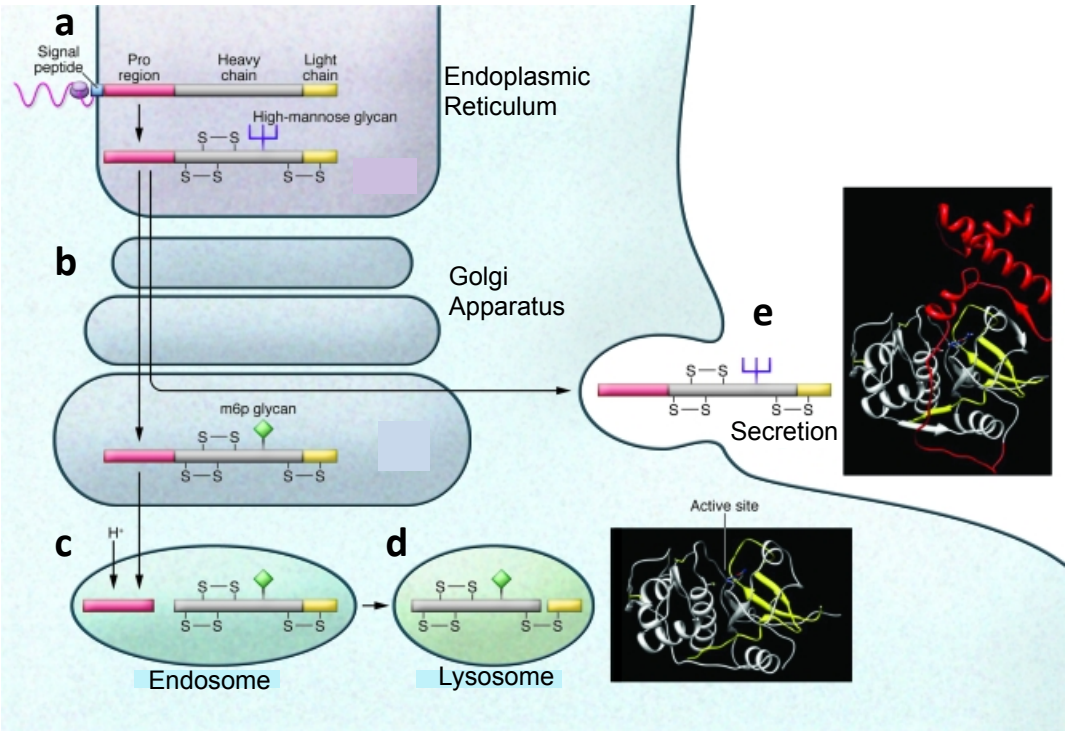


**Figure 1.6 Cathepsin activation via endosomal trafficking. a)** Within the endoplasmic reticulum, the cathepsin N-terminus signaling peptide is cleaved and the cathepsin is glycosylated then trafficked to **b)** the golgi apparatus where it undergoes mannose-6-phosphate modification inducing binding of the cathepsin to the mannose-6-phosphate receptor (M6PR). Binding to the receptor sorts the cathepsin to the early and late endosomes. **c)** Decreased pH within the early endosomes induces cathepsin release from the M6PR and unfolding of the protein exposing the active site. Once in the late endosome, the inhibitory propeptide is cleaved producing the active single-chain form of the cathepsin. **d)** Trafficking of the single-chain active cathepsin to the lysosome, which is even more acidic, induces additional cleavage producing the double-chain active form of the cathepsin. **e)** In cancer cells, increased expression of cathepsins is accompanied by decreased affinity for the M6PR within the golgi , which prevents cathepsin targeting to the endosomes subsequently causing the procathepsin to be trafficked to the plasma membrane and secreted into the extracellular environment. Figure adapted from: Reiser, et al. J Clin Invest. Oct 1, 2010; 120(10): 3421–3431.

decreased affinity for the M6PR within the golgi preventing cathepsin targeting to the endosomes [39]. The excess procathepsins within the golgi are then trafficked to theplasma membrane and secreted into the extracellular environment [37]. Once secreted, the acidic extracellular tumor environment promotes cathepsin activation [39]. Several mechanisms have been reported for extracellular cathepsins to promote invasion and metastasis. First, they directly cleave and degrade the basement membrane and extracellular matrix components such as type IV collagen, laminin and fibronectin [37]. Second, they can activate other proteases involved in ECM degradation such as the matrix metalloproteinases (MMP-1 and MMP-3) [40] and the urokinase plasminogen activator system [37]. Finally, they cleave and inactivate cell adhesion proteins such as E-cadherin, which promotes a more invasive migratory phenotype [40]. Intracellular cathepsins have also been reported to affect tumorigenicity, although they have been reported to both promote and prevent tumor progression. Intracellular cathepsins can promote tumorigenesis by degrading collagen which is endocytosed [37]. Conversely, they can inhibit tumorigenesis by degrading the anti-apoptotic protein Bcl-2, thus activating mitochondrial-induced apoptosis [37]. Data demonstrating specific intracellular substrates for cathepsins L and B are scarce, and it is generally accepted that cathepsin overexpression within these tumors promotes an invasive, metastatic phenotype. Therefore, undiscovered intracellular functions of cathepsins may exist promoting breast cancer and melanoma.

## Abl Family Kinases

*Structure and Regulation*

Oncogenes are proteins that drive the progression of cancer. The oncogenes c-Abl and Arg (Abl Related Gene) comprise the Abl family of non-receptor tyrosine kinases. These kinases are highly conserved in the amino-terminal region, which contains an SH3, SH2, and kinase domain (Figure 1.7a) [42]. The c-Abl and Arg genes, ABL1 and ABL2, generate two isoforms, 1a and 1b, with distinct N-terminal sequences due to alternative splicing of the first exons [43]. Isoform 1b contains a myristolation site at the N-terminus, which targets the kinases to the plasma membrane, whereas the 1a isoform originates from an alternative promoter yielding a truncated N-terminus, which lacks the myristolation site [44]. Although both kinases contain F-actin binding domains in the carboxyl terminus, c-Abl and Arg are less conserved in the C-terminus. Arg contains an additional F-actin domain and lacks the nuclear localization signals (NLS)

**Figure 1.7 Abl Family Kinase Structure and Activation**

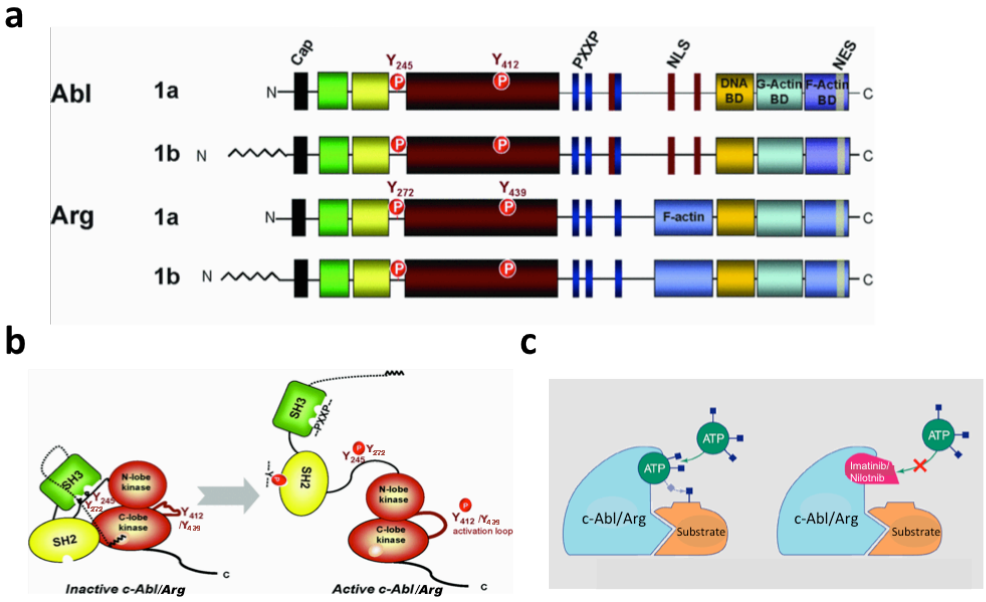


**Figure 1.7 Abl family kinase structure and activation. a)** c-Abl and Arg kinases are highly conserved in the amino-terminal region, which contains an SH3, SH2, and kinase domain. The c-Abl and Arg genes, ABL1 and ABL2, generate two isoforms, 1a and 1b, with distinct N-terminal sequences due to alternative splicing of the first exons. Isoform 1b contains a myristolation site at the N-terminus, which targets the kinases to the plasma membrane, whereas the 1a isoform originates from an alternative promotor yielding a truncated N-terminus, omitting the myristolation site. In the carboxyl terminus, Arg contains an additional F-actin domain and lacks the nuclear localization signals (NLS) and nuclear export signals (NES) present in c-Abl. **b)** Intramolecular inhibition of the Abl family kinases occurs when the SH3 domain interacts with the linker region between the SH2 and kinase domains or the myristolation residue binds the C-lobe within the kinase domain creating a folded conformation. Tyrosine phosphorylation on Y412/Y439 (c-Abl/Arg) in the activation loop and Y245/Y272 (c-Abl/Arg) in the SH2 linker region unfolds the proteins and stabilizes the active conformation. Imatinib and nilotinib are competitive inhibitors of the ATP-binding pocket of the kinase domain, preventing ATP from binding, and thus, preventing the transfer of the γ-phosphate from ATP to a substrate. **a,b)** Figures adapted from Sirvent, et al. Biol Cell. 2008 Nov;100(11):617-31 **c**) Figure adapted from http://www.cancer.govnewscenter/newsfromnci/2001/gleevecpressrelease

and nuclear export signals (NES) present in c-Abl (Figure 1.7a) [44]. Intramolecular inhibition of the Abl family kinases occurs when the SH3 domain interacts with the linker region between the SH2 and kinase domains or the myristolation residue binds the C-lobe within the kinase domain creating a folded conformation (Figure 1.7b) [43, 45]. Tyrosine phosphorylation on Y412/Y439 (c-Abl/Arg) in the activation loop and Y245/Y272 (c-Abl/Arg) in the SH2 linker region unfolds the proteins and stabilizes the active conformation [46]. Proteins that have been identified as c-Abl and Arg activators are EGFR, PDGFR, PLC-γ, and Src kinases [47, 48]. Endogenous inhibitors of c-Abl/Arg include the Abl interactor proteins (Abi-1 and Abi-2), peroxiredoxin-1 (PRDX-1/PAG1), phosphatidyl inositol bisphosphate ($PIP_2$) and the tumor suppressor FUS1; however, high expression of c-Abl and Arg, such as following transfection in 293T cells, can titrate out the inhibitors leading to constitutive activation [42, 49].

c-Abl and Arg localize to the cytoplasm and plasma membrane. Additionally, due to the presence of three NLS and a NES, c-Abl can also translocate to the nucleus in response to DNA damage and promote transcription and promote apoptosis [50]. Although nuclear c-Abl promotes apoptosis, cytoplasmic c-Abl and Arg have an opposing oncogenic, anti-apoptotic role. Furthermore, activation of cytoplasmic c-Abl and Arg in fibroblasts promotes cytoskeletal reorganization by phosphorylating N-Wasp, WAVE and cortactin allowing these proteins to interact with the Arp2/3 complex stimulating actin polymerization [46, 51]. c-Abl and Arg mediate actin polymerization in fibroblasts in response to growth factor stimulation which leads to cytoskeletal reorganization, membrane ruffling, and lamellipodia formation [46, 47].

c-Abl has been studied extensively in chronic myelogenous leukemias (CML), where a portion of the BCR gene fuses to the c-ABL gene. This fusion causes a deletion of the c-Abl gene resulting in a constitutively active BCR-Abl fusion protein which promotes the development of leukemia [49]. Imatinib mesylate (Gleevec, STI571) is an inhibitor of the Abl family kinases developed for patients with CML [52]. Imatinib stabilizes the inactive non-ATP binding form of c-Abl/Arg by binding to the ATP-binding pocket of the protein, and thus, prevents the transfer of the γ-phosphate from ATP to a substrate (Figure 1.7c) [52]. A second-generation inhibitor, nilotinib, was been developed for patients with resistance or adverse side effects to imatinib therapy [53]. Nilotinib binds and inhibits the Abl family kinases with greater specificity and potency coupled with less toxicity as compared to imatinib [53]. Imatinib and nilotinib also target cKIT and PDGFRα/β [52, 53] while nilotinib also inhibits DDR-1/2 and CSF-1R [54]. In addition to

nilotinib, other c-Abl/Arg inhibitors such as dasatinib and bosutinib have been developed to treat tumors with resistance to imatinib therapy [55]. A T315I mutation within the kinase domain of c-Abl/Arg causes a conformational change that induces resistance to most c-Abl/Arg inhibitors; however, ponatinib and DCC-2036 are multi-target inhibitors that inhibit all mutant forms of c-Abl/Arg, including T315I mutations [55].

*Role in Solid Tumors*

Our laboratory was the first to observe increased c-Abl and Arg activity in invasive breast cancer and melanoma cells as compared to human mammary epithelial cells (HMEC) and primary melanocytes [56, 57]. Subsequently, Abl family kinase activation has also been reported in glioblastoma, non-small cell lung cancer, gastric carcinoma and hepatocarcinomas [58-60]. In solid tumors, Abl family kinases are sequestered within the cytoplasm, where deregulated activation via EGFR, HER2, SRC, or IGF1R promotes proliferation, anchorage independent growth, invasion and metastases [56, 57, 61]. Our lab demonstrated that Abl family kinases promote invasion in melanoma cells via upregulation of matrix metalloproteinases (MMP-1, MMP-3, and MT1-MMP) and upregulation of MMP-1 occurs via a STAT3 dependent mechanism [57]. However, re-expression of these MMPs only partially rescued the decrease in invasion observed in c-Abl and Arg knockdown cells [57], indicating that c-Abl and Arg likely promote invasion in melanoma and breast cancer cells via multiple mechanisms. The success that c-Abl/Arg inhibitors, imatinib and nilotinib, have achieved in CML patients has yet to be translated to use in solid tumors cancers due to clinical trials not selecting for patients expressing high c-Abl/Arg activity. Regardless of the design pitfalls in the solid tumor clinical trials for these drugs, imatinib and nilotinib have proven to be invaluable for the investigation of c-Abl and Arg mechanisms in normal and cancer cells.

*Involvement in Protein Turnover*

c-Abl and Arg also have a role in endocytosis and protein turnover. Protein turnover within the cell occurs via two common mechanisms: ubiquitin-proteasomal degradation or lysosomal degradation. In hematopoietic cells, BCR/Abl promotes the proteasomal degradation of the Abi-2 proteins via tyrosine phosphorylation [62]. Additionally, c-Abl promotes the proteasomal-mediated degradation of DNA binding proteins in a kinase independent manner [63]. In addition to proteasomal degradation, c-Abl also has a role in vesicular movement and lysosomal degradation. c-Abl promotes B-cell receptor endocytosis via cytoskeletal remodeling [64] and both c-Abl and Arg were

found to promote autophagosome movement and delivery of proteins to the lysosome [65]. Conversely, c-Abl and Arg can also prevent the degradation of proteins, as c-Abl/Arg- mediated tyrosine phosphorylation of EGFR prevents endocytosis, which increases its stability and promotes cell surface expression [66]. Furthermore, c-Abl/Arg-mediated phosphorylation of galectin-3 prevents lysosomal degradation of galectin-3 [67]. Thus, it is clear that c-Abl/Arg affect protein turnover, and whether they promote or inhibit turnover may depend the cell type, the targeted substrate, and the activation level of the Abl family kinases. Therefore, although these reports provide evidence for c-Abl and Arg involvement in these processes, there remains much to be discovered about the involvement of Abl family kinases in mechanisms of vesicular trafficking and lysosomal mediated degradation.

## NM23-H1

*Role and Functions in Cancer*

Oncogenes drive the progression of cancer; however, proteins such as NM23-H1 reduce the metastatic capabilities of cancer cells. The human NM23 (NME, NDPK) family of proteins currently contains 10 homologs (H1-H10), with NM23-H1 being the most studied due to its anti-metastatic role [68]. High NM23-H1 RNA expression was first observed in murine melanoma cell lines with low metastatic potential as compared to highly metastatic cell lines having low NM23-H1 expression [69]. Transfection of NM23 into melanoma cells reduced motility and metastasis with little effect on tumor growth, supporting NM23-H1 as a metastasis suppressor rather than a tumor suppressor [70-72]. Since this discovery, reduction in NM23-H1 expression has been associated with aggressive tumors in gastric carcinoma, ovarian cancer, breast cancer, and melanoma tumors [68]. However, interestingly, in some diseases such as neuroblastoma, osteosarcoma, and hematological cancers, increased NM23-H1 expression correlates with poor survival [68]. Three biological functions have been identified for NM23-H1: nucleoside diphosphate kinase (NDPK), histidine kinase [68], and 3'->5' exonuclease [73].

As a nucleoside diphosphate kinase, NM23-H1 has the ability to add the terminal phosphate from a nucleotide triphosphate (NTP) onto a nucleotide diphosphate (NDP) [74]. Most of the NDPK functions reported for NM23-H1 involve G-protein regulation. NM23-H1 binds the Rac1 GEF, TIAM1 [75], and the CDC42 GEF, Dbl-1, which inhibits the activation of Rac1 and CDC42, preventing lamellipodia and filopodia formation, and

thus promoting an adhesive, less migratory phenotype [76]. Conversely, interaction with another GTPase, ARF6, suggests a promigratory role for NM23-H1. ARF6 recruits and interacts with NM23-H1, promoting-dynamin dependent endocytosis of E-cadherin, a process involved in epithelial to mesenchymal transition (EMT) [77]. However, ARF6 recruitment of NM23-H1 also inhibited TIAM1, reducing Rac1 activation, which would typically promote a more adhesive, less migratory phenotype [77]. It is worth noting that these experiments were conducted in polarized, epithelial MDCK cells; therefore NM23-H1 effects could differ between well-differentiated and less-differentiated cells and also could be different in mesenchymal (invasive) vs. epithelial (non-invasive) cancer cells. The NDPK function of NM23-H1 is necessary for its histidine kinase function, in which NM23-H1 transfers a phosphate from an NTP onto a histidine residue within NM23-H1 and then subsequently transfers the phosphate to another protein [74]. This histidine kinase activity is involved in phosphorylation-induced degradation of the Kinase Suppressor of Ras (KSR), which results in ERK pathway inhibition [74]. NM23-H1 also interacts with a host of other proteins, including inhibiting the ERα transcription factor causing downregulation of ERα specific genes [78]. Furthermore, NM23-H1 expression downregulates the GPCR, EDG2, decreasing motility and metastatic lung colonization [74]. Finally, NM23-H1 also has DNA-binding functions as it has been identified as a 3'->5' exonuclease [73, 79]. NM23-H1 promotes nucleotide excision repair of UVR-induced (6-4) photoproducts [79]. Furthermore, mice with a deficiency in murine *nm23-m1* and *nm23-m2* ($HGF^+$ x [*m1m2*] $^{+/-}$) have increased incidence of UVR-induced metastases as compared to $HGF^+$ mice [80], demonstrating that the 3'->5' exonuclease function of NM23-H1 is required for metastasis suppression.

*Upstream Regulators of NM23-H1*

The list of NM23-H1 binding partners and potential mechanisms for metastasis suppression is extensive; however, less information is available regarding upstream regulators of NM23-H1. Phosphorylation of NM23-H1 by casein kinase I (CKI) promotes binding of NM23-H1 to the phosphodiesterase (PDE), h-prune, subsequently preventing NM23-H1 from suppressing metastasis [81]. This interaction is speculated to promote metastasis by h-prune sequestering NM23-H1, thus, preventing its metastatic functions or by enhancing the PDE activity of h-prune leading to increased motility [82]. Aurora-A kinase has also been demonstrated to phosphorylate NM23-H1, however the functional significance of this interaction has yet to be determined [81]. Furthermore, plakoglobin, which has tumor suppressive activity via tethering cadherin-catenin complexes to actin,

increases NM23-H1 expression by binding and activating the NM23-H1 promoter when exogenously expressed [83, 84]. As previously mentioned, NM23-H1 was found to regulate the activation of ERα, and interestingly ERα in turn increases NM23-H1 transcription [85].

NM23-H1 is a potent metastasis suppressor; therefore, the ability to therapeutically increase NM23-H1 expression in patient tumors could decrease metastasis leading to increased survival. As such, compounds are being pursued in the clinic to decrease metastasis through upregulation of NM23-H1. Several compounds have been demonstrated to increase NM23-H1 expression including the glucocorticoid receptor (GR) agonists, dexamethasone, prednisolone and medroxyprogesterone acetate (MPA) [74]. Of the three GR agonists tested, only MPA increased NM23-H1 expression in the presence of serum, indicating it would be effective within the bloodstream [86]. MPA decreased anchorage-independent colonization and pulmonary metastases *in vivo* [86, 87]; however, increased survival was seen primarily in older patients; indicating that MPA treatment is limited to post-menopausal women [74, 86, 87]. An alternative to drug-induced NM23-H1 expression is delivery of NM23 protein by fusion of NM23 to a hydrophobic macromolecule transduction domain (MTD) creating a cell-permeable NM23 (CP-NM23) [88]. Delivery of CP-NM23 within metastatic cell lines reduced cell migration, invasion, MEK signaling and EDG2 expression (known downstream effects of NM23-H1) [88]. Furthermore CP-NM23 inhibited formation of lung metastases, cleared existing metastases, and increased survival as compared to MPA-treated mice [88].

In summary, these data demonstrate that re-expression of NM23-H1 within tumors dramatically reduces metastases and increases overall prognosis. Therefore, there is a need to expand upon the information we have regarding the cell signaling pathways involved in the regulation of NM23-H1 expression to rationally identify compounds that could increase NM23-H1 expression to improve patient survival.

## Project Objectives

Our lab previously demonstrated that c-Abl and Arg are highly activated in invasive melanoma and breast cancer cells and showed their activation is required for proliferation, anchorage independent growth, invasion and metastasis [57, 61]. Therefore, we hypothesize that Abl family kinases are highly activated in aggressive melanoma and breast cancer primary tissue. To test this hypothesis we will analyze c-

Abl/Arg activity in primary melanomas as compared to benign nevi, young vs. aged, and within sun-exposure subtypes (Chapter 2). Additionally, we will examine whether c-Abl/Arg activation is increased in high-grade or triple-negative breast cancers (Chapter 2).

We also demonstrated that c-Abl/Arg promote invasion via upregulation and activation of MMP1, MMP-3, and MT1-MMP in melanoma cells. However, as we stated above, overexpression of these MMPs only partially rescued the decrease in invasion observed in c-Abl/Arg knockdown cells [57]. This partial rescue leads us to hypothesize that c-Abl/Arg promote invasion via multiple pathways in breast cancer and melanoma. Here, we describe an additional mechanism by which c-Abl/Arg promote invasion via downregulation of the metastasis suppressor, NM23-H1 (Chapter 3).

# Chapter 2: Abl Family Kinase Activity and NM23-H1 Expression in Solid Tumors

## Introduction

Previously, our laboratory studied the role of c-Abl and Arg in solid tumor progression within melanoma and breast cancer cell lines. Melanoma and invasive breast cancer cells had higher c-Abl/Arg kinase activity in comparison to primary melanocytes and non-invasive breast cancer cells, respectively [56, 57]. Interestingly, protein expression of c-Abl and Arg in these cells did not correlate with their overall activity [56, 57]. Furthermore, inhibition of c-Abl and Arg with imatinib mesylate attenuated their ability to invade matrigel *in vitro*, and nilotinib treatment attenuated metastatic burden *in vivo* [56, 57, 89]. These findings demonstrate c-Abl and Arg activation promotes melanoma and breast cancer progression, and provide evidence that c-Abl/Arg activities cannot be assessed via their expression levels alone.

Several groups have examined c-Abl expression in primary solid tumors such as lung, lymphoma, colon, brain, renal, ovarian, stomach, and melanoma [90-94]. However, since c-Abl/Arg kinase activity is essential for c-Abl and Arg driven progression, and expression is not an indicator of activity, these studies failed to properly depict the role of c-Abl and Arg in these carcinomas. In an attempt to assess c-Abl and Arg kinase activity in endometrial cancer tissue, one group used an antibody directed against tyrosine phosphorylation sites on c-Abl [95], however, commercially available phospho-Abl antibodies cross-react with phospho-EGFR and phospho-PDGFR. Given that phospho-Abl antibodies lack specificity for c-Abl and Arg, one can't be certain that the investigators were analyzing activity specific to c-Abl/Arg. Although there is no way to directly measure c-Abl/Arg activity in tissues, we believe that indirect measurement of activity using an antibody directed against c-Abl and Arg tyrosine phosphorylation sites on a known c-Abl/Arg substrate such as Crk/CrkL might be a viable alternative method. Although other proteins have been shown to phosphorylate Crk/CrkL *in vitro*, c-Abl/Arg double knockout cells had undetectable phosphorylation on Y221 (Crk) and Y207 (CrkL) [96], and silencing or inhibiting c-Abl and Arg, in cancer cells, completely inhibits Crk/CrkL phosphorylation [56]. Singer, et al. stained several primary tumor types for pCrk/CrkL and found staining in brain, ovarian, colon, lymphoma, lung, and of particular interest to our studies, melanoma (57% stained positive) and breast cancer (49% stained positive) [97]. However, they did not compare the intensity of pCrk/CrkL staining

within the subtypes of these cancers. Therefore the need exists for assessment of c-Abl/Arg kinase activity via pCrk/CrkL staining in melanoma subtypes such as age and sun-exposure and breast cancer subtypes such as grade and receptor expression (HER2/ER/PR/triple-negative) to determine if c-Abl and Arg activity within a particular subtype is associated with an aggressive, metastatic phenotype.

In contrast to the few studies depicting c-Abl/Arg activity in primary melanomas and breast cancers, NM23-H1 expression has been investigated in several primary tumor tissues such as ovarian, gastic, melanoma and breast cancer [98, 99]. In melanoma, high NM23-H1 mRNA/protein expression has been linked to increased survival rates [100], decreased tumor thickness [100-102], decreased invasion [100, 101] and fewer metastases [102-104]. However, there are also conflicting reports that demonstrate high NM23-H1 expression associated with decreased survival [105], invasion, and metastases [105, 106]. Additionally, although there is data linking increased NM23-H1 expression with increased patient age [105], there is little data examining expression of NM23-H1 in various melanoma sun-exposure subtypes. A correlation between NM23-H1 expression and sun-exposure subtypes could further define the molecular differences in these subtypes and provide evidence for utilizing NM23-H1 expression as a prognostic marker for assessing the metastatic potential within a patient tumor.

In breast cancers, high NM23-H1 mRNA and protein levels were observed in low-grade tumors lacking lymph node involvement, and NM23-H1 expression was associated with increased overall survival [107-112]. To evaluate whether NM23-H1 could be a potential prognostic marker, other studies compared NM23-H1 protein expression with stage, grade, and HER2/ER/PR expression. High NM23-H1 expression was associated with smaller tumor size, lower stage, and in some cases, lower grade [111, 112]. Overall, there was no correlation with NM23-H1 and HER2 expression [113], whereas, although not significant, there was a trend towards a positive correlation between NM23-H1 and ER expression [111-114]. Interestingly, findings which oppose the data mentioned above have documented higher NM23-H1 mRNA and protein levels associate with higher grades [112, 115], larger tumors [113] and decreased survival [115]. Therefore, due to conflicting data in the literature and the lack of statistical significance for the data in some of these studies, it is worthwhile to examine NM23-H1 protein levels in melanoma and breast cancer tumor tissue to determine whether NM23-H1 expression could serve as a prognostic marker.

In summary, examination of c-Abl/Arg kinase activity and NM23-H1 expression in melanoma and breast cancer primary tumors will provide a better understanding of the roles these proteins play in tumor progression and metastasis. Therefore, in this Chapter, we will evaluate whether c-Abl/Arg activity and NM23-H1 expression are correlated within particular disease subtypes in primary melanoma and breast cancer tissue.

## Materials and Methods

*Immunohistochemistry*

Tissue microarrays (Biomax BR1502, BR10010a, BC08118, ME1003 and NCI melanoma progression array) were incubated 60°c overnight. All of the following steps were performed at room temperature unless otherwise noted. Slides were rehydrated sequentially in xylene and ethanol (100%, 95%, 70%) for 5' (x3) and subsequently rinsed in deionized water. Endogenous peroxidase activity was blocked (Dako K4010) for 10' followed by antigen retrieval in Diva Decloaking solution (Biocare Medical) within a Biocare Medical Decloaking Chamber (30" at 125°c, 10" at 90°c). After cooling for 10', slides were rinsed in deionized water, and left in TBST buffer (Dako S3306) for 5'. Primary antibody was diluted in antibody diluent (Dako S0809) and incubated for 2 hours. Antibodies used were pCrk/CrkL (1:10, Cell signaling 3181), NM23-H1 (1:75, Cell Signaling D98), or negative control (Dako N1699). After being washed 3x in TBST and left for 5' the slides were incubated for 30' in Dako Envision+ System-HRP (K4010). After being washed 3x in TBST, left for 5', the slides were incubated for 5-10' in either Diaminobenzine (DAB, (brown color) Dako 4011), for breast cancer slides, or AEC+ Red (Dako K3461) was used for melanoma slides in order to be able to differentiate between chromagen and brown melanin pigmentation. The DAB/AEC+ chromagen was deactivated in tap water and subsequently dipped in hematoxylin stain (Dako S3302), deionized water, bluing Reagent (Richard-Allan Scientific), and again deionized water. For the DAB chromagen, slides were dehydrated sequentially in ethanol (70%, 95%, 100%) and xylene for 5' (2x), and mounted in xylene-based mount (Vector H5000). For AEC+ chromagen slides, the dehydration steps were bypassed and the slides were immediately mounted in aqueous mountant (Vector H5501). A pathologist (Michael Cibull, M.D., Dana Richards, M.D., Janna Neltner, M.D.) blindly scored the slides. Each core was assigned intensity of staining (1-3+) and percentage of positively-staining

tumor cells. A score for each core was calculated by multiplying the intensity of stain by the percentage of positively-stained tumor cells.

*Statistics*

One (single) sample T-tests, One-way Anovas, Chi squares and Fisher's exact tests were all performed using the VassarStats website (http://vassarstats.net).

## Results

*c-Abl and Arg are Activated in Primary, Early-onset and Intermittent Sun-exposed Melanomas*

In previous studies, our lab demonstrated that activated c-Abl and Arg promote invasion, *in vitro,* and metastasis, *in vivo* [56, 57]. Therefore, to determine if c-Abl/Arg activation is linked to progression in human melanoma primary tissue, we performed immunohistochemistry on a melanoma tissue microarray (TMA) (ME1003, Biomax). To assess c-Abl/Arg activity we used an antibody against the phosphorylation sites on the c-Abl/Arg substrates Crk/CrkL (Figure 2.1a). A blinded pathologist read the cores assigning each core with intensity of stain (1-3+) and percentage of stained cells. We calculated a score for each core by multiplying the intensity of the stain by the percentage of positively-staining tumor cells. We observed that 60% of primary melanomas had high pCrk/CrkL staining in comparison to only 33% of the benign nevi (Figure2.2a, Table 2.1). These results were confirmed using a second melanoma TMA obtained from NCI, which showed 41% positive staining in melanomas vs. 16% in nevi (Figure 2.2 a,b). We also observed increased pCrk/CrkL scores in primary tumor, lymph metastases and organ metastases in comparison to benign nevi (Figure 2.2b), suggesting that c-Abl and Arg are likely to have a role in promoting melanoma tumor progression to a metastatic phenotype.

Next, to determine if c-Abl/Arg activity correlates with patient age in primary melanomas, we assessed pCrk/CrkL staining within the following age groups: young (39 years and younger), middle-aged (40-59 years) and elderly (60 years and older). Although not significant, we found a trend towards higher pCrk/CrkL staining in the young age group as 86% of cores had intense staining whereas only 47% of the elderly age group cores had intense staining (Figure 2.2c, Table 2.1). These data suggest that early-onset melanoma could be driven, in part, by activated c-Abl and Arg. Additionally, we examined c-Abl and Arg activity in melanoma sun-exposure subtypes and observed that mucosal melanomas had the largest percentage of cores with intense pCrk/Crk

**Figure 2.1 pCrk/CrkL Staining in Primary Melanomas**

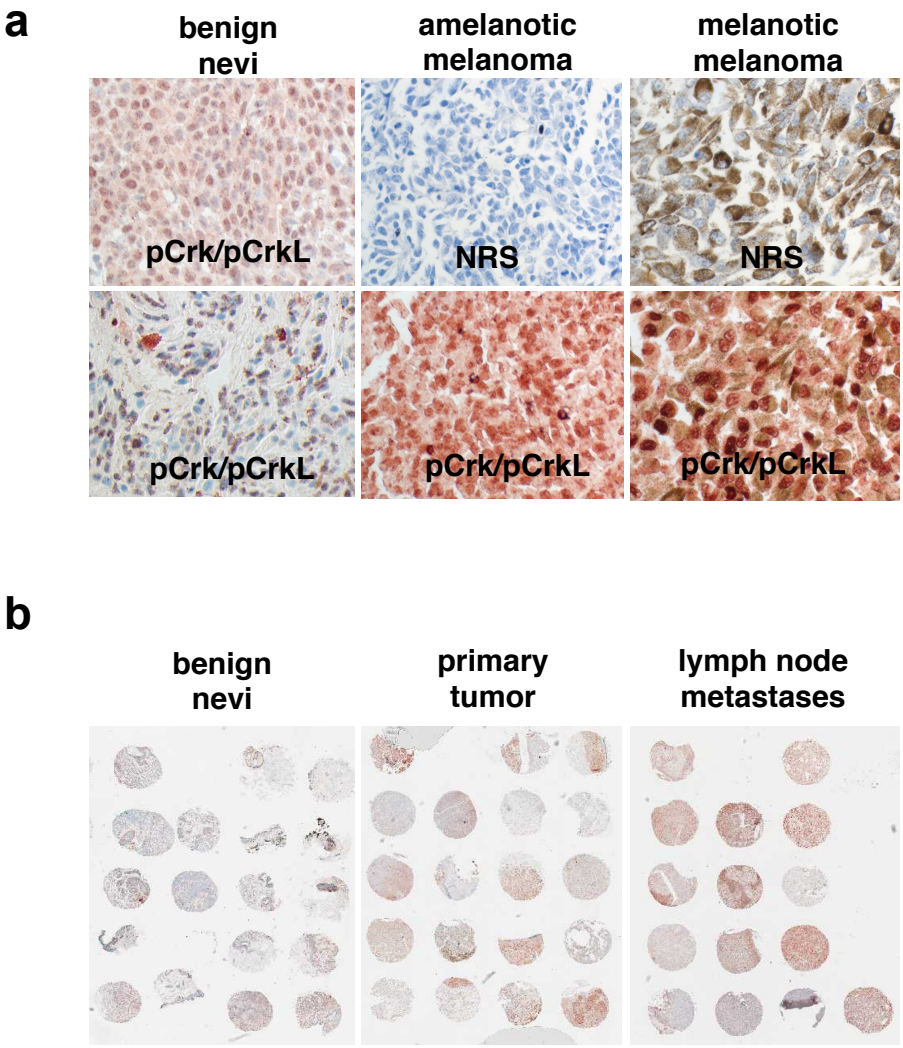


**Figure 2.1 pCrk/CrkL staining in primary melanomas. (a, b)** Melanoma tissue microarrays were stained with pCrk/CrkL or IgG antibody and visualized with Dako AEC+ Red chromagen. **(a)** ME1003 (Biomax) TMA incubated in normal rabbit serum (NRS) or phospho-Crk/CrkL antibody, 400x magnification. **(b)** Representative regions of a large NCI tissue microarray stained with pCrk/CrkL antibody.

**Figure 2.2 c-Abl/Arg Activation in Primary Melanoma Tissue**

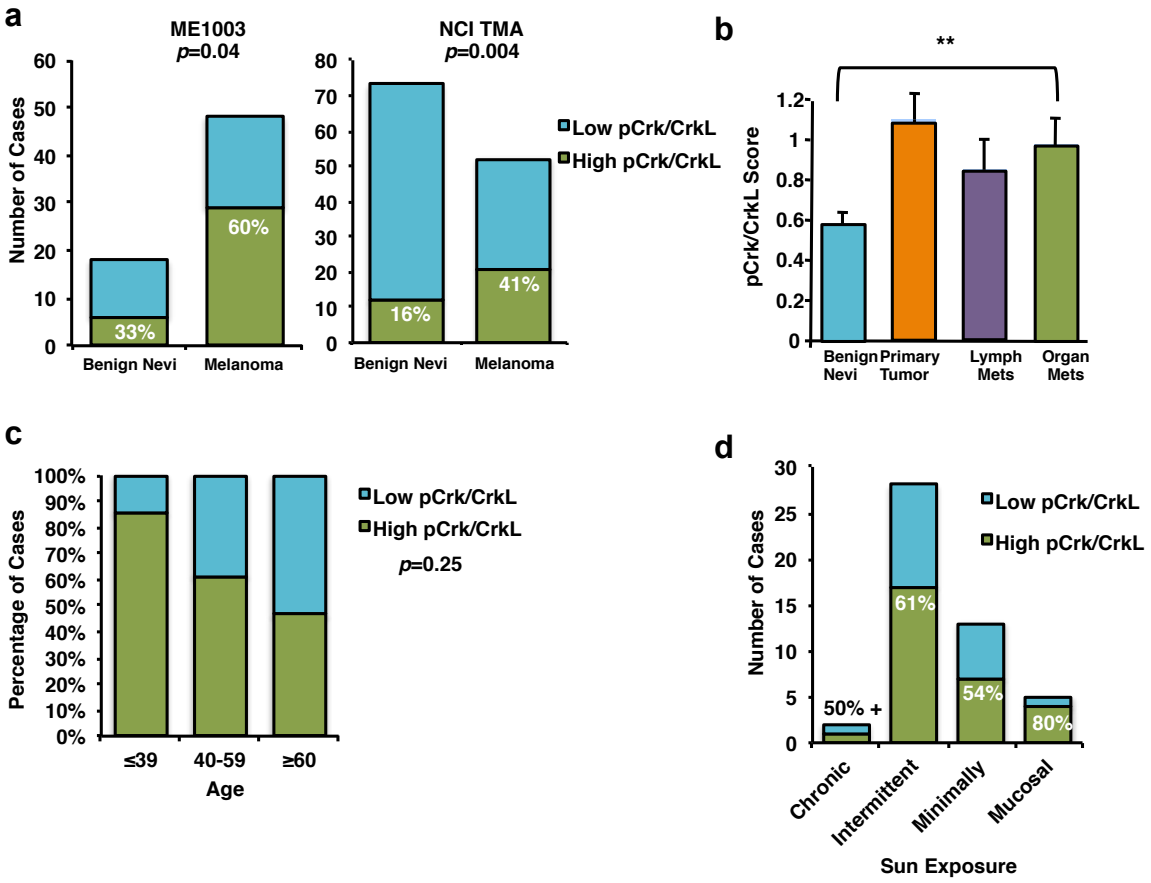


**Figure 2.2 c-Abl/Arg activation in primary melanoma tissue. (a-d)** Melanoma tissue microarrays were stained with pCrk/CrkL (Y221/Y207) or IgG antibody and visualized with Dako AEC+ Red chromagen and blindly read by a pathologists Michael L. Cibull, M.D. (ME1003) and Dana Richards, M.D. (NCI Array). Cores of bad quality were not scored. Score= intensity (1-3+) x proportion of positively stained tumor cells. **(a,c,d)** Percentage of cores with high and low pCrk/CrkL staining was graphed from ME1003 (a, c, d) and NCI melanoma TMA (a) . Cores were scored as high if the score was ≥1.4 **(a,c)** p-values obtained with Fisher's Exact Test. **(b)** Average scores from a large NCI melanoma TMA were graphed. Bracket indicates overall difference among groups: ***p*=<0.01 with one way Anova.

**Table 2.1 c-Abl/Arg Activation in Primary Melanoma Tissue**

| Tumor Type | Total n | High pCrk/ CrkL Score | Low pCrk/ CrkL Score | *p*-value |
|---|---|---|---|---|
| Benign Nevi | 18 | 6 (33%) | 12 | 0.04 |
| Primary Melanomas | 48 | 29 (60%) | 19 | |
| Lymph Node Metastases | 19 | 9 (47%) | 10 | |
| **Age** | | | | |
| ≤39 | 7 | 6 (86%) | 1 | 0.25 |
| 40-59 | 26 | 16 (62%) | 10 | |
| ≥60 | 15 | 7 (47 %) | 8 | |
| **Sun-Exposure** | | | | |
| Chronic | 2 | 1 (50%) | 1 | |
| Intermittent | 28 | 17 (61%) | 11 | |
| Minimally Exposed | 13 | 7 (54%) | 6 | |
| Mucosal | 5 | 4 (80%) | 1 | |

Melanoma tissue microarray (ME1003, Biomax) stained with pCrk/CrkL (Y221/Y207) antibody and visualized with Dako AEC+ Red chromagen was blindly scored by pathologist Michael L. Cibull, M.D. Scores described in Figure 2.2 legend. p-value is with Fisher's Exact test. Percentages are % of cores staining high for pCrk/CrkL in each tumor type.

staining (80%) followed by melanomas of intermittent sun-exposure (61%) and the lowest percentage of positive cases were observed in melanomas from minimal sun-exposed (54%) and chronically sun exposed (50%) areas (Figure 2.2d, Table 2.1). Early-onset melanomas often arise from intermittently-sun exposed areas; therefore intense pCrk/CrkL staining in both of these melanoma subtypes suggests c-Abl/Arg activity could promote early-onset melanoma or are the result of genetic events that occur during the development of these melanomas.

*c-Abl and Arg are Activated in High-grade and Triple-negative Breast Cancer*

Previously, we found that in addition to promoting invasion, c-Abl and Arg activation was highest in invasive breast cancer cell lines compared to non-invasive lines [89]. Therefore, we investigated whether c-Abl and Arg activity is increased in aggressive, invasive, high-grade breast cancers. We stained BC08118 and BR1502 breast cancer TMAs (Biomax) with pCrk/CrkL antibody (Figure 2.3a) and found that Grade III tumors had a significantly higher percentage of intensely staining cores (17%) as compared to Grade I tumors (0%) (Figure 2.3b, Table 2.2). To determine whether c-Abl/Arg activation is more frequent in a particular breast cancer subtype, we stained another TMA (BR10010a, Biomax) which contained clinical information including ER, PR and HER2 status. Although not significant due to small sample numbers, we observed a trend towards stronger pCrk/CrkL staining in the triple-negative cores as compared to HER2+ and ER/PR+ samples, which mostly stained weakly or moderately (Figure 2.3c, Table 2.2). These data suggest c-Abl and Arg could potentially be therapeutic targets in triple-negative breast cancer, which currently is the most difficult subclass to treat due to proteins not targeted by traditional chemotherapeutic drugs drive the cancers.

To determine if c-Abl and Arg activation promotes metastasis in breast cancer, we compared pCrk/CrkL staining intensity in breast cancer metastases with their matched primary tumor tissue. Although not statistically significant, the majority of metastases had higher pCrk/CrkL staining than their matched primary tumor tissue (Figure 2.3d), suggesting tumors with c-Abl/Arg activation might be more likely to invade and metastasize.

*NM23-H1 Expression is Decreased in Benign Nevi, Early-onset and Intermittent Sun-exposed Melanomas*

To examine whether NM23-H1 expression levels vary in benign nevi, primary melanoma tumors, and metastases, we stained the melanoma ME1003 TMA (Biomax)

**Figure 2.3 c-Abl/Arg Activation in Matched Primary and Metastatic Breast Cancers**

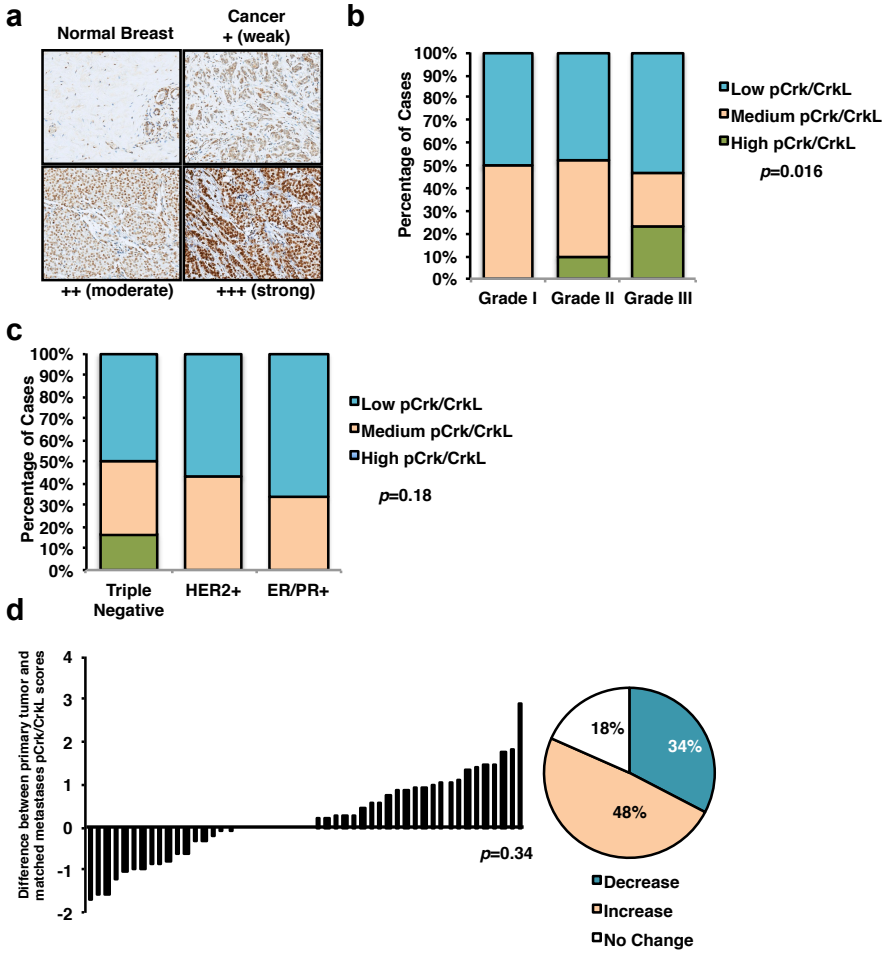


**Figure 2.3 c-Abl/Arg activation in matched primary and metastatic breast cancers.** **(a-d)** Breast tissue microarrays were stained with pCrk/CrkL or IgG antibody and visualized with DAB chromagen. Slides were blindly read by pathologist Michael L. Cibull, M.D**.** Score= intensity (1-3+) * proportion of positively stained tumor cells. **(a)** Breast cancers representing weak, moderate and strong intensity staining are shown (TMA: BC08118). **(b)** TMAs BC08118 and BR1502, scores >2.7 were scored as high and <1.4 scored as low **(c)** TMA BR1001a**,** scores >2.5 were scored as high and <1 scored as low. **(b,c)** *p*-value with Fisher's Exact Test **d)** Difference between the pCrk/CrkL scores from primary tumors and matched metastases (left). Percentage of metastatic cores with increased, decreased or no change in pCrk/CrkL staining as compared to primary tumor cores shown in pie chart (right) (TMA:BR10010a). *p*=0.34 using a single sample t-test.

**Table 2.2 c-Abl/Arg Activation in Breast Cancer Subtypes**

| Grade | Total n | High pcCrk/CrkL Score | Medium pCrk/CrkL Score | Low pCrk/CrkL Score | *p*-value |
|---|---|---|---|---|---|
| I | 26 | 0 (0%) | 13 | 13 (50%) | 0.016 |
| II | 63 | 6 (9.5%) | 27 | 30 (48%) | |
| III | 71 | 12 (17%) | 13 | 28 (39%) | |
| **Tumor Type** | | | | | |
| Triple Negative | 12 | 2 (16%) | 4 | 6 (50%) | 0.18 |
| HER2+ | 23 | 0 (0%) | 10 | 13 (57%) | |
| ER/PR+ | 24 | 0 (0%) | 8 | 16 (67%) | |

Breast cancer tissue microarrays were stained with pCrk/CrkL antibody and visualized with DAB chromagen. Slides were blindly read by pathologist Michael L. Cibull, M.D. Scores described in Figure 2.3 legend. p-value is with Fisher's Exact test.

with an NM23-H1 antibody (Figure2.4a). Overall, primary melanoma cores had more intensely staining cores as compared to benign nevi cores, which primarily had weak staining (Figure 2.5a, left; Table 2.3). In fact, the average NM23-H1 score within the primary melanomas was 9x that of the nevi (Figure 2.5a, right). In addition, the lymph node metastases, had 10% fewer cores staining intensely with NM23-H1 antibody as compared to the primary melanomas (Figure 2.5a, left; Table 2.3), and there was no significant difference between the mean scores of the two groups (Figure 2.5a, right), which was unexpected given the known anti-metastatic properties of NM23-H1.

There are few studies that have investigated whether NM23-H1 expression correlates with age in primary melanoma tissue. We found that melanomas from younger patients have less NM23-H1 staining as compared to those derived from older individuals (Figure 2.5b, left; Table 2.3). Additionally, we found the average NM23-H1 score of the melanomas from the younger age group to be significantly lower than both the middle age and elderly age groups (Figure 2.5b, right), which indicates that NM23-H1 downregulation may be necessary for the progression of early-onset melanoma or may result from genetic changes that occur in this subgroup. This is in contrast to pCrk/CrkL staining, which was highest in melanomas from the younger age group, suggesting that there may be a possible inverse correlation between c-Abl/Arg activity and NM23-H1 in primary melanoma tissue (assessed in Chapter 3).

Finally, we determined whether NM23-H1 expression varies among melanoma sun-exposure subtypes. Chronic sun-exposed melanomas had the smallest percentage of intensely staining cores (0%) followed by melanomas in the intermittent sun-exposure (28%) category as compared to more intense staining in melanomas from minimally sun-exposed areas (38%) and mucosal melanomas (50%) (Figure 2.5c, Table 2.3). As previously mentioned, early-onset melanoma tumors are usually of the intermittent sun-exposed subtype. Therefore, our data demonstrating low NM23-H1 staining in both intermittent sun-exposed melanomas and in melanomas from younger patients strengthens the hypothesis that NM23-H1 loss may be a contributing factor in the development of early-onset melanoma.

*NM23-H1 Expression is Decreased in High-grade, Advanced-stage Breast Cancer and is Positively Correlated with ER Expression*

In an attempt to confirm or deny conflicting reports regarding the expression of NM23-H1 in breast cancer, we stained breast cancer TMAs BR01001a and BC08118

**Figure 2.4 NM23-H1 Expression in Primary Melanomas and Breast Cancers**

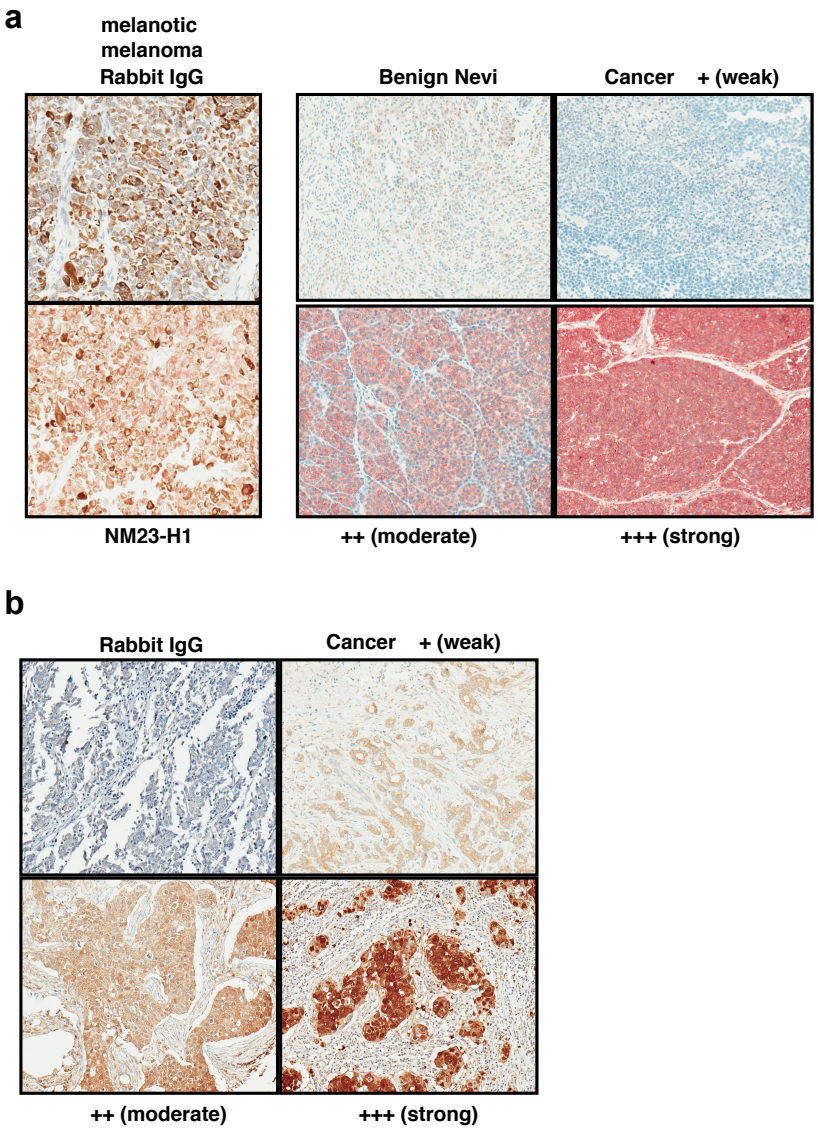


**Figure 2.4 NM23-H1 expression in primary melanomas and breast cancers. (a)** ME1003 melanoma tissue microarrays were stained with NM23 or IgG antibody and visualized with Dako Red chromagen (left). Benign nevi and representative weak, moderate, and strong staining primary tumors are shown (right). **(b)** Breast cancer tissue microarray BR1001a was stained with NM23 or IgG antibody and visualized with DAB chromagen. Breast cancer representing weak, moderate and strong NM23 staining are shown. 400x maginification.

**Figure 2.5 NM23-H1 Expression in Primary Melanomas**

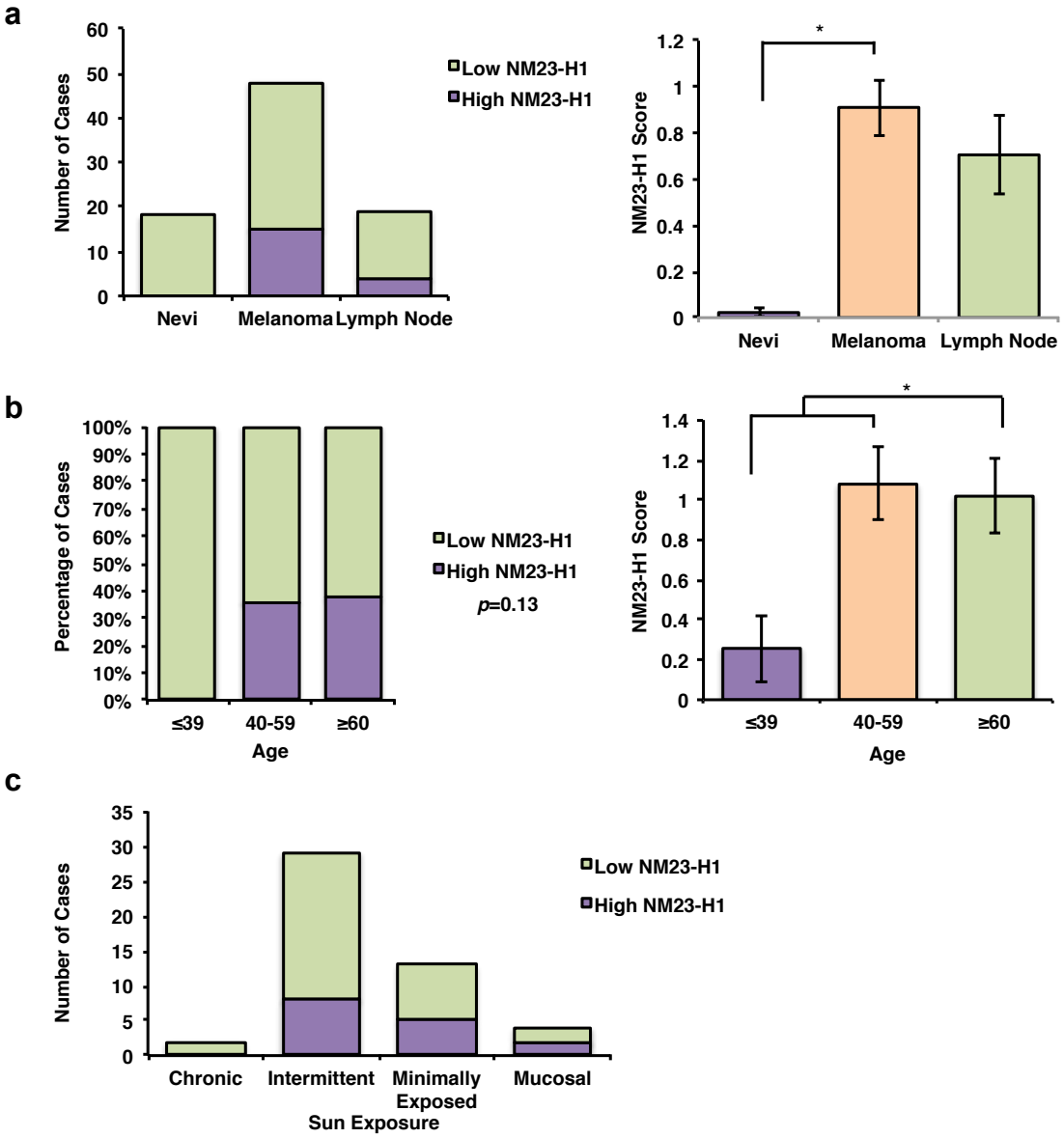


**Figure 2.5 NM23-H1 expression in primary melanomas. (a-c)** ME1003 melanoma tissue microarrays were stained with NM23 or IgG antibody and visualized with Dako Red chromagen. Cores of bad quality were not scored. Slides were blindly read by pathologist Michael L. Cibull, M.D. Score= intensity (1-3+) * proportion of positively stained tumor cells. Cores were scored as either low or high staining NM23 (left) and the average NM23 score of each group was determined (right) Cores were scored as high if the score was ≥1.4**. (b, left)** $p$=0.13 with Fishers exact test. **(a, b right)** *$p$=<.01 with Single Sample T-test.

**Table 2.3 NM23-H1 Expression in Primary Melanomas**

| Tumor Type | Total n | High NM23-H1 | Low NM23-H1 | *p*-value |
|---|---|---|---|---|
| Benign Nevi | 18 | 0 (0%) | 18 | 0.006 |
| Primary Melanomas | 48 | 15 (31%) | 33 | |
| Lymph Node Metastases | 19 | 4 (21%) | 15 | |
| **Age** | | | | |
| ≤39 | 7 | 0 (0%) | 7 | 0.13 |
| 40-59 | 25 | 10 (38%) | 16 | |
| ≥60 | 16 | 6 (40%) | 9 | |
| **Sun-Exposure** | | | | |
| Chronic | 2 | 0 (0%) | 2 | |
| Intermittent | 28 | 8 (28%) | 20 | |
| Minimally Exposed | 13 | 5 (38%) | 8 | |
| Mucosal | 5 | 2 (50%) | 3 | |

Melanoma tissue microarray (ME1003, Biomax), stained with NM23 antibody and visualized with Dako AEC+ Red chromagen, and blindly read by pathologist Michael L. Cibull, M.D. Scores described in Figure 2.5 legend. p-value is with Fisher's Exact Test.

(Biomax) with an NM23-H1 antibody (Figure 2.4b). We found no significant correlation between NM23-H1 expression and grade (Figure 2.6a); however, we observed a trend towards an inverse correlation between NM23-H1 expression and stage (Figure 2.6b, Table 2.4), confirming studies linking higher NM23-H1 expression to better survival [100]. Additionally, ER/PR+ and HER2+ breast cancers had the largest percentage of cores with intense NM23-H1 staining (25% and 23% respectively) as compared to triple-negative cores, which had moderate and weak staining exclusively (0% intense staining) (Figure 2.6c, Table 2.4). Consistent with other reports [111, 112, 114], we found a significantly higher percentage of ER+ tumors stained intensely with NM23-H1 antibody (33%) as compared to the ER- group (6%) (Figure 2.6d, Table 2.4). These data suggest a potential role for NM23-H1 in regulation of ER expression or vice versa.

## Discussion

In this Chapter, we provide evidence for increased c-Abl and Arg activity in melanoma primary tumors compared to benign nevi, as assessed by pCrk/CrkL staining. This is the first report demonstrating increased c-Abl/Arg activity in primary melanomas as previous studies only analyzed c-Abl and Arg protein expression. These findings support our *in vitro* and *in vivo* data demonstrating that c-Abl/Arg activity is required for melanoma invasion and metastasis [56, 57]. Immunohistological staining for NM23-H1 in melanoma tissue revealed a trend similar to pCrk/CrkL in that NM23-H1 expression was increased in primary melanomas as compared to benign nevi. Although overexpression of a metastasis suppressor in primary tumors was unexpected, these data are consistent with reports in the literature demonstrating increased NM23-H1 mRNA levels in melanoma primary tumors compared to nevi [103]. Likewise, in colorectal carcinoma, lower stage tumors (0-II) had much higher NM23-H1 expression as compared to normal mucosa [116]. Interestingly, in advanced colorectal tumors (stage III-IV), NM23-H1 expression was significantly decreased compared to lower stage tumors (0-II) [116], suggesting that NM23-H1 expression is likely upregulated in the initial stages of tumorigenesis and subsequently downregulated during progression. In support of this hypothesis, NM23-H1 has been shown to promote proliferation and DNA synthesis in breast cancer primary tumor cells [117]. Furthermore, a positive correlation has been observed between NM23-H1 expression and proliferation in primary neoplastic tumors, however this is likely occurring exclusively in early stage tumors as no correlation was

**Figure 2.6 NM23 Expression in Primary Breast Cancers**

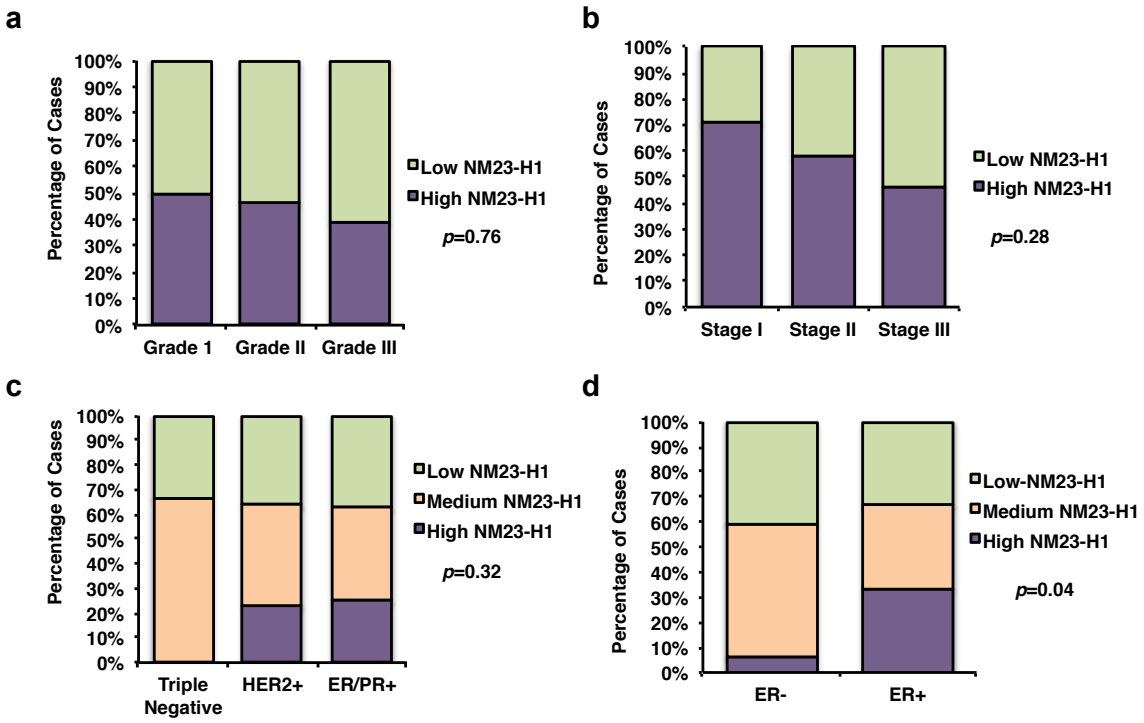


**Figure 2.6 NM23 expression in primary breast cancers. (a-d)** Breast tissue microarrays were stained with NM23 antibody and visualized with DAB chromagen. Slides were blindly read by pathologist Michael L. Cibull, M.D. Cores of bad quality were not scored. Score= intensity (1-3+) * proportion of positively stained tumor cells. **(a,b)** TMAs BC08118 and BR1502 (Biomax), scores >1.8 were scored as high and ≤ 1.8 scored as low. **(c,d)** TMA BR1001a (Biomax) Scores >2.5 were scored as high and < 1 scored as low. p-values are Fisher's exact test.

**Table 2.4 NM23-H1 Expression in Primary Breast Cancers**

| Grade | Total n | High NM23-H1 | Medium NM23-H1 | High NM23-H1 | *p*-Value |
|---|---|---|---|---|---|
| I | 22 | 11 (50%) | | 11 | |
| II | 82 | 38 (46%) | | 44 | 0.76[a] |
| III | 18 | 7 (39%) | | 11 | |
| **Stage** | | | | | |
| I | 17 | 12 (71%) | | 5 | |
| II | 99 | 57 (58%) | | 42 | 0.28[a] |
| III | 24 | 11 (42%) | | 13 | |
| **Tumor Type** | | | | | |
| Triple Negative | 12 | 0 (0%) | 8 | 4 (33%) | |
| HER2+ | 22 | 5 (23%) | 9 | 8 (36%) | 0.32[a] |
| ER/PR+ | 24 | 6 (25%) | 9 | 9 (38%) | |
| **Tumor Type** | | | | | |
| ER- | 32 | 2 (6%) | 17 | 13 (41%) | 0.04[b] |
| ER + | 18 | 6 (33%) | 6 | 6 (33%) | |

Breast cancer tissue microarrays were stained with NM23-H1 antibody and visualized with DAB chromagen. Slides were blindly read by pathologist Michael L. Cibull, M.D. Scores described in Figure 2.6 legend. p-values with Fisher's Exact Test[a] or Chi Square Analysis[b].

observed in primary tumors known to metastasize [118] further supporting a role for NM23-H1 in early stage tumorigenesis. The molecular switch for NM23-H1 downregulation in advanced stage tumors is unknown, however we observed increased c-Abl/Arg activity in tumor types containing low NM23-H1 expression such as age and sun-exposure in melanomas, and grade and receptor expression subtypes in breast cancer. These data suggest that the activities of c-Abl/Arg and NM23-H1 may be linked during melanoma and breast cancer progression (investigated in Chapter 3).

We observed only a slight decrease in the percentage of cores (10%) staining intensely with NM23-H1 antibody in melanoma lymph node metastases as compared to primary tumors. We expected a more significant decrease in NM23-H1 expression in metastases given that NM23-H1 has metastasis suppressor functions. We believe that these unexpected results are due to ascertainment bias, as most of the lymph node metastases were derived from melanomas in minimal (79%) or chronic (21%) sun-exposure subtypes, which tend to have higher NM23-H1 expression than the other subtypes. Additionally, minimal and chronic sun-exposed melanomas often have cKIT upregulation and activation [7, 119]. Therefore we hypothesize that NM23-H1 and cKIT expression may be related. Although NM23-H1 has been linked to transcriptional activation of EBNA3C (Epstein-Barr virus nuclear antigen 3C) [120], its role in transcription remains undefined. However, NM23-H2, which shares 88% homology with NM23-H1 at the protein level, is a transcriptional activator and binds G4 motifs within the *c-Myc* promoter [121, 122]. Therefore, given that the cKIT promoter also contains G4 motifs [123], and NM23-H1 is highly homologous to NM23-H2, we hypothesize that NM23-H1 may bind G4 motifs within the *cKIT* promoter thereby promoting cKIT overexpression in minimally and chronically sun-exposed melanomas.

Interestingly, we observed higher c-Abl and Arg activity in melanomas from young patients (<40 years of age) as compared to older patients. Early-onset melanomas often arise from areas with sunburn-induced intermittent sun-exposure caused by frequent tanning bed use in younger individuals [124, 125]. Intermittent sun-exposure does not activate the protective tanning response often seen with chronic sun-exposure and instead leads to production of reactive oxygen species, which causes genetic mutations, such as BRAF [1, 7]. Our observation of high c-Abl and Arg activity in melanomas from young patients and in melanomas from the intermittent sun-exposed subtype suggests c-Abl and Arg may be involved in promoting early-onset melanomas caused by intermittent sun-exposure. Given that c-Abl/Arg activity is high and BRAF

mutations are common in this melanoma subtype, it is possible that activities of BRAF and c-Abl/Arg may be linked.

In contrast to c-Abl and Arg activity being increased in early-onset melanoma, we observed a significant decrease in NM23-H1 expression in melanomas from young patients. As mentioned above, early-onset melanomas typically arise from areas of intermittent sun-exposure, and contain genetic mutations such as BRAF [1, 124]. Low NM23-H1 expression in early-onset melanoma is further supported by our observation that 72% of intermittent sun exposed melanomas (the majority of which tend to have early onset) also have low NM23-H1 expression. NM23-H1 functions in DNA repair as NM23-H1 activity (NDPK, HisK and 3'->5' exonuclease) was found to be essential for the repair of polymerase blocking lesions induced by UV radiation and, additionally, deletion of mouse NM23-M1/M2 was shown to increase UV- induced metastatic lesions, *in vivo* [79, 80]. Therefore, the low NM23-H1 expression that we observed in intermittent sun-exposed, early-onset melanomas, which are prone to genetic mutations, may contribute to melanoma progression due to lack of NM23-H1-mediated DNA repair.

In addition to elevated c-Abl/Arg activity in primary melanomas, we also observed a significant increase in activity with increasing breast cancer grade. Increased grade has been linked with decreased disease-free survival and together with stage, is highly prognostic [19, 20]. These data suggest that, in addition to grade and stage, elevated c-Abl/Arg activity also could potentially be a prognostic marker for breast cancer. In addition, we found the largest percentage of cores staining intensely for c-Abl/Arg activity were classified as triple-negative, complementing our previously published data in breast cancer cell lines, demonstrating that Arg activity was highest in triple-negative lines and c-Abl activity was highest in triple-negative and HER2+ cell lines [56]. Considering the trends we observed of increased c-Abl/Arg activity in high-grade, ER/PR-negative tumors, we hypothesize that activated c-Abl and Arg promote an aggressive, triple-negative phenotype. Furthermore, although not significant, we found that the majority of lymph node metastases had higher pCrk/CrkL staining (c-Abl/Arg activity) as compared to the matched primary tumors, further supporting a role for c-Abl/Arg activity in promoting an aggressive tumor type leading to metastasis.

Surprisingly, we found no significant correlation between NM23-H1 expression and grade in primary breast cancers. However, we did observe a trend towards decreased NM23-H1 expression with increasing stage that will likely approach significance with increased sample numbers. Breast cancer grade is indicative of tumor

growth rate whereas tumor staging not only takes into account primary tumor size but spread to the lymph nodes and distant organ sites. Our previously mentioned data demonstrated increased NM23-H1 expression in primary tumors as compared to benign nevi indicated NM23-H1 may promote the early stages of tumorigenesis, therefore we were surprised to observe no correlation between NM23-H1 expression and grade. However, decreased NM23-H1 expression with breast cancer stage is consistent with our melanoma data in which we observed decreased NM23-H1 expression in lymph node metastases as compared to primary tumors. These data suggest that the downregulation of NM23-H1 expression is required for progression to an advanced stage tumor.

We also observed a significant positive correlation between NM23-H1 and estrogen receptor expression confirming the trend previously reported by other investigators [111, 112, 114]. This data supports a model in which the ER increases NM23-H1 expression levels. In fact, it has been previously reported that ERα reduces invasion via transcriptional upregulation of NM23-H1 expression in several breast cancer cell lines, including MCF-7 cells [85]. Interestingly, also in MCF-7 cells, another group found that increased NM23-H1 expression decreased transactivation of ERα [78], suggesting a potential feedback mechanism between ERα and NM23-H1. Although ERα promotes proliferation, it has also been shown to suppress cell motility and invasion, thus making ER+ tumors less aggressive than triple-negative tumors. Taken together, these data indicate that ER may upregulate NM23-H1 in early stage tumors to promote proliferation, and downregulation of ERα in advanced stage tumors may lead to decreased NM23-H1 expression, thereby promoting an aggressive, metastatic phenotype.

In summary, in this Chapter we showed that increased c-Abl and Arg activity correlates with progression to an aggressive tumor type in two solid tumor cancers. Based on our observations in primary melanoma and breast cancer tissue discussed in this chapter, we hypothesize two mechanisms by which c-Abl/Arg promote progression and metastasis in these cancers. First, we propose activation of c-Abl/Arg kinases in intermittently sun-exposed melanomas inhibits DNA repair mechanisms via downregulation of NM23-H1 expression leading to tumor progressing mutations. Second, we hypothesize that c-Abl/Arg activation in late stage breast cancers subsequently causing loss of NM23-H1 expression thereby leading to an aggressive, triple-negative tumor type. In conjunction with our already published *in vitro* and *in vivo*

data, these data presented in this Chapter support the use of pCrk/CrkL and NM23-H1 expression as diagnostic markers for early-onset, intermittent melanomas and triple-negative breast cancers.

# Chapter 3: c-Abl and Arg Induce Degradation of the NM23-H1 Metastasis Suppressor

## Introduction

Metastatic spread of the primary tumor to a secondary location is the primary cause of mortality in melanoma and breast cancer patients. Metastasis requires the cell to detach from the primary tumor, invade the basement membrane, degrade the extracellular matrix, travel through the vasculature, and survive and proliferate at a secondary site. This process is driven by upregulation and activation of oncogenes, which promote proliferation, anchorage independent growth, cell-survival, migration and invasion [126]. In conjunction with oncogenes, downregulation of metastasis suppressors promotes these pro-metastatic mechanisms. Resistance to conventional therapies often results in metastasis and death; therefore, discovery of alternative treatment options for patients is necessary to promote survival.

The Abl family kinases, c-Abl and Arg, drive the progression of chronic myelogenous leukemia in which the BCR gene is translocated next to the Abl gene creating a constitutively active kinase [49]. Our laboratory was the first to identify the role of these kinases in solid tumor progression. We demonstrated that c-Abl and Arg are highly activated in melanoma and breast cancer cell lines promoting proliferation, anchorage-independent growth, survival, invasion and metastasis [56, 61]. We also demonstrated that c-Abl/Arg promote invasion via the upregulation and activation of MMP1, MMP3, and MT1-MMP [57]. However, recombinant MMPs only partially rescued the block in invasion observed following c-Abl/Arg knockdown [57], suggesting that c-Abl/Arg likely promote invasion via multiple mechanisms.

NM23-H1 was the first gene identified as a metastasis suppressor gene due to the observation that metastatic murine cell lines had low NM23-H1 mRNA expression as compared to non-metastatic cell lines having high NM23-H1 mRNA expression [69]. Furthermore, transfection of NM23-H1 into invasive melanoma cells reduced motility and metastasis and high NM23-H1 expression was demonstrated to correlate with better prognosis and increased survival [70-72, 100, 107]. Three NM23-H1 biological functions have been identified: nucleoside diphosphate kinase activity, histidine kinase activity, and a 3'->5' exonuclease function [68, 73]. Most of the research on NM23-H1 has focused on binding partners and mechanisms of metastasis suppression; therefore, very little information has been obtained regarding upstream regulators of NM23-H1. In fact,

the mechanism of NM23-H1 protein turnover and downregulation has yet to be identified. Given the decreased incidence of metastasis in tumors with high NM23-H1 expression, identification of the mechanisms that regulate NM23-H1 expression could help define new treatment options that increase NM23-H1 expression and benefit patients.

In Chapter two, we analyzed NM23-H1 expression and c-Abl/Arg activity in melanoma and breast cancer primary tissue. Interestingly, in melanoma tissue, we observed high c-Abl/Arg activity in tumors from younger patients and with intermittent sun-exposure, whereas NM23-H1 expression was low in these same subtypes. Furthermore, in breast cancer tissue, in tumors classified as triple-negative and high-grade we observed c-Abl/Arg activity to be high and NM23-H1 expression to be low. These data suggest c-Abl/Arg activity and NM23-H1 may be inversely correlated in melanoma and breast cancer primary tumors, suggesting a potential link between c-Abl/Arg and NM23-H1.

Given that c-Abl/Arg and NM23-H1 have opposing functions in cancer progression led us to hypothesize that c-Abl/Arg downregulate NM23-H1 expression to promote invasion and metastasis. Therefore, in this Chapter, we investigate the regulation of NM23-H1 protein expression, the novel link between c-Abl/Arg activity and NM23-H1, and the role this mechanism plays in invasion and metastasis.

## Materials and Methods

*Cell Lines and Reagents*

MDA-MB-435s cells were obtained from University of North Carolina Tissue Culture Facility (Chapel Hill, NC), BT-549 cells were from Rolf Craven (University of Kentucky), and A375 were from Dr. Suyan Huang (M.D. Anderson Cancer Center, Houston, TX). All sources obtained the lines from ATCC (Manassas, VA). 435s is genetically identical to melanoma M14, and BT-549 cells are identical to ATCC BT-549 [127]. WM melanoma cells were obtained from Dr. Meenhard Herlyn (Wistar Institute, Philadephia, PA). A375 was from Dr. Suyun Huang (M.D. Anderson Cancer Center, Houston, TX). Primary melanoma cells (HEMn) were purchased from Invitrogen (Carlsbad, CA) and MCF-7 cells were gifts from Dr. Vivek Rangnekar (University of Kentucky, Lexington, KY). Breast cancer cell lines were obtained from University of North Carolina Tissue Culture Facility (Chapel Hill, NC). Human mammary epithelial cells (HMEC) were purchased from Cambrex (Baltimore, MD).

The following antibodies were purchased commercially: c-Abl (K12; kinase assay), Arg (9H5-western blot), α-tubulin, LAMP1, cathepsin L1, HRP-conjugated secondary antibodies (Santa Cruz Biotechnology; Santa Cruz, CA); pCrk/CrkL (Y221/Y207), c-Abl (8E9; western blotting), GAPDH, (BD Biosciences; Chicago, IL); EEA1, Rab7, p27, NM23-H1 (D98-immunohistochemistry, D14H1-western blotting), (Cell Signaling; Danvers, MA); cathepsin B (R&D; Minneapolis, MN), cyclin D1 (Millipore; Billerica, MA); Rab5 (Abcam, Cambridge, England) and β-actin (Sigma Aldrich, St. Louis, MO). The Arg kinase Assay Antibody was previously described [47]. Imatinib and nilotinib were provided by Novartis (Basel, Switzerland); IGF-1 was from Upstate Biotechnology (Charlottesville, VA), matrigel invasion chambers were from BD Biosciences; chloroquine and ammonium chloride were from Sigma; MG132 and proteasome inhibitor I (PS1) were from Millipore; and E64d was from Tocris (Minneapolis, MN).

*Western Blots and Kinase Assay*

Cell lines were lysed in RIPA buffer (50mM Tris pH 7.5, 150mM NaCl, 1% triton-X 100, 0.1% SDS, 1% sodium deoxycholate, 1mM pefabloc, 1mM sodium orthovanadate, 25mM sodium fluoride, 10μg/ml leupeptin, 10μg/ml aprotinin, 10μg/mL pepstatin). Total protein was quantitated using Lowry (Biorad, Hercules, CA) or Pierce BCA protein assay (Thermo Scientific, Waltham, MA). Equal protein was loaded onto SDS-PAGE gels. Immunoblotting was performed using antibody manufacturer protocols. Kinase assays were performed as previously described [47]. Bands were quantified using ImageQuant (GE Healthcare, Piccataway, NL) or ImageJ (NIH, Bethesda, MA).

*Transfection and RNAi*

Cells were plated in 60mm dishes and transfected two consecutive days to increase knockdown efficiency. Cells were transfected with the following siRNAs (Applied Biosystems; Carlsbad, CA) using Lipofectamine 2000 (Invitrogen). Abl #1,2 (1336, s886, respectively; 20nM), Arg #1,2 (1478, s872; 20nM), NM23-H1 (s9588; 1nM); cathepsin L1 (s3753; 5nM); cathepsin B (s3740; 5nM), scrambled control #1 (control for non-silencer select), silencer select scrambled #1 (for silencer select). Cells were infected with PLK01-NM23-H1 shRNA (182s1c1) or PLK01 non-targeted shRNA Mission lentiviruses (MOI6; Sigma;12h; 8mg/ml polybrene) and selected with puromycin (1mg/ml).

*RT-PCR*

Semi-quantitative. Cells were plated in 60mm dishes and treated per experiments. RNA was extracted using RNAeasy kit (Qiagen, Valencia, CA). RNA was then DNase treated with TURBO DNA-free (Life Technologies, Grand Island, NY). RNA was reverse transcribed into cDNA using SuperScript reverse transcriptase and random primers (Invitrogen, Carlsbad, CA) and PCR was performed using specific primers (below) together with β-actin control primers. Aliquots were taken at cycles 27-35 to check for linearity. Bands were quantified with ImageQuant (GE Healthcare) and normalized to b-actin. c-Abl, Arg and β-actin primers were previously described [56].

cathepsin B forward primer, 5'TGGACAAGAAAAGGCCTGGTT3'

cathepsin B reverse primer, 5'CCCAGTCAGTGTTCCAGGA3'

cathepsin L1 forward primer, 5'CTGTGAAGAATCAGGGTCAGTG3'

cathepsin L1 reverse primer, 5'CTGGCTGCTGAGGCAATTC3'.

Real-Time RT-qPCR (Bio-Rad; Hercules, CA). DNase-treated cDNA (50ng; iScript; Bio-Rad) was amplified using SYBER green and specific primers (500 ng, 40 cycles, 62$^{o}$C annealing temperature). Results were analyzed with CFX Manager (Bio-Rad). Primers were designed and blasted with Primer Blast (NCBI website) to rule out homology to other genes. RPS13 was found to be the most stable reference gene out of five tested (RPS13, β-actin, HSP13, RPS11, and RP2).

NM23-H1 forward primer, 5'CAGTGTTACCATCCCCGACC3'

NM23-H1 reverse primer, 5'CAACAATATGAAGTAACCAACTCA3'

RPS13 forward primer, 5'CGAAAGCATCTTGAGAGGAACA3'

RPS13 reverse primer, 5'GCACCACGTCCAATGACAT3'

β-actin forward primer, 5' GAGCACAGAGCCTCGCCTTT3'

β-actin reverse primer, 5' CGAGCGCGGCGATATCATCA3'

HSP13 forward primer, 5'TCTGGGTATCGGAAAGCAAGCC3'

HSP13 reverse primer, 5'GTGCACTTCCTCAGGCATCTTG3'

RPS11 forward primer, 5' GCCGAGACTATCTGCACTAC3'

RPS11 reverse primer, 5' ATGTCCAGCCTCAGAACTTC3'

RP2 forward primer 5'GCACCACGTCCAATGACAT3'

RP2 reverse primer 5'GTGCGGCTGCTTCCATAA3'

*Percoll-Gradient Fractionation*

Cells were plated to 60% confluency in six 150cm plates per treatment group. Plates were washed 2x in PBS+ (0.5mM MgCl2 and 1mM CaCl2) and scraped into PBS without $MgCl_2$ and $CaCl_2$. Cells were spun 5', 1,200 RPM, 4°C. Cell pellets were resuspended in homogenization buffer (20mM HEPES, 150mM NaCl, 2mM CaCl2, pH 7.4, 1mM pefabloc, 1mM sodium orthovanadate, 25mM sodium fluoride, 10µg/ml leupeptin, 10µg/ml aprotinin, 10µg/mL pepstatin). Cells were spun again as before, followed by dounce homogenization. Cells were spun again and supernatant was collected (PNS). A sample of the PNS was solubilized, and protein levels were determined using Pierce BCA protein assay (Thermo Scientific, Waltham, MA). Eleven mL of Percoll solution (10mM Tris pH 8.0, 150mM NaCl, 20% Percoll, +inhibitors) was added to a 12 mL centrifuge tube and equal PNS protein amounts across treatment groups added to top of Percoll solution. Tubes were centrifuged for 50', 15,000RPM (20,000g), 4°c in a 70.1Ti fixed angle rotor (Beckman Coulter, Indianapolis, IN). Fractions (1mL) were collected and incubated in CHAPS (10mM final) for 1 hour. SDS-PAGE sample buffer (4x) was added to fractions, boiled for 5' and supernatant collected. Equal volumes of fractions were loaded on an SDS-PAGE gel and immunoblotted (EEA1, LAMP1, RAB7, NM23-H1).

*Matrigel Boyden Chamber Invasion Assays*

Cells were serum-starved for 24hrs and treated with nilotinib for 8hrs. Cells were trypsinized and resuspended in invasion assay media (basal media + 1% BSA + vehicle/inhibitor) and washed 3x in invasion assay media. Invasion assay media was added to a well in a 24 well plate with 10nM IGF-1 added as a chemoattractant. The matrigel invasion chamber (BD Biosciences, Chicago, IL) was placed into the well, and $2.5x10^5$ cells were plated in the top of the chamber. After the cells were allowed to migrate through the matrigel for 48hr (37°C), the membrane was rinsed in PBS, cells on the upper surface of the membrane were removed, cells on the lower surface were fixed in methanol, stained (1% toluidine blue in 1% borax), and mounted on a slide. Cells on the lower surface were counted using a 20x objective.

*Lung Colonization/Metastasis Assays*

The metastasis assay is described in detail in Ganguly, et al. [57]. 435s-GFP/luciferase cells, were injected ($2x10^6$ cells/100ml Hanks Balanced Salt Solution; Invitrogen) into the tail vein of 7- 8-week old SCID-beige mice. Mice were treated with vehicle (0.5%

hydroxymethylcellulose/0.05% Tween-80) or nilotinib (33mg/kg in vehicle; b.i.d.) by oral gavage. Fluorescence was measured by IVIS Xenogen Spectrum (Caliper Life Sciences, Hopkinton, MA). Mice were euthanized on day 24 and lungs were removed, fixed in 100% formalin, paraffin embedded (see below), sectioned and immunostained (see below) with pCrk/CrkL or NM23-H1 antibody, and scanned on an Aperio Scanscope. The University IACUC Committee approved all studies.

*Paraffin Embedding*

Formalin-fixed lungs were dehydrated in 70% ethanol, 100% ethanol, 1:1 ethanol/xylene, followed by 100% xylene for 2 hours at room temperature. Lungs were placed in melted paraplast X-tra:xylene (1:1) for 1hr at 54$^{o}$C followed by room temperature incubation overnight. Paraplast (100%) was then added to the tissue for 1h at 54$^{o}$C, incubated at room temperature for 2h, paraffin was melted, and mounted at room temperature. The mounted lungs were sectioned on a ThermoShandon Finesse microtome (University of Kentucky Histology Facility) and stored in a desiccator for long-term storage.

*Immunohistochemistry*

Tissue microarrays (Biomax BR1502, BR10010a, BC08118, ME1003 and NCI melanoma progression array) were incubated at 60°C overnight. All of the following steps were performed at room temperature unless otherwise noted. Slides were rehydrated sequentially in xylene and ethanol (100%, 95%, 70%) for 5' (x3) and subsequently rinsed in deionized water. Endogenous peroxidase activity was blocked (Dako K4010) for 10' followed by antigen retrieval in Diva Decloaking solution (Biocare Medical) within a Biocare Medical Decloaking Chamber (30" at 125°c, 10" at 90°c). After cooling for 10', slides were rinsed in deionized water, and left in TBST buffer (Dako S3306) for 5'. Primary antibody was diluted in antibody diluent (Dako S0809) and incubated for 2 hours. Antibodies used were pCrk/CrkL (1:10, Cell signaling 3181), NM23-H1 (1:75, Cell Signaling D98), or negative control (Dako N1699). After being washed 3x in TBST and left for 5' the slides were incubated for 30' in Dako Envision+ System-HRP (K4010). After being washed 3x in TBST, left for 5', the slides were incubated for 5-10' in either Diaminobenzine (DAB, (brown color) Dako 4011), for breast cancer slides, or AEC+ Red (Dako K3461) was used for melanoma slides in order to be able to differentiate between chromagen and brown melanin pigmentation. The DAB/AEC+ chromagen was deactivated in tap water and subsequently dipped in hematoxylin stain (Dako S3302), deionized water, bluing Reagent (Richard-Allan

Scientific), and again deionized water. For the DAB chromagen, slides were dehydrated sequentially in ethanol (70%, 95%, 100%) and xylene for 5' (2x), and mounted in xylene-based mount (Vector H5000). For AEC+ chromagen slides, the dehydration steps were bypassed and the slides were immediately mounted in aqueous mountant (Vector H5501). A pathologist (Michael Cibull, M.D.) blindly scored the slides. Each core was assigned intensity of staining (1-3+) and percentage of positively-staining tumor cells. A score for each core was calculated by multiplying the intensity of stain by the percentage of positively-stained tumor cells.

*Statistics*

Statistical analyses were performed with SAS or the Vassar Website (http://vassarstats.net). Some data were normalized to vehicle or scrambled control, and analyzed with one-sample t-tests. Two-tailed values are shown. $0.01 \leq *p<0.05$; $0.001 \leq **p<0.01$; $***p<0.001$.

## Results

*Abl Family Kinases Downregulate NM23-H1 Expression*

c-Abl/Arg and NM23-H1 have opposing functions; therefore, we investigated if a correlation exists between c-Abl and Arg kinase activity and NM23-H1 expression in melanoma and breast cancer cell lines. We analyzed c-Abl and Arg activity indirectly, by examining phosphorylation of the c-Abl/Arg kinase substrate, pCrk/Crk, or we directly assessed c-Abl and Arg kinase activity by *in vitro* kinase activity assay utilizing GST-Crk as substrate in panels of melanoma and breast cancer cell lines, respectively. We observed NM23-H1 expression to be the lowest in cell lines with the highest c-Abl and Arg activity (Figure 3.1a,b). In fact, when we plotted pCrk/CrkL expression or c-Abl/Arg kinase activity against NM23-H1 protein expression we observed a significant negative correlation in both the melanoma and breast cancer cell line panels (Figure 3.1a,b,bottom), indicating a potential link between Abl family kinase activity and NM23-H1 expression.

To determine if c-Abl and Arg lie upstream of NM23-H1, we transfected two independent siRNAs directed against c-Abl and Arg into the invasive 435s and BT-549 cells lines, which have endogenously high c-Abl/Arg activity and low NM23-H1 expression. Knocking down c-Abl or Arg resulted in a significant increase in NM23-H1 expression (Figure 3.2a,b, left). Furthermore, inhibition of c-Abl and Arg activity with

**Figure 3.1 Abl Kinase Activity and NM23-H1 Expression are Inversely Correlated in Cell Lines**

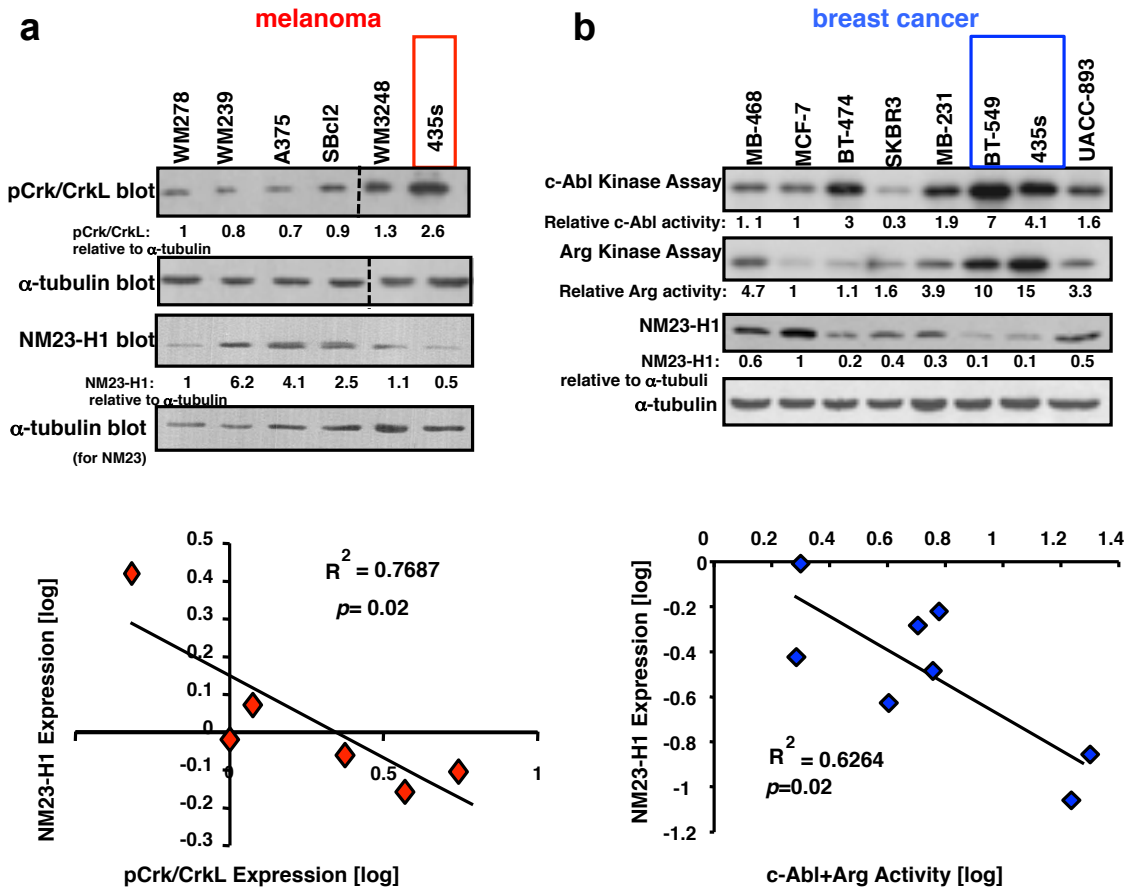


**Figure 3.1 Abl kinase activity and NM23-H1 expression are inversely correlated in cell lines. (a,b)** c-Abl/Arg activities were assessed indirectly via phosphorylation of substrates, Crk/CrkL **(a)** or directly by *in vitro* kinase assay (b)**,** in melanoma (a) or breast cancer (b) cell lines, bands quantitated, and log-transformed values for c-Abl+Arg activity plotted against log-transformed NM23-H1 expression values. The inverse correlations were statistically significant. **(a)** Pearson's correlation coefficient =-0.79, 95% confidence interval -0.96 to -0.19, *p*=0.02. **(b)** Pearson's correlation coefficient =-0.85, 95% confidence interval -0.98 to -0.139, *p*=0.03. Panel (a) Performed by W. Friend and S. Ganguly and panel (b) performed by R. Plattner and D. Srinivasan.

**Figure 3.2 c-Abl and Arg Activation Induces Loss of NM23-H1 Expression**

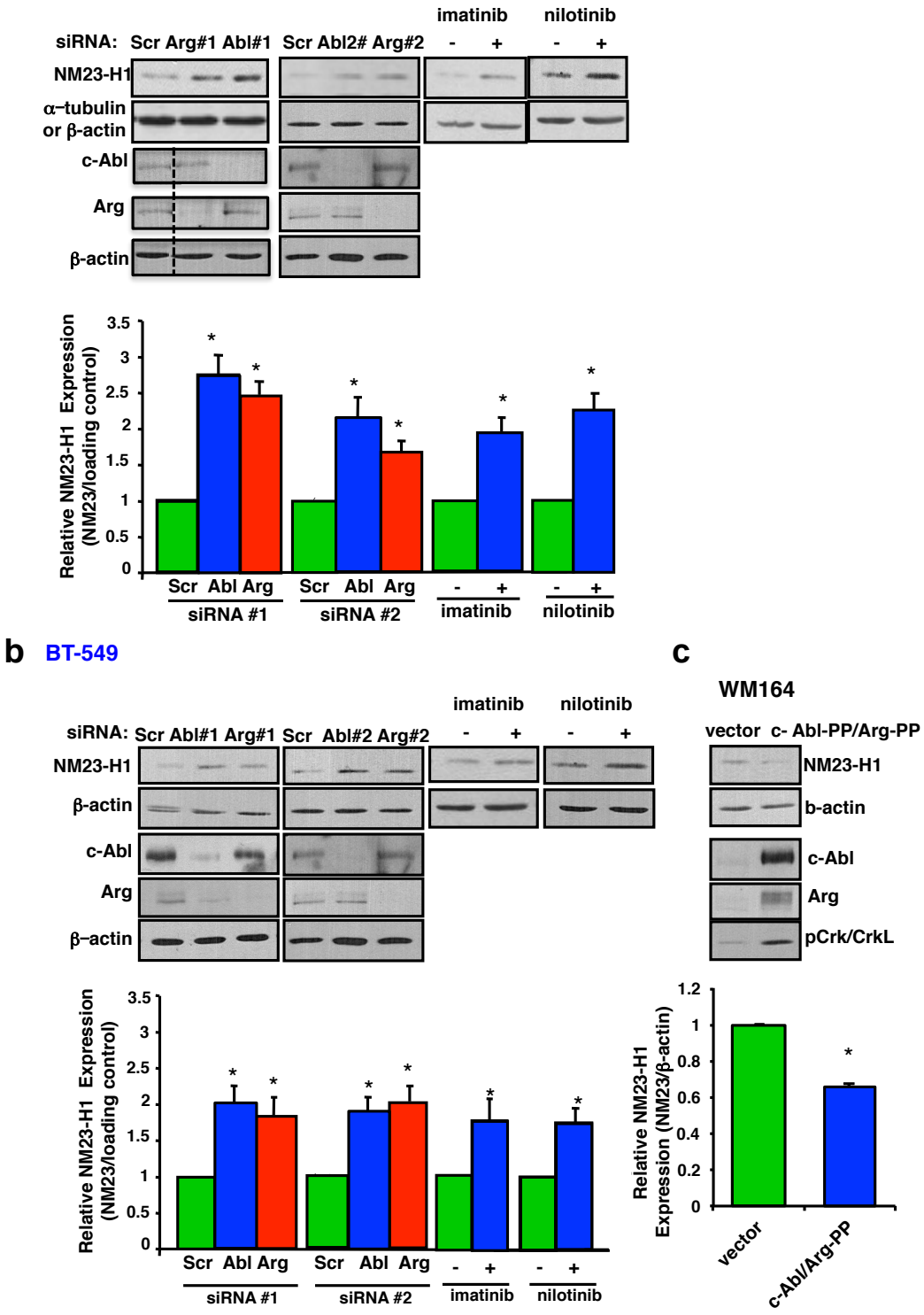

**Figure 3.2 c-Abl and Arg activation induces loss of NM23-H1 expression. (a,b)** Cell lines containing highly active c-Abl/Arg were transfected with c-Abl or Arg siRNAs (#1 and #2 are independent siRNAs) or treated with imatinib (10μM) or nilotinib (5μM) for 8h, and NM23-H1 expression examined by western blot. Bands were quantitated, and values expressed relative to loading controls and to scrambled- or vehicle-treated cells. **(c)** Western blot analysis of lysates from WM164 melanoma cells transiently transfected with constitutively active c-Abl and Arg (PP) (72h). Relative NM23-H1 expression (relative to loading control) was graphed (Mean±SEM for 3-4 independent experiments). *$p$<0.05, **$p$<0.01, ***$p$<0.001 using one-sample t-tests. R. Plattner performed siRNA #1 experiment in panel a,b S. Ganguly performed siRNA#2 experiment in panel a,b, and experiment in panel c.

imatinib or the second generation c-Abl/Arg inhibitor, nilotinib, significantly increased NM23-H1 expression (Figure 3.2 a,b, right). Next, we transfected constitutively active c-Abl-PP and Arg-PP into the non-invasive WM164 melanoma cell line and observed a decrease in NM23-H1 expression (Figure 3.2 c). Taken together, these data demonstrate that c-Abl and Arg kinases downregulate NM23-H1 expression in a kinase-dependent manner.

*Abl Family Kinases Do Not Transcriptionally Regulate NM23-H1*

Several studies have linked NM23-H1 mRNA expression to loss of invasive and metastatic potential [69, 128], while others have shown the opposite [106, 129] or no correlation [115, 130]. Therefore, we investigated if the decrease in NM23-H1 protein expression we observed with c-Abl/Arg knockdown/inhibition could be due to mRNA regulation. First, to determine if NM23-H1 mRNA and protein levels are correlated in melanoma and breast cancer cells, we analyzed NM23-H1 mRNA levels using quantitative real-time RT-PCR on melanoma and breast cancer cell lines. We determined RPS13 to be the most stable reference gene among the five that we tested (β-actin, HSP13, RP2, RPS11, and RPS13) (Figure 3.3a). There was no significant correlation between NM23-H1 mRNA and protein levels in melanoma and breast cancer cell lines (Figure 3.3 b-d) suggesting that protein levels of NM23-H1 are regulated independently of mRNA expression. To confirm that c-Abl and Arg do not regulate NM23-H1 mRNA expression, we performed semi-quantitative RT-PCR in 435s cells, and observed no change in NM23-H1 mRNA following siRNA-mediated knockdown of c-Abl and Arg (Figure 3.3e). Taken together, these results indicate that c-Abl and Arg do not regulate NM23-H1 mRNA expression; therefore, c-Abl/Arg downregulation of NM23-H1 is likely a post-transcriptional mechanism.

*NM23-H1 is Degraded via Lysosomal Cathepsins*

To determine the mechanism in which c-Abl and Arg post-transcriptionally regulate NM23-H1, we determined the previously unknown mechanism of NM23-H1 protein degradation. Abl family kinases have been shown to promote proteasomal degradation [63]; therefore, we investigated if NM23-H1 could be a substrate of the proteasome. Treatment with proteasome inhibitors MG132 and Proteasome Inhibitor 1 (PS1) resulted in no change in NM23-H1 expression levels regardless of dose or length of treatment, whereas proteasomal substrates such as p27 and cyclin D1 were significantly

**Figure 3.3 c-Abl and Arg Do Not Trancriptionally Regulate NM23-H1**

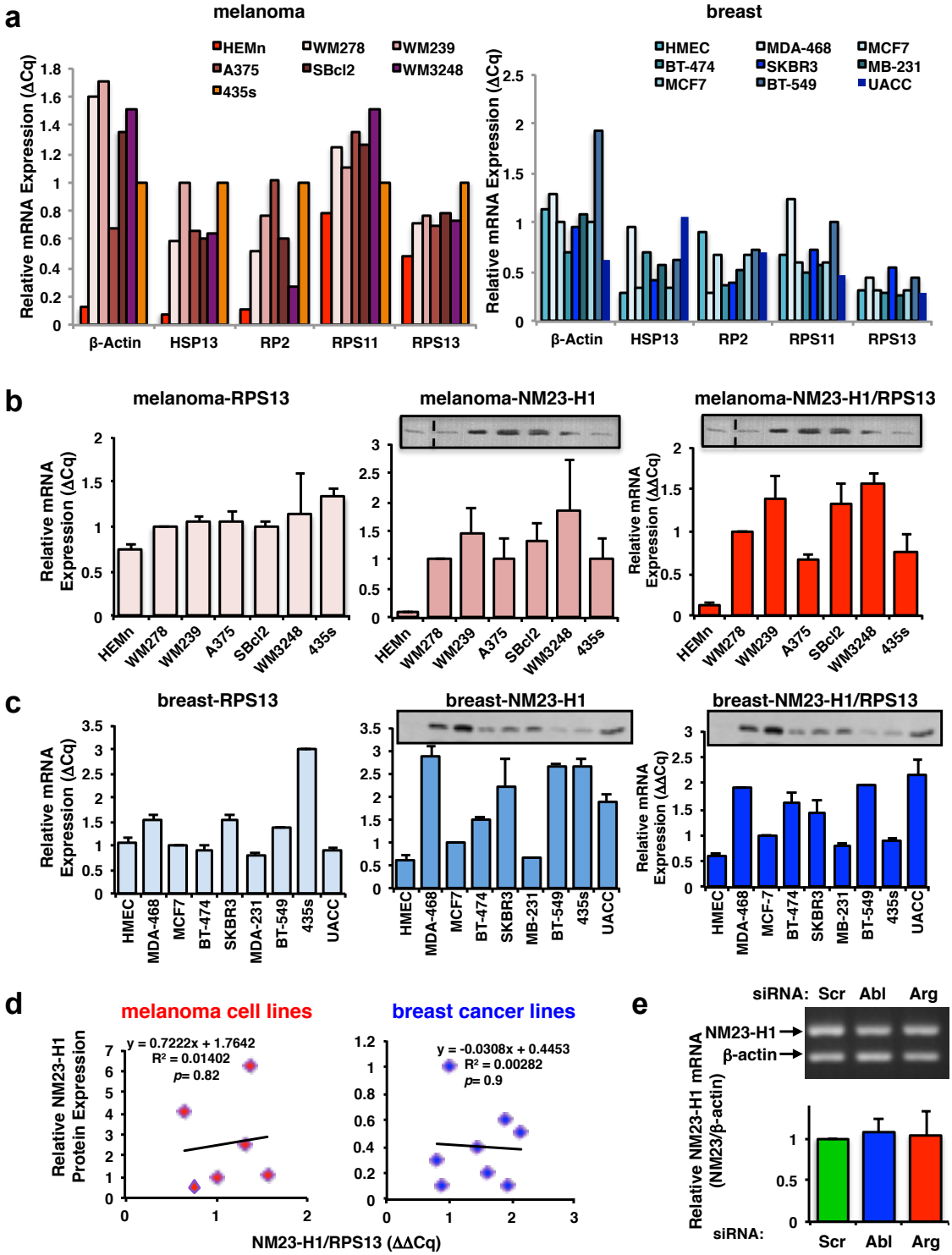

**Figure 3.3 c-Abl and Arg do not transcriptionally regulate NM23-H1. (a-c)** mRNA levels of were quantitated by real-time RT-qPCR **(a)** loading controls in a panel of serum-starved melanoma (left) or breast cancer (right) cell lines **(b,c)** Graphs are panel of serum-starved melanoma **(b)** or breast cancer **(c)** cell lines. Mean±SEM for 2 independent experiments performed in duplicate. Reference (RPS13), NM23-H1 (non-normalized), and NM23-H1 relative to RPS13 are shown. NM23-H1 protein is above graphs. HMEC=human mammary epithelial cells. HEMn=human epidermal melanocytes (light pigment). **(d)** NM23-H1 mRNA was graphed relative to protein for all cancer lines (excludes non-cancer cells). **(e)** cDNA was prepared from RNA from 435s cells expressing c-Abl or Arg siRNAs, and subjected to semi-quantitative RT-PCR using NM23-H1 primers and internal control b-actin primers. Relative NM23-H1 expression (relative to loading control) was graphed (Mean±SEM for 3 independent experiments).

upregulated (Figure 3.4a,b). These results indicate c-Abl and Arg downregulate NM23-H1 in a proteasome-independent manner.

Next, we investigated if NM23-H1 degradation occurred via the lysosome, a protease-filled organelle involved in protein turnover. Inhibition of the lysosome with ammonium chloride ($NH_4Cl$) or chloroquine in 435s and BT-549 cells increased NM23-H1 expression levels, suggesting lysosomal involvement in NM23-H1 degradation (Figure 3.5a,b). Within the lysosome, cathepsin proteases are responsible for the degradation of proteins. To determine the specific proteases responsible for NM23-H1 degradation, we treated 435s and BT-549 cells with a cell-permeable cysteine protease inhibitor, E64d, and observed increased NM23-H1 expression (Figure 3.5 a,b). Of the 11 cysteine proteases, cathepsins L and B are the most documented in promoting invasion and metastasis [37, 41]; therefore, we hypothesized they may be responsible for degrading a metastasis suppressor. Indeed, siRNA-mediated knockdown of cathepsins L and B significantly increased NM23-H1 expression in both 435s and BT-549 cells (Figure 3.5c). Furthermore, using an *in vitro* cleavage assay, we demonstrated that cathepsins L and B directly cleave NM23-H1 [89]. These data indicate that lysosomal cathepsins L and B are responsible for the degradation of NM23-H1.

*Abl Family Kinases Increase Cathepsin L/B Expression and Activation*

We demonstrated that c-Abl/Arg and cathepsins L/B are both involved in the downregulation of NM23-H1; therefore, we investigated if c-Abl and Arg affect cathepsin L and B activation and/or expression. Cathepsins are expressed as proforms in the golgi apparatus and early endosomes, then cleaved to an intermediate active form in the late endosome, and subsequently cleaved again to a double chain active form within the lysosome [37]. Inhibition of c-Abl/Arg with imatinib or nilotinib in 435s cells, resulted in decreased double-chain active cathepsin L/B and increased procathepsin L/B (Figure3.6a, left), indicating that c-Abl/Arg activity promote the cleavage of cathepsin L and B to their double-chain mature form in melanoma cells. In BT-549 cells, we also observed the cleavage of cathepsin L to be blocked following c-Abl/Arg inhibition; however, very little double chain active form is present in these cells, thus, we observed a reduction in the cleavage of the pro-form to the single chain active form instead (Figure 3.6b, left). These data demonstrate that c-Abl/Arg promote cleavage of cathepsins L/B to their active forms.

Next, we investigated the effects of siRNA-mediated knockdown of c-Abl and Arg on cathepsin L and B expression/activation. In 435s cells, we observed decreased

**Figure 3.4 NM23-H1 is Not a Proteasomal Substrate**

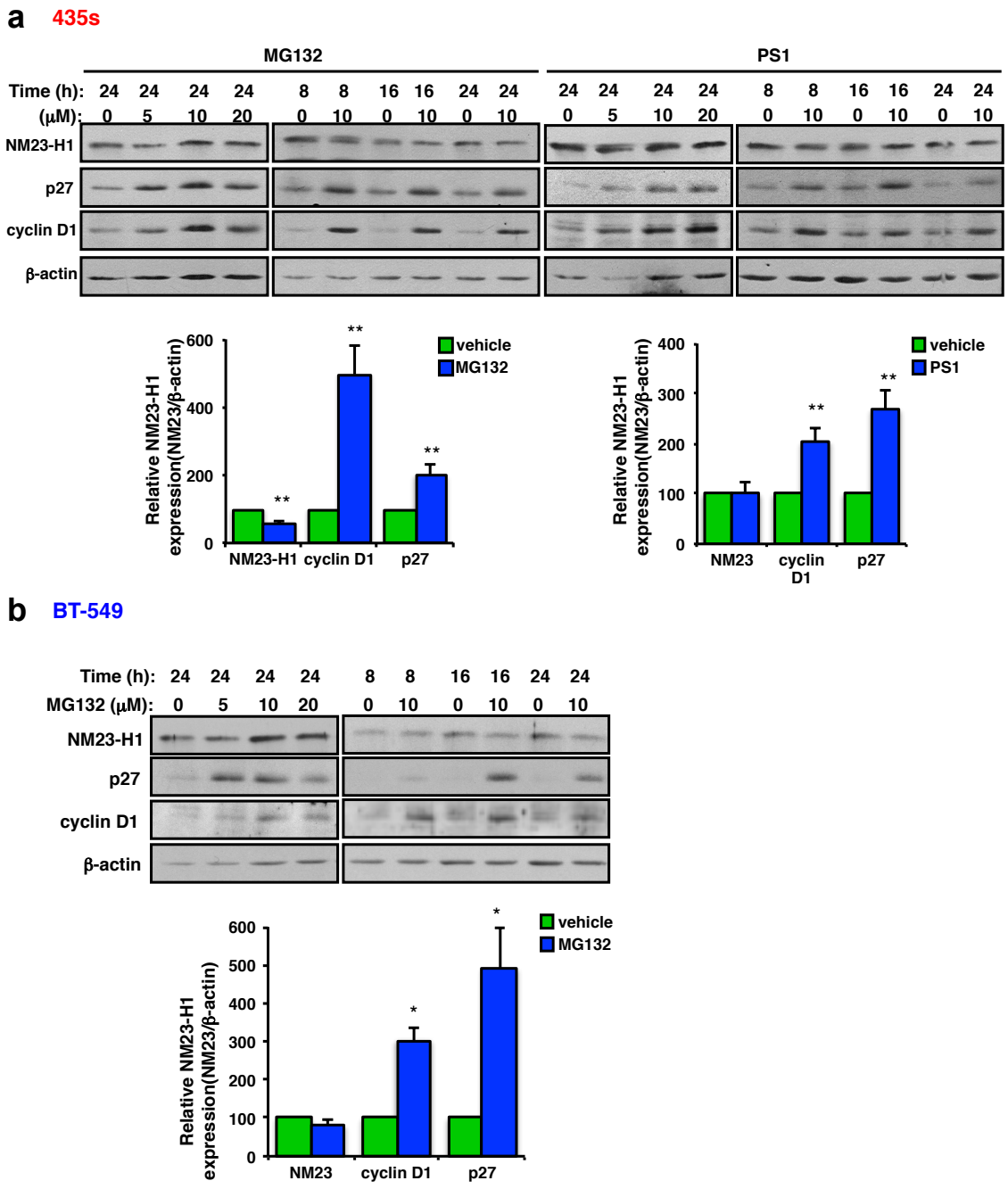


**Figure 3.4 NM23-H1 is not a proteasomal substrate. (a,b)** Lysates from 435s (a) and BT-549 (b) detached and attached cells treated with the proteasome inhibitor, MG132 and proteasome inhibitor 1 (PS1) and probed with antibodies. Relative NM23-H1 expression (relative to loading control) was graphed with mean ±SEM for three independent experiments. $p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ using one-sample t-tests.

**Figure 3.5 NM23-H1 is Degraded by Lysosomal Cysteine Proteases, Cathepsins L and B**

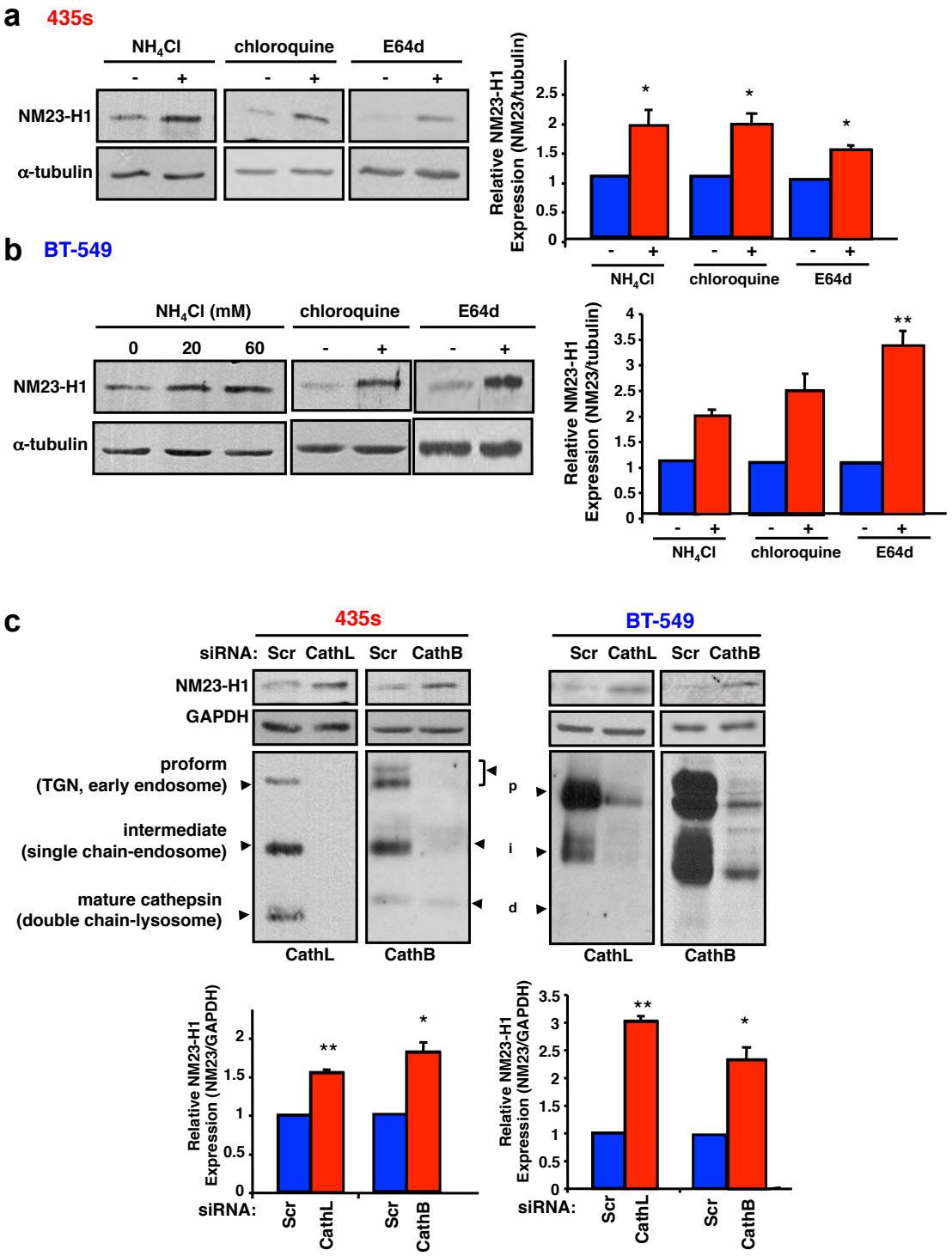

**Figure 3.5 NM23-H1 is degraded by lysosomal cysteine proteases, cathepsins L and B. (a,b)** Western blot analysis of lysates from cells treated with lysosomal inhibitors (ammonium chloride, 20mM (BT-549) or 60mM (435s, BT-549); chloroquine, 100mM) or the cysteine protease inhibitor, E64d (20mM) for 8h. **(c)** Lysates from cell lines transfected with siRNAs were blotted with antibodies 72h after the initial transfection. Relative NM23-H1 expression (relative to loading control) was graphed with the mean±SEM for 3 independent experiments. *$p$<0.05, **$p$<0.01, ***$p$<0.001 using one-sample t-tests.

## Figure 3.6 c-Abl and Arg Promote Cathepsin Expression and Activation

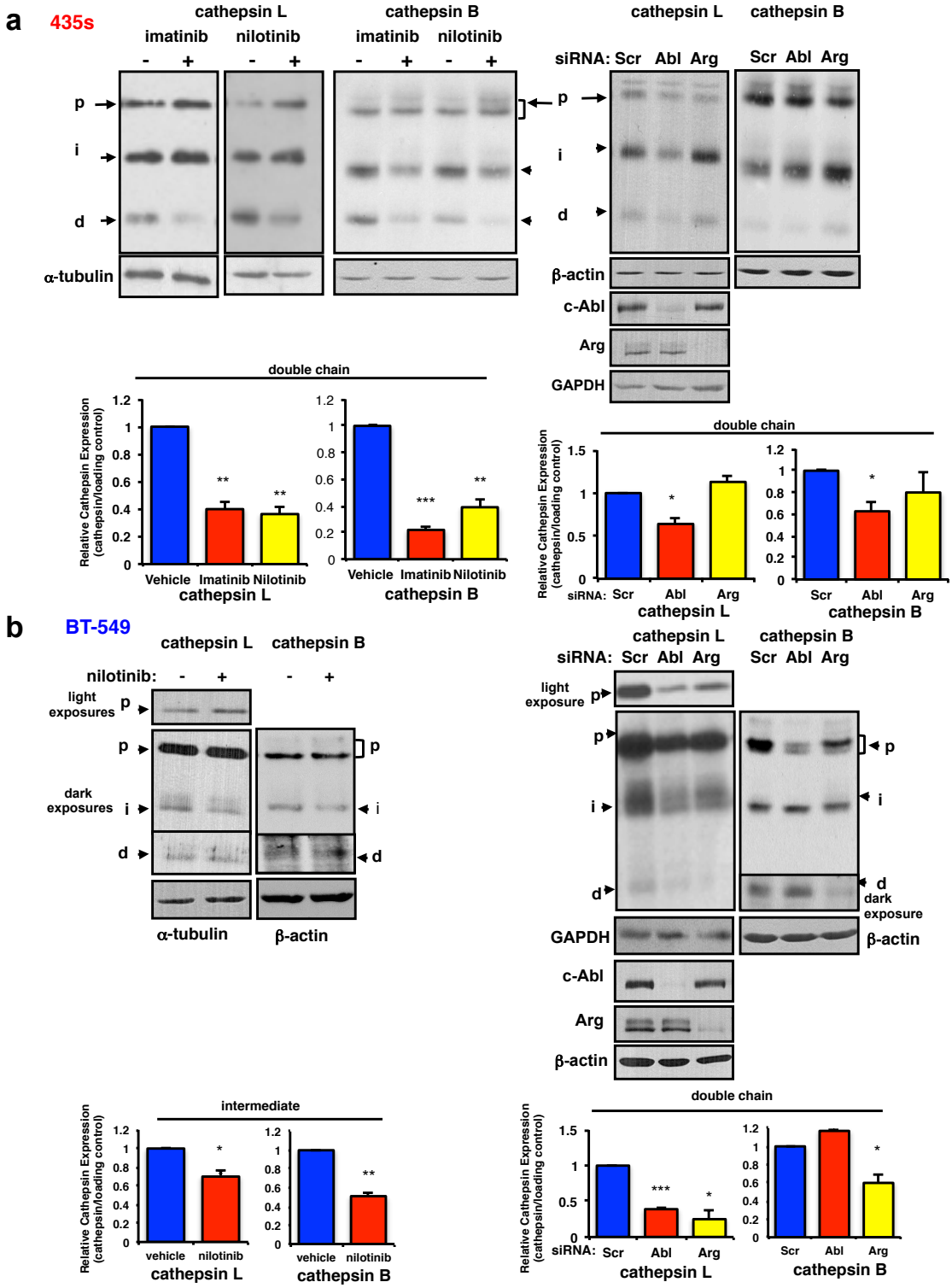

**Figure 3.6 c-Abl and Arg promote cathepsin expression and activation. (a,b)** Western blot analysis of lysates from 435s (a) and BT-549 (b) cells treated with imatinib (10μM) or nilotinib(5μM) for 48h (left) or transfected with siRNAs (right), and replated 24h prior to lysis such that densities were equivalent. Relative cathepsin expression levels (relative to loading control) were graphed with the mean±SEM for 3 independent experiments. *$p$<0.05, **$p$<0.01, ***$p$<0.001 using one-sample t-tests. p=procathepsin, i=intermediate, d-double chain. siRNA experiment in panel (a) performed by S. Ganguly.

expression of the double-chain active form of cathepsins L/B following c-Abl knockdown; however, no significant change was observed with Arg knockdown, indicating that cathepsin activation is c-Abl-dependent in these cells (Figure 3.6a, right). Interestingly, in contrast to the increase in the proform of cathepsins L/B we observed with imatinib and nilotinib, silencing c-Abl and Arg in 435s cells decreased procathepsin expression, suggesting c-Abl and Arg upregulate cathepsin expression in a kinase-independent manner (Figure 3.6a, right). In BT-549 cells, silencing c-Abl and Arg decreased the double chain active form of cathepsin L, however the cathepsin B active form only decreased with Arg knockdown, indicating that activation of cathepsin B in BT-549 cells is an Arg-dependent mechanism (Figure 3.6b, right). Furthermore, similar to 435s, silencing c-Abl and Arg decreased the procathepsin form of both cathepsins L and B in BT-549 cells. In summary, in 435s cells, activation of cathepsin L and B is c-Abl-dependent, whereas in BT-549 cells it is Arg-dependent. Furthermore, c-Abl and Arg increase the expression of the proform of cathepsins L and B; however c-Abl/Arg kinase activity has no effect on proform expression, indicating that c-Abl and Arg upregulate cathepsins L and B expression in a kinase-independent manner.

Our observation that c-Abl and Arg increase expression of procathepsin L and B led us to investigate if c-Abl and Arg regulate cathepsin L/B mRNA levels. In the 435s cells, knockdown of both c-Abl and Arg reduced cathepsin L and B mRNA levels, and Arg knockdown had the most dramatic effect (Figure 3.7a). In BT-549 cells, cathepsin L mRNA levels were decreased following c-Abl but not Arg knockdown. Interestingly, in BT-549 cells, neither c-Abl nor Arg knockdown had an effect on cathepsin B mRNA levels (Figure 3.7b). Our data demonstrating that procathepsin L and B proteins are not reduced following nilotinib treatment is further supported by data demonstrating that nilotinib had no effect on cathepsin L/B mRNA levels (Figure 3.7a,b). In summary, c-Abl and Arg promote activation of cathepsin L and B in a kinase-dependent manner, whereas c-Abl and Arg promote expression of cathepsin L/B mRNA in a kinase-independent manner.

*Abl Family Kinases Promote Endosomal Trafficking of NM23-H1 to the Lysosome*

We demonstrated that inhibition of c-Abl/Arg increased expression of the early endosome form of cathepsin L/B and decreased expression of the lysosomal forms, suggesting that c-Abl and Arg may play a role in endosomal vesicular trafficking. To determine if c-Abl/Arg activity promotes endosomal trafficking and delivery of NM23-H1 to the lysosome, we treated 435s cells with nilotinib and performed a Percoll-gradient

**Figure 3.7 c-Abl and Arg Promote Cathepsin Expression in a Kinase-Independent Manner**

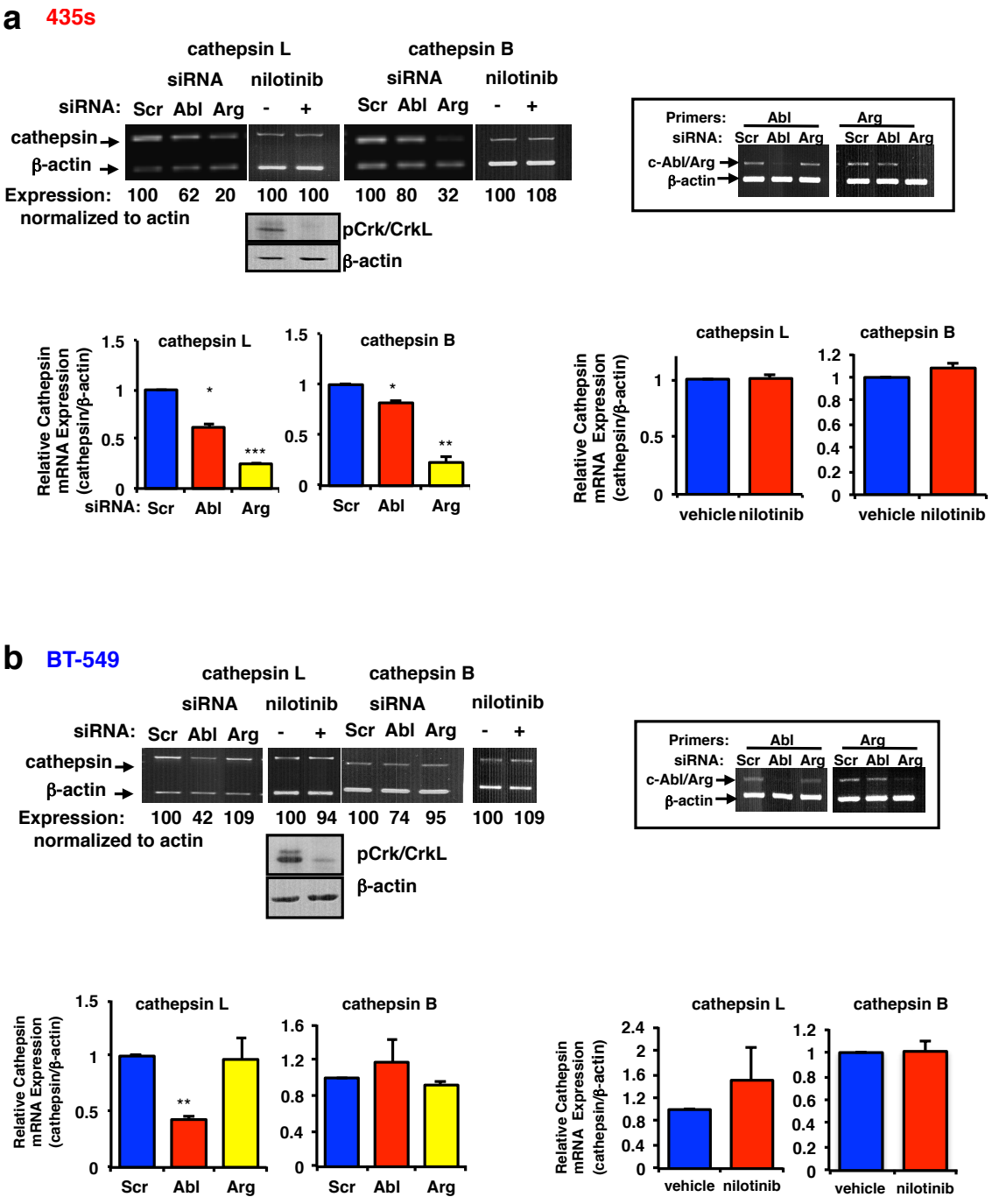

**Figure 3.7 c-Abl and Arg promote cathepsin expression in a kinase-independent manner. (a,b)** Cathepsin mRNA levels in cells treated with nilotinib (5μM, 48h) or transfected with siRNAs (72h) were examined by semi-quantitative RT-PCR. Knockdown efficiency is shown (right). pCrk/CrkL (c-Abl/Arg targets) blots indicate that nilotinib effectively inhibited c-Abl/Arg. Relative cathepsin mRNA expression levels (relative to loading control) were graphed with the mean±SEM for 3 independent experiments. *$p$<0.05, **$p$<0.01 using one-sample t-tests.

subcellular fractionation. In vehicle-treated cells, lysosomes (LAMP1=lysosome marker) and late endosomes (Rab7) were localized within high-density fractions (fractions 10-12), and the early endosomes (EEA1) were localized within low-density fractions (fractions 1-6) and high-density fractions (fractions 10-12) (Figure 3.8a, left). We believe that EEA1 localization in high-density fractions is due to the formation of hybrid vesicles as early endosomes mature to a late endosomes. In vehicle-treated cells, NM23-H1 localized in all fractions; however, expression was higher in the early endosome fractions as compared to the lysosome fractions, likely due to degradation of NM23-H1 within the lysosome (Figure 3.8a, left). Inhibition of c-Abl/Arg with nilotinib resulted in increased early endosomes (Figure 3.8a,b), decreased lysosomes (Figure 3.8a, c), and a slight decrease in late endosomes (Figure 3.8a, d). Furthermore, c-Abl/Arg inhibition increased NM23-H1 expression within endosomal fractions coupled with decreased expression in the lysosomal fractions (Figure 3.8a, e-h). Taken together, these results demonstrate that c-Abl/Arg activity induces NM23-H1 degradation via promoting endosomal trafficking to the lysosome.

*Abl Family Kinases Promote Invasion/Metastasis via NM23-H1 Downregulation*

Previously, our lab demonstrated that c-Abl and Arg kinases promote invasion in 435s and BT-549 cells [56, 89]. Therefore, we investigated if c-Abl and Arg promote invasion in these cells by inducing NM23-H1 degradation. Inhibition of c-Abl/Arg increased NM23-H1 expression and inhibited invasion in 435s and BT-549 cells (Figure 3.9a,b). However, preventing the upregulation of NM23-H1 by silencing NM23-H1 with an shRNA, partially rescued the block in invasion we observed in nilotinib-treated cells (Figure 3.9a,b), indicating c-Abl and Arg promote invasion by degrading NM23-H1.

Since c-Abl/Arg promote invasion by downregulating NM23-H1, we hypothesized that downregulation of NM23-H1 also was required for c-Abl/Arg to promote metastasis. Green fluorescent protein (GFP)/luciferase-labeled 435s cells were injected into the tail vein of SCID-beige mice. Metastatic burden was measured by IVIS imaging. Mice treated with nilotinib had significantly decreased metastatic burden as compared to mice treated with vehicle (Figure 3.10a,b). To determine if the outlier (mouse #10) with large metastases in the nilotinib-treated group responded to nilotinib treatment, we sectioned the lungs and performed immunohistochemistry assessing c-Abl/Arg activity by staining with antibody to the phosphorylated c-Abl/Arg substrate, Crk/CrkL. We observed intense pCrk/CrkL staining in the lung metastases from mouse #10 as compared to mouse #9 with low metastatic burden and low pCrk/CrkL staining (Figure 3.10c, left), indicating that

**Figure 3.8 c-Abl and Arg Kinase Activity Promotes Endosome-Lysosome Trafficking and Promotes NM23-H1 Expression in Lysosomal Fractions**

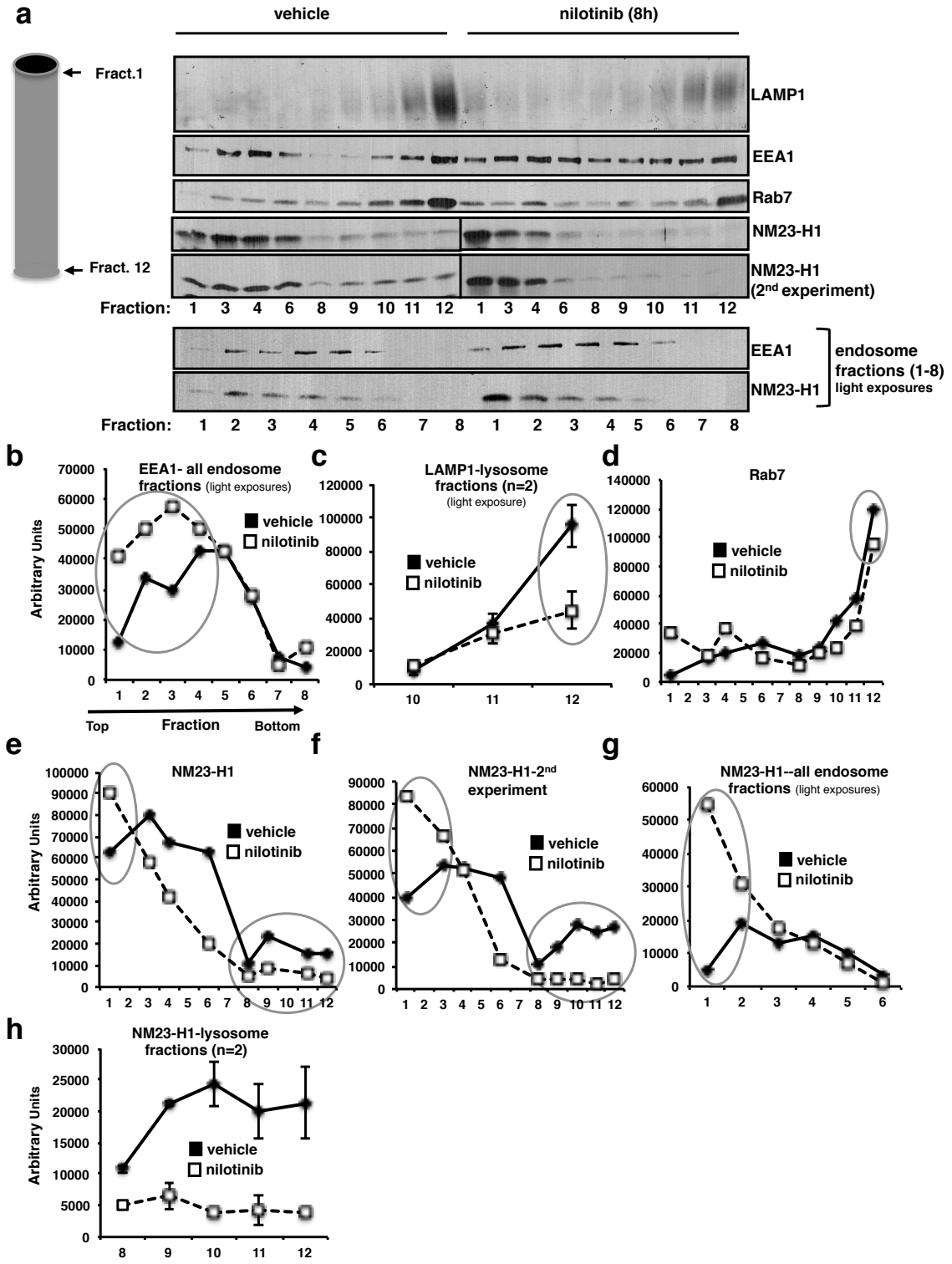

**Figure 3.8 c-Abl and Arg kinase activity promotes endosome-lysosome trafficking and promotes NM23-H1 expression in lysosomal fractions. (a)** 435s cells, treated with vehicle/nilotinib (5μM, 8h), were subjected to 20% Percoll gradient fractionation. Twelve fractions were collected, the indicated fractions (1,3,4,6,8,9-12) from vehicle- and nilotinib-treated cells were run on the same gel, and blotted with EEA1, Rab7 or LAMP-1 antibodies (top). Since all 24 fractions could not be run on one gel and fractions 2,5,7 were missing from the first gel, consecutive vehicle- and nilotinib-treated endosomal fractions (1-8) were rerun on a second gel. Western blots from one of three experiments are shown. Two independent experiments are shown for NM23-H1. **(b-h)** Bands were quantified and graphed. **(c)** Mean±SEM, n=2 for fractions 10-12 blotted with LAMP1 antibody. **(h)** Mean±SEM, n=2 for fractions 8-12 blotted with NM23-H1 antibody.

**Figure 3.9. c-Abl/Arg Promote Invasion by Inducing NM23-H1 Loss**

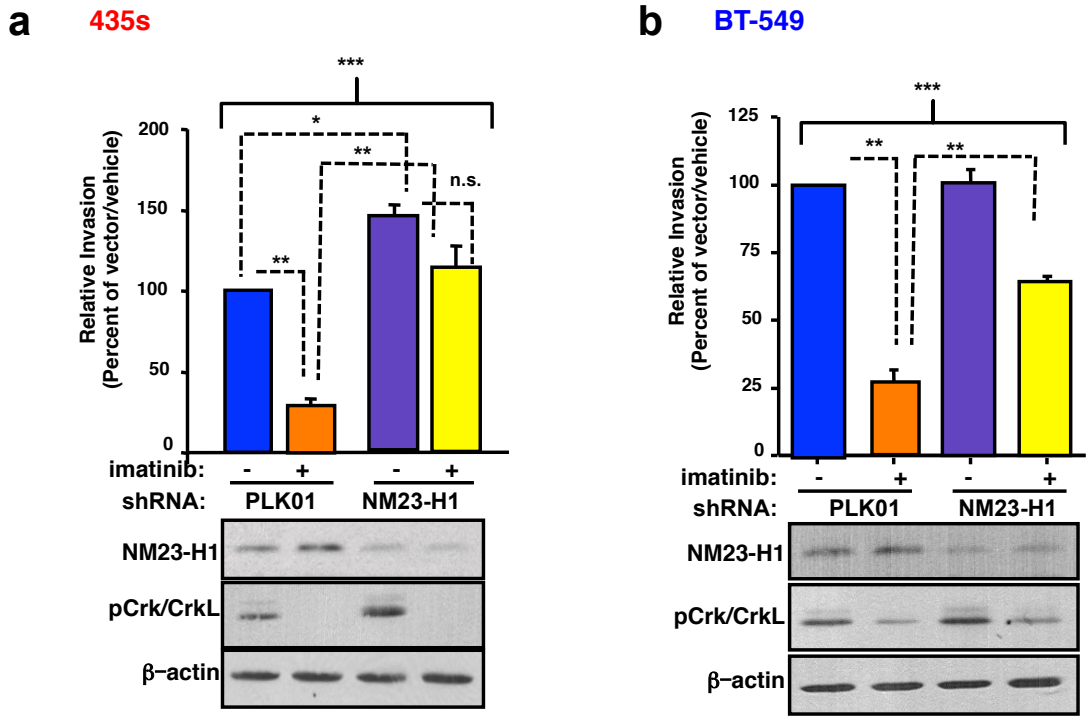


**Figure 3.9. c-Abl/Arg promote invasion by inducing NM23-H1 loss. (a,b)** 435s (a) or BT-549 (b) cells expressing non-targeting vector (PLK01) or NM23-H1 shRNA were serum-starved and treated with imatinib for 8h (435s; 10μM) or 16h (BT-549; 5μM), and utilized in a matrigel invasion assay. Lysates from a representative experiments were probed with antibodies. Brackets indicate comparisons among groups (***p<0.001), whereas dotted lines indicate post-hoc pairwise comparisons (*$p$<0.05, **$p$<0.01, or n.s. non-significant). S. Ganguly performed invasion assay experiments.

## Figure 3.10. c-Abl/Arg Promote Metastasis by Inducing NM23-H1 Loss

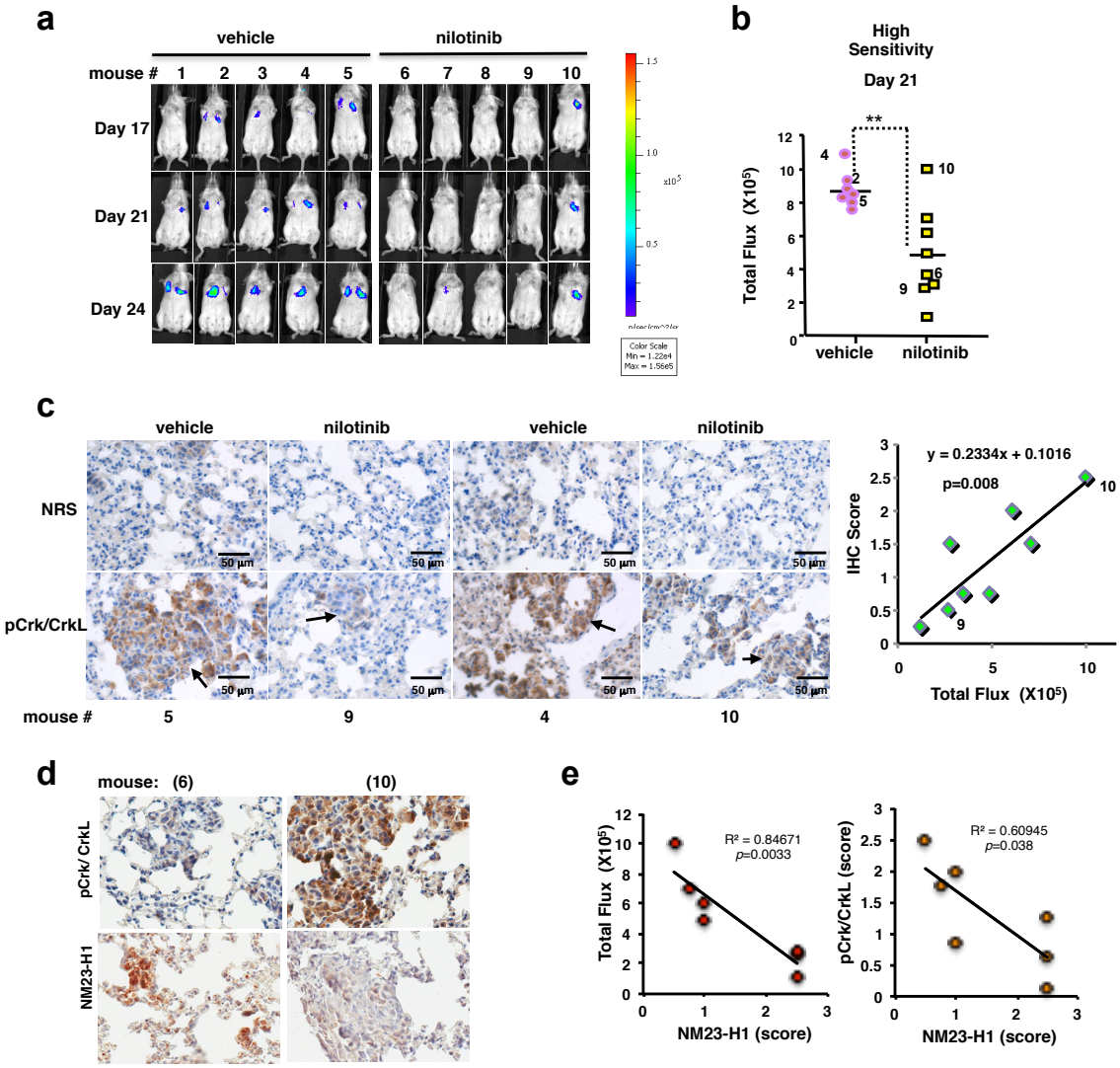

**Figure 3.10. c-Abl/Arg promote metastasis by inducing NM23-H1 loss. (a,b)** [57] SCID-beige mice were injected with 435s cells expressing GFP/luciferase and treated with nilotinib (n=7) by oral gavage and imaged with IVIS. **(a)** IVIS imaging of mice on days 17, 21, and 24. **(b)** quantitated fluorescence of mice on day 21. Each data point represents an animal and numbers correlate to numbers in panel a. **0.001≤p<0.01. **(c)** Lungs from nilotinib-treated mice were taken on day 24 and stained with normal rabbit serum or antibody directed against phosphorylated Crk/CrkL, and visualized with DAB. IVIS fluorescent values plotted against pCrk/CrkL IHC intensity scores (1-3+) in metastases (right). A positive correlation was observed between the values ($R^2$=0.72, p=0.008). **(d,e)** Lungs from nilotinib-treated mice were stained with NM23-H1 or pCrk/CrkL antibodies. Representative lungs from responding (low IVIS luminescence; mouse #6) and non-responding (high IVIS luminescence; mouse #10) mice are shown (d). NM23-H1 IHC scores were plotted against IVIS flux (left) or pCrk/CrkL scores (right) (e). NM23-H1/Flux Pearson's correlation coefficient = 0.92, 95% confidence interval -0.988 to -0.544, *p*=0.0033. NM23-H1/pCrk/CrkL Pearson's correlation coefficient =0.78, 95% confidence interval -0.965 to -0.068, *p*=0.038. Metastasis assays (a,b) performed by S. Ganguly.

c-Abl/Arg activity was not inhibited in response to nilotinib treatment in this mouse. In fact, pCrk/CrkL staining was positively correlated with metastatic burden in all mice, demonstrating that c-Abl/Arg kinase activity promotes metastasis (Figure 3.10c, right). Next, we investigated if c-Abl/Arg require the downregulation of NM23-H1 to promote metastasis. In addition to pCrk/CrkL staining, we stained serial lung sections with antibody to NM23-H1. Mouse #10, which had high metastatic burden and intense pCrk/CrkL staining, stained weakly for NM23-H1 as compared to mouse #6 with low metastatic burden, low pCrk/CrkL staining, staining intensely for NM23-H1 (Figure 3.10d, left). Furthermore, NM23-H1 expression was significantly inversely correlated to metastatic burden and pCrk/CrkL staining (Figure 3.10e), revealing that inhibition of c-Abl/Arg increases NM23-H1 expression preventing metastasis. In conclusion, c-Abl/Arg require the downregulation of NM23-H1 to promote invasion and metastasis.

*c-Abl/Arg Activity and NM23-H1 Expression are Inversely Correlated in Melanoma and Breast Cancer and Primary Tissue*

To determine if the downregulation of NM23-H1 via c-Abl/Arg that we observe in cell lines and in mice also occurs in human tissue, we stained melanoma and breast cancer tissue microarrays with pCrk/CrkL and NM23-H1 antibody to assess c-Abl/Arg activity and NM23-H1 expression. Melanoma and breast cancers that stained intensely with pCrk/CrkL antibody had low expression of NM23-H1, and vice versa (Figure 3.11a,b left). In fact, there was a significant inverse correlation between pCrk/CrkL staining and NM23-H1 expression (Figure 3.11a,b right), confirming that the mechanism of c-Abl/Arg downregulation of NM23-H1 expression observed in human cell lines and *in vivo* also exists in human melanoma and breast cancer primary tissues.

## Discussion

Here, we demonstrate a novel pathway in which the oncogenic Abl family kinases promote invasion and metastasis via cathepsin-mediated degradation of the NM23-H1 metastasis suppressor. Prior to our studies, very little was known regarding upstream regulators of NM23-H1 and the mechanism of NM23-H1 protein turnover also was unknown. We reveal a mechanism by which NM23-H1 is degraded within the lysosome via cathepsins L and B. Cathepsin involvement in invasion and metastasis often focuses on cathepsin-mediated extracellular matrix degradation; therefore, the discovery that cathepsins degrade a metastasis suppressor within the lysosome reveals

**Figure 3.11. c-Abl/Arg Activation is Inversely Correlated with NM23-H1 Expression in Primary Melanomas and Breast Cancers**

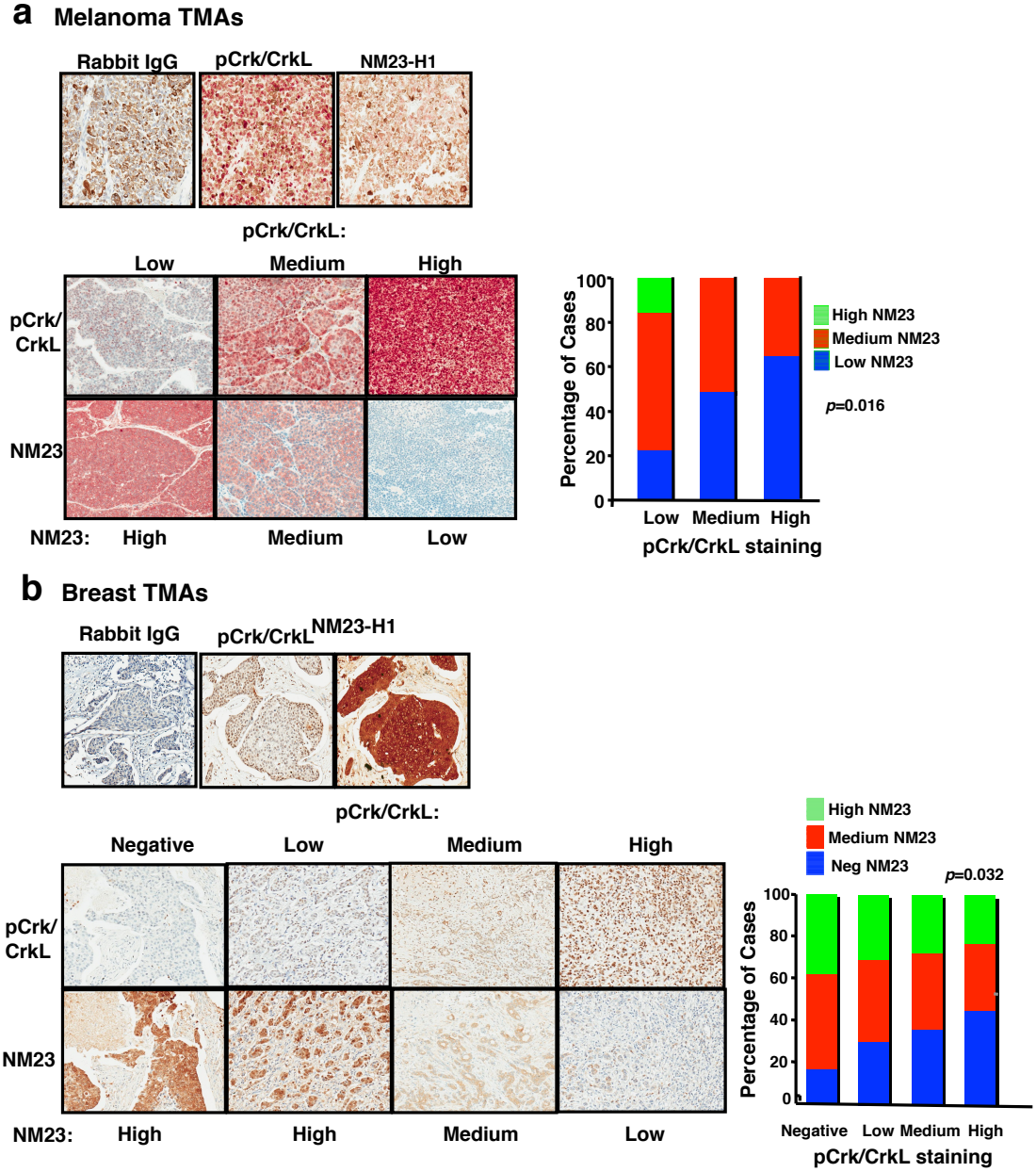

**Figure 3.11. c-Abl/Arg activation is inversely correlated with NM23-H1 expression in primary melanomas and breast cancers. (a)** Melanoma TMAs containing benign nevi (n=18), primary melanomas (n=48), and melanoma metastases (n=19) (ME1003) were stained with pCrk/CrkL, NM23-H1 (D98), or IgG antibodies; and scored by a pathologist. Representative cores are on left. Cores were grouped into low (≤0.5), medium (1-2.5) and high (>2.5) NM23 intensity of stain and pCrk/CrkL intensity of stain, and the percentage of cases containing low, medium, or high NM23-H1 staining in each pCrk/CrkL group was graphed (right). An inverse correlation was observed: $p$=0.016 with a Fisher's Exact test-3X3 and Kendall's tau-b=-0.2637, 95% CI -0.4540 to -0.0734, $p$=0.009. **(b)** Breast cancer TMA serial sections (BR10010a; n=100), were stained with pCrk/CrkL and NM23-H1 antibodies and scored by a pathologist (Michael Cibull, M.D.). Score = intensity * percentage of tumor. pCrk/CrkL scores were grouped into negative (0), Low (.1-.5), Medium (.6-1.6), and High (1.7-3). NM23-H1 scores were grouped into Low (>1), Medium (1-1.9) and High (>1.9). An inverse correlation between pCrk/CrkL and NM23-H1 was observed ($p$=0.032; Kendall's tau-b = -0.177 with 95% confidence interval -0.339 to -0.015). IgG controls shown (top).

a novel role for cathepsins as intracellular regulators of metastasis in addition to their extracellular functions. Furthermore, we demonstrate that c-Abl and Arg promote cathepsin-mediated NM23-H1 degradation revealing a novel mechanism in which an oncogene downregulates a metastasis suppressor to promote invasion and metastasis (Figure 3.12).

Inhibition of c-Abl/Arg resulted in increased expression of procathepsin L/B coupled with decreased active cathepsin L/B. Additionally, we observed an accumulation of endosomes and a decrease in lysosomes following c-Abl/Arg inhibition, indicating that c-Abl/Arg promote endosomal trafficking to the lysosome. The switch from Rab5 GTPase expression on the early endosome membrane to Rab7 GTPase on the late endosome membrane is an essential step for maturation of endosomes to lysosomes. One potential mechanism by which the Abl family kinases may promote endosome maturation is through the Rab5/Rab7 switch. PI3K activation leads to an accumulation of PI(3)P within the vesicle membrane promoting the recruitment of a Rab7-GEF, which then subsequently recruits the Rab7 GTPase [30]. Abl oncogene variants with enhanced kinase activity such as v-Abl (fusion product of Mo-MuLV gag and truncated c-Abl) [131] and BCR/Abl (fusion product of BCR and truncated c-Abl) [49] have been reported to bind and activate PI3K in NIH3T3 fibroblasts [132], whereas endogenous c-Abl does not activate PI3K [132]. However, basal c-Abl activity in NIH3T3 fibroblasts is not constitutively active unlike v-Abl and BCR/Abl. Our lab has demonstrated that melanoma and breast cancer cells such as 435s and BT-549 cells have high c-Abl and Arg activity [56]; therefore, in these cancer cells c-Abl may activate PI3K in similar manner to v-Abl and BCR/Abl in fibroblasts. Our laboratory has reported that inhibition of c-Abl/Arg had no effect on the phosphorylation of Akt (a downstream effector of PI3K) [61]; however, the PI3K/AKT/mTOR pathway is associated with class I PI3K [133], whereas endosomal trafficking is associated with class III PI3K [134]. Therefore, we hypothesize that c-Abl/Arg activate Class III PI3K leading to the accumulation of PI(3)P at the endosomal membrane promoting the Rab5/Rab7 switch and endosome maturation.

Interestingly, Plattner et al. demonstrated that c-Abl and Arg have the ability to bind to PI(3)P [135]. Therefore, we can form an alternative hypothesis that rather than c-Abl/Arg promoting the accumulation of PI(3)P, PI(3)P may recruit c-Abl and Arg to the endosomal membrane. Recruitment of c-Abl and Arg to the endosomal membrane could potentially promote actin reorganization, which is also necessary for the Rab5/Rab7 switch and endosome maturation [23]. Nucleation promoting factors (NPF) interact with

**Figure 3.12 Effect of c-Abl on Endocytic Trafficking**

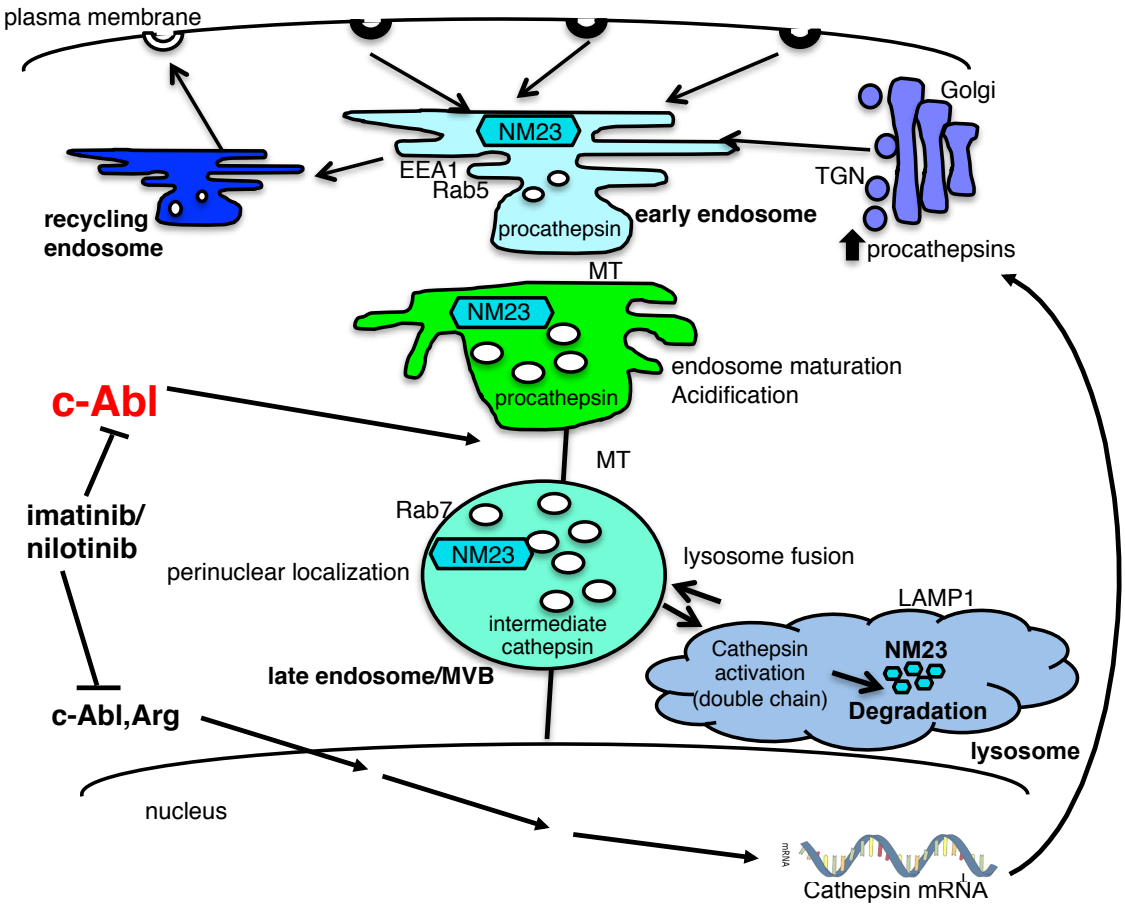


**Figure 3.12 Effect of c-Abl on endocytic trafficking.** Model for the mechanism by which c-Abl/Arg promote vesicular trafficking and NM23-H1 degradation. c-Abl and Arg promote expression of cysteine cathepsins B, L by increasing mRNA levels and c-Abl induces cathepsin activation by promoting endosome->lysosome trafficking. Endosomal NM23-H1 is transported to lysosomes and degraded by lysosomal cathepsins B,L. Inhibition or silencing c-Abl/Arg prevents cathepsin expression and activation, which leads to stabilization of NM23-H1. MT=microtubules, MVB=multi-vesicular body.

the actin-related protein, ARP2/3, to nucleate branched actin filament networks [36]. The WASH NPF is specific for actin reorganization involved with endosomal trafficking [36]. c-Abl/Arg have been found to phosphorylate several NPFs including N-Wasp, WAVE2, WAVE3 and Cortactin [136-139]. Given that c-Abl/Arg phosphorylate several NPFs, they conceivably could have a role in phosphorylating the WASH NPF and thus activate ARP2/3. thereby promoting endosomal trafficking. Additionally, it is possible that c-Abl/Arg phosphorylation of N-Wasp, WAVE2, WAVE3, or cortactin may also promote endosomal trafficking.

Cathepsins L and B are commonly over-expressed in aggressive cancers [140]. Here, we demonstrate a novel mechanism in which c-Abl and Arg increase cathepsin L/B mRNA and protein expression. Knockdown of c-Abl and Arg with siRNA resulted in decreased cathepsin L/B mRNA and protein expression whereas kinase inhibition with nilotinib had no effect indicating that c-Abl and Arg increase cathepsin L/B expression in a kinase-independent manner. The STAT3 transcription factor has been reported to have a role in promoting cathepsin L and B transcription [141] and our laboratory demonstrated that c-Abl/Arg promote STAT3 activation [61]. However, c-Abl/Arg activate STAT3 via an indirect, kinase-dependent mechanism [57], whereas the effect of c-Abl/Arg on cathepsin mRNA levels is kinase-independent. The presence of SH2 and SH3 binding domains within c-Abl and Arg may allow for kinase-independent scaffolding functions. Therefore, we propose c-Abl and Arg may have an additional indirect, kinase-independent role as a regulator of STAT3. c-Abl and Arg have been reported to be involved in protein turnover by promoting the ubiquitination and degradation of proteins. c-Abl and Arg often promote ubiquitination in a kinase-dependent manner [62, 142, 143]; however, c-Abl has also been demonstrated to have a kinase-independent function in ubiquitination and degradation. c-Abl binds and promotes the ubiquitination of the DNA binding protein, DDB2 (DNA damage-binding protein 2) [63]. This mechanism is mediated through the CUL4 E3 ligase complex. Interestingly, c-Abl kinase activity was not required for c-Abl-dependent ubiquitination and degradation of DDB2 via CUL4. [63]. Therefore, we hypothesize that independent of their kinase activity, c-Abl/Arg promote the ubiquitination and subsequent degradation of a protein inhibitor of activated STAT3, PIAS, which inhibits STAT3 DNA-binding activity [144]. c-Abl/Arg degradation of PIAS in a kinase-independent manner would allow for activation of STAT3 and upregulation of cathepsin L/B mRNA expression.

The attenuation of invasion that we observe in cells with c-Abl/Arg knockdown or inhibition was rescued with stable knockdown of NM23-H1, demonstrating that c-Abl and Arg require the downregulation of NM23-H1 to promote invasion in melanoma and breast cancer cells. Furthermore, we also observed that c-Abl/Arg inhibition increased NM23-H1 expression levels within early endosomal fractions, suggesting that the build up of NM23-H1 within the endosomes suppresses invasion. Receptor tyrosine kinases (RTK), which are endocytosed and sequestered within the endosomal compartments, often maintain their signaling capabilities [145]; therefore it is conceivable that the metastatic suppressing functions of NM23-H1 may remain intact within the endosome compartment. In fact, NM23-H1 is involved in regulating endocytosis. The *Drosophila* homolog of NM23, *awd*, increased the efficiency of dynamin, a GTPase with an essential role in endocytosis [146], indicating that NM23-H1 promotes endocytosis. Furthermore, in human MDCK cells, ARF6, which can localize to the plasma membrane and the endosome, recruits NM23-H1, inducing dynamin-dependent vesicle fission/endocytosis [77]. Given that c-Abl/Arg inhibition causes NM23-H1 accumulation within the early endosome and NM23-H1 promotes endocytosis, we hypothesize that inhibition of c-Abl/Arg attenuates invasion via ARF6 interacting with NM23-H1 and promoting the endocytosis of receptor tyrosine kinases (RTK). Since inhibition of c-Abl/Arg blocks maturation of the early endosome to the late endosome, RTKs would remain sequestered within the early endosome. Sequestering an RTK such as EGFR within the endosome would prevent cell surface mechanisms such as heterodimer formation and activation of phospholipase (PLC)-γ, thus decreasing migration.

ARF6 recruitment of NM23-H1 was also demonstrated to promote the function of NM23-H1 as a suppressor of the Rac1 GEF, Tiam1 [75, 77]. Rac1-GTP promotes membrane ruffling, invasion and metastasis [147]. ARF6 regulates the movement of Rac1 between the plasma membrane and the endosomal compartment [147]. We propose that increased levels of NM23-H1 within the endosome increases ARF6 bound to NM23-H1. This interaction promotes NM23-H1 inhibition of TIAM1 subsequently inhibiting Rac1 and thus inhibiting invasion. In fact, Rac was demonstrated to be activated downstream of Abl [148], therefore c-Abl/Arg promoting endosomal trafficking may prevent ARF6 recruitment of NM23-H1 subsequently activating TIAM1 and Rac1 promoting invasion and metastasis.

Another possible mechanism by which NM23-H1 may mediate metastatic suppression within the endosome is via inhibition of TGFβ signaling. The downstream

effects of TGFβ are dependent upon cell type and environment. In advanced cancer cells, TGFβ signaling promotes epithelial to mesenchymal transition (EMT) and metastasis [149]. Activated TGFβ receptors are internalized from the plasma membrane and localized to early endosomes [149]. NM23-H1 interaction with the TGFβ negative regulator, STRAP, was demonstrated to enhance negative regulation on the TGFβ signaling pathway [150]. Therefore, we speculate that increased expression of NM23-H1 within the endosome following c-Abl/Arg inhibition may promote the interaction of NM23-H1 and STRAP attenuating TGFβ signaling and thus invasion/metastasis. Finally, one may speculate that NM23-H1 localized within the endosome may reduce invasion via the phosphorylation and inhibition of the ERK inhibitor, Kinase Suppressor of Ras (KSR), given that KSR can localize within the endosome and is a known substrate of NM23-H1 [151, 152]. However, our laboratory observed c-Abl/Arg inhibition actually increased phosphorylation of ERK1/2 in 435s cells, suggesting that NM23-H1 is unlikely acting via KSR to suppress invasion following c-Abl/Arg inhibition in this cell type [61, 153].

Decreased invasion in cells with c-Abl/Arg knockdown/inhibition was only partially rescued with stable knockdown of NM23-H1, suggesting that c-Abl/Arg promote invasion via multiple pathways. Previously, our lab demonstrated that c-Abl/Arg increase the active forms of the proteases, MMP1 and MMP3 [57]. MMPs promote invasion/metastasis via degradation of the extracellular matrix. Interestingly, invasion was only partially rescued with recombinant MMP1 and MMP3 in c-Abl and Arg knockdown cells [57]. Thus, c-Abl and Arg promote invasion via NM23-H1 degradation and MMP1 and MMP3 activation. In fact, cathepsins L and B have been reported to cleave and activate MMP1 and MMP3 [40], suggesting that c-Abl/Arg may activate MMP1 and MMP3 via cathepsin activation. Additionally, increased cathepsin expression in cancer cells induces their secretion into the extracellular environment where they degrade the extracellular matrix [37]. Therefore, a c-Abl/Arg-dependent increase in cathepsin L/B expression/activation likely promotes invasion via three pathways: downregulation of the metastasis suppressor NM23-H1, activation of MMP1 and MMP3, and cathepsin-mediated degradation of the extracellular matrix (Discussed in Chapter 4).

Here, we present a novel pathway in which c-Abl/Arg kinases downregulate the expression of the metastasis suppressor NM23-H1 in two cell lines, *in vivo*, and in human primary tumors. Identification of agents that can induce the upregulation of NM23-H1 in tumors could decrease metastasis and increase patient survival. These data demonstrate that treatment of melanoma and triple-negative breast cancers with an

inhibitor of c-Abl/Arg such as imatinib or nilotinib may reduce metastasis and increase survival of melanoma and breast cancer patients by inducing the re-expression of NM23-H1.

# Chapter 4: Discussion and Future Directions

*Summary*

Our laboratory previously demonstrated that c-Abl and Arg are highly activated in melanoma and breast cancer cells and promote proliferation, anchorage-independent growth, migration, invasion and metastasis [56, 57, 61]. In Chapter 2, we demonstrated that activated c-Abl and Arg are relevant to human disease. In human tissue, we reported high c-Abl/Arg kinase activity in melanomas as compared to benign nevi and there was a trend towards a positive correlation between c-Abl/Arg activity and early-onset and intermittently sun-exposed melanomas. Additionally, in human breast cancer tissue, c-Abl/Arg activity correlated positively with high-grade tumors. Furthermore, we reported that triple-negative tumors have high c-Abl/Arg activity as compared to HER2/ER/PR$^+$ tumors and these data will likely reach significance with increased sample numbers. Taken together with our previously published *in vitro* and *in vivo* data demonstrating a role for c-Abl/Arg in melanoma progression [57], these data provide clinical evidence that c-Abl and Arg may be novel therapeutic targets in melanomas and breast cancers.

Overexpressed cathepsins in cancer increase invasion and metastasis due to the excess cathepsins being secreted into the extracellular environment, where they degrade the extracellular matrix (ECM) and activate other proteases (MMPs, uPa) involved in ECM degradation [37, 40]. The extracellular role of the cathepsins has been well documented; however, little was known regarding how intracellular cathepsins affect metastasis. In Chapter 3, we discovered a novel, pro-metastatic, intracellular role for these cathepsins demonstrating that the oncogenes, c-Abl and Arg, mediate overexpression and activation of cathepsins L and B, subsequently promoting the intracellular degradation of the metastasis suppressor, NM23-H1, within the lysosome. Additionally, we demonstrated that c-Abl/Arg promote the trafficking of the endosome/lysosome pathway to induce cathepsin-mediated NM23-H1 degradation. This was the first report of an oncogene downregulating a metastasis suppressor and the first reported mechanism for NM23-H1 protein turnover. We also demonstrated clinical relevance for this pathway as we observed a significant negative correlation between c-Abl/Arg kinase activity and NM23-H1 expression in human melanoma and breast cancer tissue. Therefore, inhibition of c-Abl and Arg kinases in melanoma and breast cancer tumors is likely to elevate expression of NM23-H1, thus decreasing the incidence of metastasis, which may increase survival.

*Future Directions*

Future directions for this project will focus on elucidating the mechanisms for c-Abl/Arg promoting endosome maturation and cathepsin mRNA upregulation. We demonstrated that c-Abl and Arg activity are required for the maturation of early endosomes to late endosomes and lysosomes. An essential step in endosome maturation is the switch from Rab5 GTPase expression on the early endosome membrane to Rab7 GTPase expression on the late endosome membrane [30]. Within the endosomal membrane, PI(3)P accumulation induced by Class III PI3K activation is necessary for the recruitment of the Rab7 GEF, which recruits the Rab7 GTPase [30]. As mentioned in the discussion for Chapter 3, we hypothesized that c-Abl and Arg promote endosome maturation by activating class III PI3K thus causing an accumulation of PI(3)P and subsequently inducing the Rab5/Rab7 switch. To test this hypothesis, we will utilize a PI3K kinase activity assay to determine if silencing or inhibiting c-Abl and Arg reduces class III PI3K activity. If we determine that c-Abl and Arg regulate class III PI3K activity, we will investigate if this activation promotes endosome maturation. We demonstrated that c-Abl/Arg promote the maturation of early endosomes to lysosomes by performing a Percoll-gradient fractionation following inhibition of c-Abl and Arg and we observed an accumulation of early endosomes and a decrease in lysosomes with c-Abl/Arg inhibition as compared to vehicle treatment (Chapter 3). To test if c-Abl and Arg promote endosome maturation by activating class III PI3K, we will perform a Percoll-gradient fractionation in cells overexpressing constitutively active class III PI3K. We predict expression of constitutively active class III PI3K will rescue the block in endosome maturation we observe with c-Abl/Arg inhibition, indicating that class III PI3K activation is required for c-Abl/Arg to promote endosome maturation.

If experiments reveal c-Abl and Arg do not regulate class III PI3K, an alternative hypothesis is that c-Abl/Arg phosphorylate the actin nucleation promoting factor, WASH, thus promoting actin reorganization, which also is essential for endosome maturation [36]. To test this hypothesis, we will perform western blot analysis and observe the tyrosine phosphorylation levels of immunoprecipitated WASH in cells with c-Abl/Arg inhibition or knockdown. If the inhibition or knockdown of c-Abl and Arg reduces WASH tyrosine phosphorylation levels, this would indicate c-Abl/Arg promote WASH phosphorylation. To confirm that c-Abl/Arg phosphorylation of WASH is necessary for endosome maturation, we would identify and mutate the c-Abl/Arg tyrosine phosphorylation sites within WASH to glutamates, creating a WASH phosphorylation

mimic. We will perform the previously mentioned Percoll-gradient fractionation overexpressing the WASH phosphorylation mimic. If the WASH phosphorylation mimic rescues the block in endosome maturation we observe with c-Abl/Arg inhibition, this would indicate that c-Abl and Arg require the phosphorylation of WASH to promote endosome maturation. To confirm these results, we will also mutate the c-Abl/Arg tyrosine phosphorylation sites within WASH to phenylalanines, preventing phosphorylation of WASH by c-Abl/Arg. In cells with endogenously low c-Abl/Arg activity, such as melanoma WM164 and breast cancer MCF-7 cells, we will overexpress constitutively active c-Abl/Arg-PP and mutant WASH and perform Percoll-gradient fractionation. We anticipate that blocking c-Abl and Arg from phosphorylating WASH will prevent the constitutively active c-Abl/Arg from promoting endosome maturation.

We also reported for the first time that c-Abl and Arg upregulate cathepsin mRNA expression in melanoma and breast cancer cell lines in a kinase-independent manner. Cathepsins L and B are regulated by the transcription factor STAT3 [141] and our lab previously demonstrated that c-Abl/Arg promote STAT3 phosphorylation [61]; therefore, it is conceivable that c-Abl/Arg could promote cathepsin L and B mRNA expression through activation of STAT3. Additionally, our lab has also previously demonstrated that c-Abl/Arg activation of STAT3 occurs via an indirect mechanism [57]. These data led us to hypothesize that c-Abl and Arg activate STAT3 by promoting the degradation of the STAT3 inhibitor, PIAS, inducing the upregulation of cathepsin L and B mRNA. We will perform several experiments to test our hypothesis. First, we will investigate if c-Abl/Arg upregulate cathepsin expression via STAT3 activation. We demonstrated that knockdown of c-Abl or Arg decreased cathepsin mRNA levels (Chapter 3); therefore, to determine if STAT3 activation is required for c-Abl and Arg cathepsin upregulation, we will overexpress constitutively active STAT3 to observe if STAT3 activation rescues the decrease in cathepsin mRNA we observe in c-Abl/Arg knockdown cells. Next, we will investigate if c-Abl and Arg downregulate the expression of the STAT3 inhibitor, PIAS, by performing western blot analysis on whole cell lysates from c-Abl/Arg knockdown cells. We expect c-Abl/Arg knockdown will upregulate PIAS protein expression. Finally, to confirm that PIAS downregulation by c-Abl/Arg induces STAT3 activation and upregulation of cathepsin mRNA, we will use PIAS shRNA to create stable PIAS knockdown cells. If c-Abl/Arg require the downregulation of PIAS to activate STAT3 and upregulate cathepsin expression, silencing of c-Abl and Arg in cells with stable PIAS knockdown will maintain activated STAT3 and cathepsin expression levels as compared

to cells expressing endogenous PIAS. However, c-Abl and Arg may be regulating cathepsin mRNA expression levels by mechanisms other than transcriptional regulation such as promoting mRNA stability by inhibiting deadenylation of the poly (A) tail [154].

*Projects in Progress*

As previously mentioned, in cancer, upregulated cathepsins are often secreted into the extracellular environment [38]; therefore, our discovery that c-Abl and Arg dramatically upregulate cathepsin expression led us to speculate whether c-Abl and Arg may have a role in procathepsin secretion. As described in Chapter 1, procathepsins are trafficked through the endoplasmic reticulum to the golgi apparatus [37]. In the golgi, cathepsins are targeted to early endosomes which subsequently mature to late endosomes [37]. Once in late endosomes, procathepsins are cleaved to their single chain active form and trafficked to acidic lysosomes where the cathepsins are cleaved again to the double chain active form [37]. In cancer cells, overexpressed procathepsins are secreted from the cell rather than the being trafficked to the endosome/lysosome pathway [38]. Procathepsins follow the conventional pathway of secretion in which proteins localized within the golgi are trafficked via vesicles budding from the golgi to the plasma membrane to be secreted (Figure 4.1a) [155]. To examine if c-Abl and Arg promote cathepsin secretion, we silenced c-Abl and Arg in BT-549 cells and blotted the conditioned media with antibodies to cathepsins known to have a role in invasion and metastasis: procathepsin L, B and D [37]. Interestingly, both c-Abl and Arg promote the secretion of cathepsins L and D. c-Abl had a more dramatic effect on cathepsin L secretion as compared to Arg and cathepsin B secretion was only c-Abl-dependent (Figure 4.2 left, middle). These results confirm that c-Abl and Arg mediate procathepsin secretion.

Given that secretion of cathepsins is linked to their increased expression [38, 156], we examined if cathepsin secretion induced by c-Abl and Arg was due to increased cathepsin transcript levels in nutrient-deprived conditions. Using quantitative real-time RT-PCR, we observed reduced cathepsin L and D mRNA levels following silencing of c-Abl or Arg; however, decreased levels of cathepsin B mRNA were observed following silencing of c-Abl but not Arg, indicating that cathepsin B mRNA upregulation is c-Abl-dependent (Figure 4.2 right). Although the changes in cathepsin expression that we observe with c-Abl and Arg knockdown are similar between the mRNA and conditioned media expression levels, we observed a more dramatic effect on procathepsin expression levels in the lysate and media as compared to the mRNA (Figure 4.2). These

**Figure 4.1 Conventional and Unconventional Pathways of Secretion**

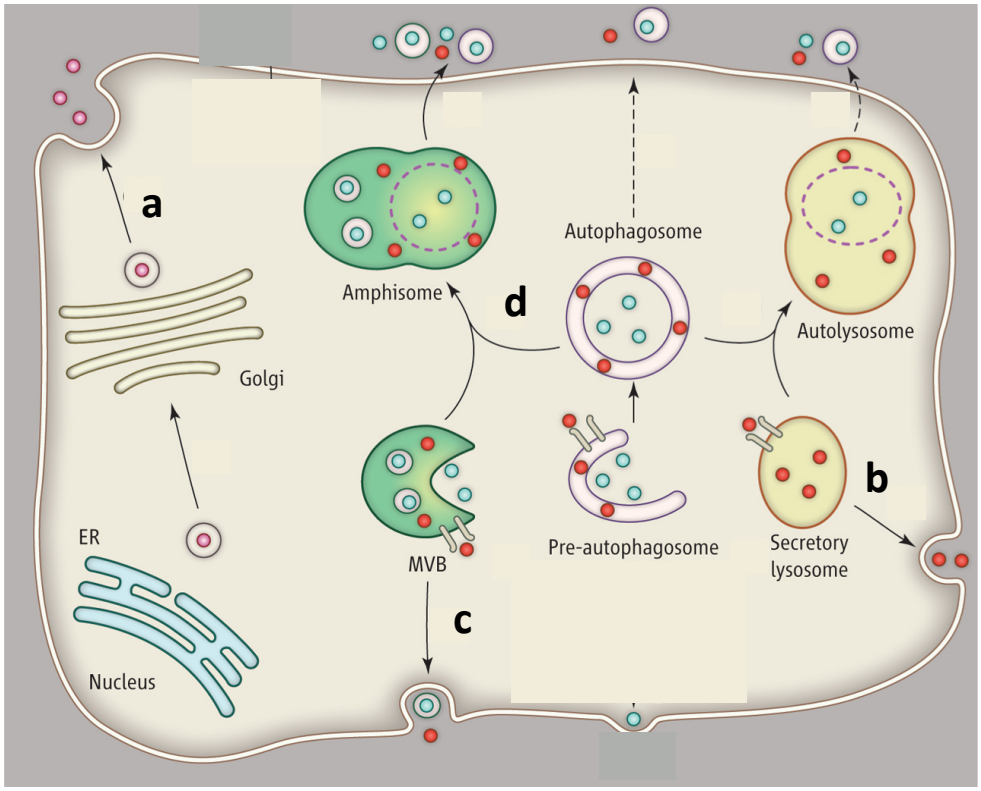


**Figure 4.1 Conventional and unconventional pathways of secretion. (a)** Conventional Secretion of proteins localized within the golgi are trafficked via vesicles budding from the golgi to the plasma membrane to be secreted into the extracellular environment **(b-d)** Unconventional secretion pathways. **(b)** Secretory lysosomes are modified lysosomes that can function in secretion by fusing with the plasma membrane and releasing their contents into the extracellular environment. **(c)** Multivesicular bodies are intermediate vesicles that form as late endosomes mature into lysosomes. Multivesicular bodies contain endosomal membrane invaginations known as intraluminal vesicles (ILVs) that encapsulate proteins. Multivesicular bodies can fuse with the plasma membrane and release the ILVs into the extracellular environment as exosomes. **(d)** A phagophore (pre-autophagosome) engulfs and encapsulates a cytoplasmic protein forming an immature autophagosome. The immature autophagosome fuses with early endosomes, late endosomes, or multivesicular bodies to form an amphisome vesicle. The amphisome can then fuse with the plasma membrane to release its contents into the extracellular environment. Figure adapted from Zhang, et al. Science. 2013 May 3;340(6132):559-61.

**Figure 4.2 c-Abl and Arg Increase Cathepsins L, B, and D Expression in Whole Cell Lysate, Conditioned Media and mRNA**

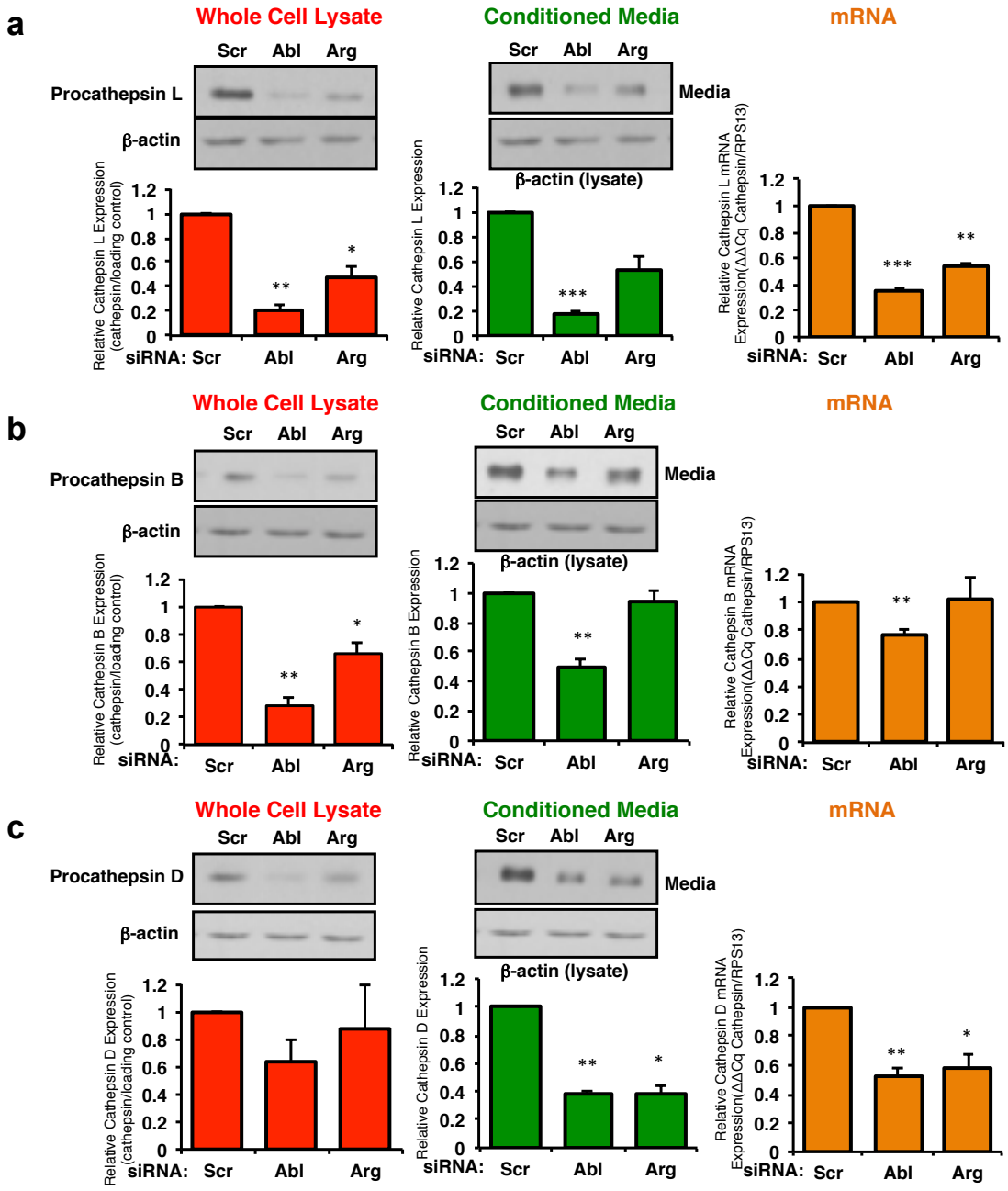

**Figure 4.2 c-Abl and Arg increase Cathepsins L, B, and D expression in whole cell lysate, conditioned media and mRNA.** (a-c) BT-549 cells were transfected with c-Abl or Arg siRNA in 60mm dishes and serum starved for 24 hours. (a-c, left) Cells were lysed and blotted with antibodies to procathepsins L (a) , B (b) , or D (c). (a-c, middle) Conditioned media (2mL) was collected and filtered through .2 µm filter to remove floating cells. Media was concentrated with Millipore Amicon .5mL filter to 200µL. Sample buffer (4x) was added to media and boiled for 5'. Equal protein concentrations (determined by whole cell lysate protein concentration) were loaded onto a SDS-page gel and blotted for prcathepsin L (a) , B (b), or D (c). (a-c, right) Quantitative real-time RT-PCR was performed with primers for procathepsin L (a), B (b), D (c) and RPS13 as a loading control. **(a-c)** Graphs represent mean±SEM for 3 independent experiments. *$p$<0.05, **$p$<0.01, ***$p$<0.001 using one sample t-tests.

data suggest that in addition to increasing transcript levels, c-Abl and Arg also may promote procathepsin expression/secretion via an additional mechanism.

In addition to increased transcript levels, increased expression of procathepsin L and B could be due to the presence of multiple transcript variants [156]. Both cathepsins L and B have 5 transcript variants all yielding the same protein product due to the translation initiation site being located in exon 2 and alternative splicing occurring within exon 1 [156]. Interestingly, the shortest transcript is translated more efficiently as compared to the longer transcripts due to the shortest transcript containing fewer stem-loop structures within the mRNA [156]. Furthermore, in comparison to the other transcript variants, the shortest transcript has a higher expression level in malignant cells [156]. Therefore, in addition to regulating the overall expression of cathepsin L and B mRNA, it would be interesting to determine if c-Abl and Arg promote alternative splicing of cathepsins L and B, producing the shortest transcript variant and thus, increasing the translation efficiency and expression of the cathepsins. Consistent with this hypothesis, the constitutively active Abl kinase, BCR-Abl, upregulates expression of multiple genes involved in pre-mRNA splicing in hematopoietic cells [157]. Furthermore, inhibition of BCR-Abl resulted in splicing patterns consistent with normal cells as compared to malignant hematopoietic cells [157]. In solid tumors, such as melanoma and breast cancer, we propose c-Abl and Arg may promote the pre-mRNA splicing of cathepsins L and B yielding the shortest transcript variant, subsequently increasing translation efficiency and procathepsin expression/secretion. Since alternative splicing of cathepsins occurs within exon 1, we will test this hypothesis by designing primers to span the alternative splicing sites within exon 1 and perform semi-quantitative RT-PCR to observe expression changes of the five cathepsin L and B transcript variants with c-Abl and Arg knockdown. An alternative hypothesis to consider which also may account for the more dramatic effect we observe on secretion as compared to the mRNA levels could be that c-Abl and Arg increase the trafficking of vesicles involved in secreting cathepsins from the cell. As previously mentioned, procathepsins are trafficked to the plasma membrane via vesicles budding from the golgi [155]. A block in golgi vesicular trafficking induced by Brefeldin A causes extensive golgi tubulation [158]; therefore, if c-Abl and Arg promote golgi vesicular trafficking to the plasma membrane, we anticipate that silencing c-Abl and Arg will induce golgi tubulation, which can be visualized by fluorescent staining with antibodies against the golgi marker, mannosidase A, or by electron microscopy. Interestingly, activation of protein kinase D (PKD) is essential for

the fission of golgi vesicles destined for the plasma membrane [159] and PKD is a substrate of c-Abl [160]; therefore, we hypothesize that c-Abl and Arg promote cathepsin secretion by activating PKD, and thus, inducing vesicle budding from the golgi to the plasma membrane. If c-Abl and Arg activate PKD to promote trafficking of cathepsins from the golgi to the cell surface, we anticipate that overexpression of constitutively active PKD will rescue the decrease in cathepsin secretion we observe in c-Abl and Arg knockdown cells.

NM23-H1 has been observed in the extracellular environment; therefore, our findings that c-Abl and Arg regulate NM23-H1 expression led us to investigate a role for c-Abl and Arg in regulating NM23-H1 secretion. Surprisingly, c-Abl and Arg knockdown dramatically decreased NM23-H1 expression in the conditioned media of 435s cells (Figure 4.3), indicating that c-Abl and Arg promote the secretion of NM23-H1. Interestingly, c-Abl and Arg increase the extracellular expression of NM23-H1 while downregulating intracellular levels. These data contrast with c-Abl- and Arg-mediated upregulation of cathepsins in which expression is increased both extracellularly and intracellularly. Furthermore, c-Abl and Arg increase cathepsin transcript levels, whereas c-Abl and Arg do not affect the transcript levels of NM23-H1 (Chapter 3); therefore, c-Abl and Arg promote NM23-H1 secretion in a transcription-independent manner. c-Abl and Arg-dependent secretion of NM23-H1 may be an additional mechanism by which c-Abl and Arg decrease intracellular protein levels of NM23-H1, in addition to lysosomal degradation. NM23-H1 lacks the signal-peptide required for conventional secretion [155, 161]; therefore, c-Abl and Arg are likely promoting NM23-H1 secretion via unconventional secretion. Indeed, in Chapter 3, we demonstrated that c-Abl and Arg regulate intracellular trafficking of NM23-H1 within endosomes and lysosomes, vesicles associated with unconventional secretion.

Unconventional secretion is defined as secretion which involves mechanisms other than vesicles budding from the golgi apparatus [155]. There are several mechanisms by which proteins undergo unconventional secretion. These mechanisms involve secretory lysosomes, secretion of multivesicular bodies [155], and autophagosome-induced secretion [162]. Secretory lysosomes are modified lysosomes that can function in secretion by fusing with the plasma membrane and releasing their contents into the extracellular environment (Figure 4.1b) [163]. This is an unlikely mechanism for NM23-H1 secretion because secretory lysosomes are reportedly only found in hematopoietic cells and melanocytes [163]. Although we observe NM23-H1

**Figure 4.3 c-Abl and Arg Increase Expression of NM23-H1 in Conditioned Media**

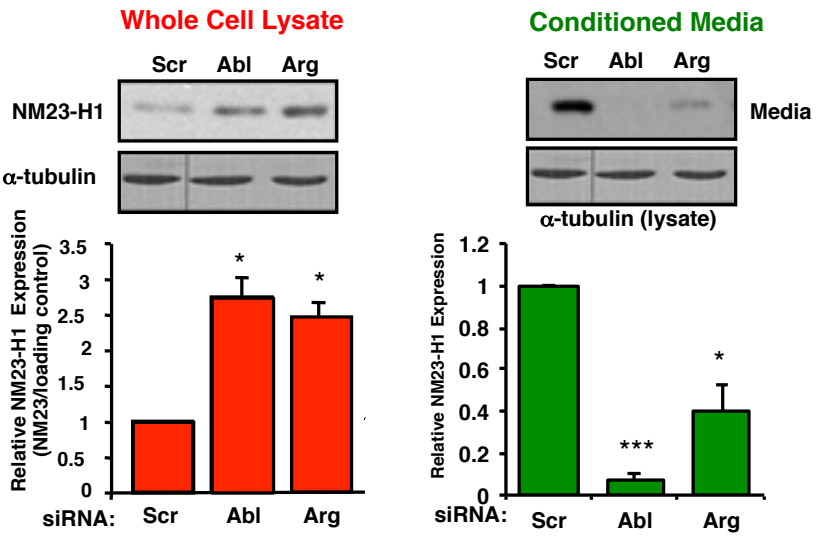


**Figure 4.3 c-Abl and Arg increase expression of NM23-H1 in conditioned media.** 435s cells were transfected with c-Abl or Arg siRNA in 60mm dishes and serum starved for 24 hours. Cells were lysed and blotted for NM23-H1, left. Conditioned media (2mL) was collected and filtered through .2 μm filter to remove floating cells. Media was concentrated with Millipore Amicon .5mL filter to 200μL. Sample buffer (4x) was added to media and boiled for 5'. Equal protein concentrations (determined by whole cell lysate protein concentration) were loaded onto a SDS-page gel and blotted for NM23-H1, right. Graphs represent mean±SEM for 3 independent experiments. *$p$<0.05, ***$p$<0.001 using one-sample t-tests. Media prepared by S. Ganguly.

secretion in melanomas, the melanocyte secretory lysosomes are described as melanosomes which function to secrete melanin [163]; therefore, these would be unlikely to contain NM23-H1. Multivesicular bodies are intermediate vesicles that form as late endosomes mature into lysosomes. Multivesicular bodies contain endosomal membrane invaginations known as intraluminal vesicles (ILVs) that encapsulate proteins [37]. Multivesicular bodies can fuse with the plasma membrane and release the ILVs into the extracellular environment as exosomes (Figure 4.1c) [155]. As previously mentioned, we demonstrated that c-Abl/Arg promote the trafficking of late endosomes to lysosomes and NM23-H1 is localized within these vesicles (Chapter 3); therefore, it is possible that c-Abl and Arg may promote NM23-H1 secretion via trafficking of multivesicular bodies to the plasma membrane. Autophagosomes can also be involved in unconventional protein secretion [164]. Within the cytoplasm, a phagophore (pre-autophagosome) can engulf and encapsulate a cytoplasmic protein forming an immature autophagosome [164]. During autophagy, this autophagosome fuses with lysosomes introducing the engulfed proteins to the lysosomal compartment to be degraded [164]. Alternatively, this immature autophagosome can also fuse with early endosomes, recycling endosomes, late endosomes, or multivesicular bodies to form an amphisome vesicle [164-166]. The amphisome can then fuse with the plasma membrane to release its contents into the extracellular environment (Figure 4.1d) [164]. Thus, it would be interesting to evaluate if, in addition to endosomes and lysosomes, NM23-H1 also localizes within autophagosomes. To test this hypothesis, we will perform immunocytochemistry and subcellular fractionation to observe if NM23-H1 and the autophagosome marker LC3 colocalize. Furthermore, to examine if autophagosome maturation is required for NM23-H1 secretion, we can inhibit autophagosome maturation by treatment with bafilomycin A, or silencing ATG5, an E3 ligase essential for autophagosome maturation, and observe if extracellular levels of NM23-H1 are reduced. If we confirm NM23-H1 is being secreted via autophagosome induced unconventional secretion, we hypothesize that c-Abl and Arg promote NM23-H1 secretion by promoting autophagosome maturation. Consistent with this hypothesis, c-Abl and Arg have been shown to promote autophagosome trafficking and autophagosome-lysosomal fusion [65]. Additionally, c-Abl and Arg phosphorylate the autophagosomal SNARE, syntaxin 17, which is involved in the fusion of autophagosomes with other vesicles [167-169]; further supporting a role for c-Abl and Arg in promoting NM23-H1 secretion via autophagosome trafficking. Interestingly, NM23-H1 uptake by pre-autophagosomes and

delivery to the endosomal compartment could not only explain c-Abl and Arg promoting NM23-H1 secretion but may also explain the mechanism by which c-Abl and Arg promote delivery of NM23-H1 to the endosome for subsequent degradation within the lysosome.

It is well documented that increased cathepsin expression in the extracellular environment is an indicator of increased metastasis and poor survival. As previously mentioned, activated cathepsins in the extracellular environment increase invasion and metastasis by activating proteases such as uPA and MMPs that degrade the ECM [37]. Furthermore, cathepsins can also directly degrade the ECM [37]. Inactive procathepsins may also promote invasion in the extracellular environment by binding to cell surface receptors. In fact, procathepsin D promotes fibroblast outgrowth by binding to the LRP1 receptor on the cell surface [170]. Additionally, procathepsin B binds to the annexin II receptor on the cell surface of breast cancer and glioma cells [171]. Annexin II has been demonstrated to promote exocytosis [172, 173]; therefore, it would be interesting to examine if c-Abl and Arg promote NM23-H1 secretion via inducing procathepsin B exocytosis/binding to annexin II on the cell surface. Consistent with this hypothesis, cathepsin B has been shown to promote the unconventional secretion of IL-1β in macrophages [174]. To test if extracellular cathepsin B increases NM23-H1 secretion in melanoma cells, we will silence cathepsin B and observe if there is a decrease in extracellular NM23-H1 levels. To confirm that cathepsin B-mediated NM23-H1 secretion is specific to extracellular procathepsin B, we will add procathepsin B to the media in cathepsin B knockdown cells and examine if extracellular NM23-H1 expression is rescued with extracellular cathepsin B expression. Finally, to investigate if extracellular procathepsin B requires annexin II to promote NM23-H1 secretion, we will silence annexin II in cathepsin B knockdown cells and observe if adding procathepsin B to the media fails to rescue extracellular NM23-H1 levels in annexin II knockdown cells as compared to cells expressing endogenous annexin II.

Interestingly, even though intracellular NM23-H1 has metastasis suppressing functions, some data indicate that extracellular NM23-H1 may promote tumor growth, angiogenesis, and its secretion has been linked with poor survival in breast cancer and hematological malignancies [161, 175, 176]. Given that extracellular NM23-H1 promotes proliferation in hematopoietic cells and our lab demonstrated that c-Abl and Arg promote proliferation [57] and NM23-H1 secretion in melanoma cells, we hypothesize that c-Abl/Arg promote proliferation in melanoma cells by increasing NM23-H1 secretion. To

test this hypothesis, we will perform a $^{3}$H-thymidine proliferation assay and observe if recombinant NM23-H1 added to the media of c-Abl and Arg knockdown cells rescues the inhibition of proliferation we observe following silencing c-Abl and Arg in melanoma cells. To investigate if extracellular NM23-H1 levels may contribute to the decreased survival in melanoma patients, we will perform an ELISA to examine if there is a positive correlation between NM23-H1 levels in serum from melanoma patients and overall patient survival. The results of these experiments may provide evidence for the use of NM23-H1 serum levels as a biomarker for melanoma prognosis.

*Clinical Significance*

Melanoma can be categorized into many subtypes, each with their own challenges in treatment due to complex signaling pathways promoting tumor progression and metastasis. Melanoma prognostic techniques include staging and identifying the presence of molecular biomarkers [177]. Although staging indicates how invasive and advanced the tumor may be, the complexity and uniqueness of each individual tumor requires additional information for accurate diagnosis and treatment options. Metastatic malignant melanomas are diagnosed using several biomarkers such as S100, MelanA, tyrosinase, and MITF [178]. Once malignant melanoma is diagnosed, identification of the tumor molecular profile driving the disease provides clinicians with information to provide tailored treatment options for patients. For example, the presence of the V600E BRAF mutation is a biomarker for the use of a BRAF inhibitor for melanoma treatment; however, not all tumors respond to treatment due to intrinsic or acquired resistance [8]. Resistance to BRAF inhibitors does not occur due to secondary mutations within BRAF, but instead via upregulation of other pathways, which activate the CRAF isoform [9, 10, 87]. Although many mechanisms of resistance to BRAF inhibitors have been identified, overall survival rates of patients the metastatic melanoma have failed to improve [14]. Further investigation and understanding of mechanisms and biomarkers of resistance could provide better treatment options for patients. Our lab has previously shown that c-Abl/Arg promote melanoma progression, [57, 61] and the c-Abl/Arg phosphorylated substrates, Crk/CrkL, are intensely stained in melanoma tumors as compared to benign nevi (Chapter 2). Furthermore, we examined pCrk/CrkL staining in melanoma subtypes and found intense pCrk/CrkL staining in tumors from young patients and with intermittent sun-exposure, which commonly present with BRAF activating mutations [1], indicating that BRAF mutations may be linked to c-Abl/Arg activity. Interestingly, long-term inhibition of BRAF is associated with increased IGFR1 activation and PDGFRα

upregulation [179, 180]. IGFR1 and PDGFR are both upstream regulators of c-Abl [47, 61]; therefore, tumors resistant to BRAF inhibitors may require the activation of c-Abl and Arg. These data provoked us to ask the following questions: Is intense pCrk/CrkL staining a biomarker for melanoma progression and survival? Are BRAF and c-Abl/Arg activity linked in primary melanomas? If so, is the activation of c-Abl and Arg a predictor of tumors harboring acquired/intrinsic BRAF resistance?

Correlating pCrk/CrkL staining with patient survival in a large sample of melanoma tumors would provide further evidence for the use of pCrk/CrkL as a biomarker for melanoma progression. Furthermore, staining serial sections of tumors with pCrk/CrkL and BRAF V600E antibody would address the question whether there is a connection between BRAF mutations and c-Abl/Arg activation in primary melanoma tumors. Analysis of pCrk/CrkL staining (c-Abl/Arg activity) within tumors resistant to treatment with BRAF inhibitors may indicate a link between c-Abl/Arg activity and BRAF inhibitor resistance. If tumors staining intensely for pCrk/CrkL are less responsive to treatment as compared to tumors with low pCrk/CrkL staining, this suggests that c-Abl/Arg kinase activation may contribute to intrinsic tumor resistance to BRAF inhibitors. However, if tumors resistant to BRAF inhibitors present with increased post-treatment pCrk/CrkL staining as compared to pre-treatment staining, c-Abl/Arg activation may contribute to acquired resistance to BRAF inhibitors. If there is a correlation between pCrk/CrkL staining and BRAF mutation and/or resistance to BRAF inhibitors, we will then use a melanoma cell line resistant to BRAF inhibitors to determine if the c-Abl and Arg inhibitor, nilotinib, sensitizes cells to the BRAF inhibitor. If we find that c-Abl/Arg contribute to BRAF resistance, this would be of particular significance clinically, as combination treatment with c-Abl/Arg inhibitors and BRAF inhibitors could likely improve treatment response rates and survival.

Similar to melanoma, breast cancer tumor progression can be assessed by stage, grade, and molecular biomarkers. Breast cancer tumors are categorized based on the presence/absence of receptors: ER/PR$^{+}$, HER2$^{+}$, or triple-negative lacking all three receptors [15]. The presence of ER, PR or HER2 receptors provides targeted treatment options against the receptor present. In addition to their aggressive phenotype, triple-negative tumors have limited therapeutic options due to the lack of targetable receptors, and thus, have a poor prognosis and decreased rates of survival [17]. Staining of breast cancer tumor tissue revealed intense pCrk/CrkL staining in triple-negative tumors and a significant positive correlation between pCrk/CrkL staining and high-grade tumors

suggests that pCrk/CrkL staining (c-Abl/Arg activity) could be a potential biomarker for advanced, aggressive, triple-negative tumors. To test the reliability of pCrk/CrkL as a biomarker for breast cancer progression, we will determine if a positive correlation exists between pCrk/CrkL staining and patient survival in breast cancer tumor samples. Furthermore, high pCrk/CrkL staining in triple-negative tumors as compared to ER/PR/HER2$^{+}$ tumors suggests c-Abl/Arg may be a targetable protein for treatment in this aggressive tumor type. To test if inhibition of c-Abl/Arg activity is a viable treatment option for patients with triple-negative tumors, we will perform *in vivo* metastasis experiments using human triple-negative breast cancer cell lines with high c-Abl/Arg kinase activity and allow tumors to form in the mammary fat pad. Following the formation of lung metastases we will administer nilotinib and observe if metastatic burden in nilotinib-treated mice is diminished as compared to vehicle-treated mice. Reduced metastatic burden in mice with c-Abl/Arg inhibition would provide convincing evidence that c-Abl/Arg could be targeted therapeutically in triple-negative breast cancer.

Taken together, the data presented in this dissertation build upon a body of work demonstrating that c-Abl and Arg are potent oncogenes in solid tumors. We present novel mechanisms in which c-Abl and Arg downregulate the metastasis suppressor NM23-H1, upregulate the pro-tumorigenic proteases, cathepsins L and B, and promote the secretion of proteins such as cathepsins L/B/D and NM23-H1. Furthermore, these data positively impact both the Abl family kinase and NM23-H1 research fields, and lead to a number of new hypotheses, which need to be investigated. Our data strongly support the use of FDA-approved c-Abl/Arg inhibitors in the clinic to prevent tumor progression and metastasis in tumors with elevated c-Abl/Arg activity.

# References


1. Miller, A.J. and M.C. Mihm, Jr., *Melanoma.* N Engl J Med, 2006. **355**(1): p. 51-65.
2. Kabbarah, O. and L. Chin, *Revealing the genomic heterogeneity of melanoma.* Cancer Cell, 2005. **8**(6): p. 439-41.
3. Bertolotto, C., *Melanoma: From Melanocyte to Genetic Alterations and Clinical Options.* Scientifica (Cairo), 2013. **2013**: p. 635203.
4. Patton, E.E., et al., *BRAF mutations are sufficient to promote nevi formation and cooperate with p53 in the genesis of melanoma.* Curr Biol, 2005. **15**(3): p. 249-54.
5. Bandarchi, B., et al., *Molecular biology of normal melanocytes and melanoma cells.* J Clin Pathol, 2013. **66**(8): p. 644-8.
6. Palmieri, G., et al., *Main roads to melanoma.* J Transl Med, 2009. **7**: p. 86.
7. Palmieri, G., et al., *Targeted Therapies in Melanoma: Successes and Pitfalls*, in *Melanoma - From Early Detection to Treatment*. 2013, InTech.
8. Kudchadkar, R.R., et al., *Targeted therapy in melanoma.* Clin Dermatol, 2013. **31**(2): p. 200-8.
9. Smalley, K.S., K.L. Nathanson, and K.T. Flaherty, *Genetic subgrouping of melanoma reveals new opportunities for targeted therapy.* Cancer Res, 2009. **69**(8): p. 3241-4.
10. Klinac, D., et al., *Advances in personalized targeted treatment of metastatic melanoma and non-invasive tumor monitoring.* Front Oncol, 2013. **3**: p. 54.
11. Ugurel, S., et al., *Lack of clinical efficacy of imatinib in metastatic melanoma.* Br J Cancer, 2005. **92**(8): p. 1398-405.
12. Hodi, F.S., et al., *Major response to imatinib mesylate in KIT-mutated melanoma.* J Clin Oncol, 2008. **26**(12): p. 2046-51.
13. Lutzky, J., J. Bauer, and B.C. Bastian, *Dose-dependent, complete response to imatinib of a metastatic mucosal melanoma with a K642E KIT mutation.* Pigment Cell Melanoma Res, 2008. **21**(4): p. 492-3.
14. Jemal, A., et al., *Cancer statistics, 2010.* CA Cancer J Clin, 2010. **60**(5): p. 277-300.
15. Tinoco, G., et al., *Treating breast cancer in the 21st century: emerging biological therapies.* J Cancer, 2013. **4**(2): p. 117-32.
16. Rivenbark, A.G., S.M. O'Connor, and W.B. Coleman, *Molecular and cellular heterogeneity in breast cancer: challenges for personalized medicine.* Am J Pathol, 2013. **183**(4): p. 1113-24.
17. Engebraaten, O., H.K. Vollan, and A.L. Borresen-Dale, *Triple-negative breast cancer and the need for new therapeutic targets.* Am J Pathol, 2013. **183**(4): p. 1064-74.
18. Rakha, E.A., J.S. Reis-Filho, and I.O. Ellis, *Basal-like breast cancer: a critical review.* J Clin Oncol, 2008. **26**(15): p. 2568-81.
19. Rakha, E.A., et al., *Breast cancer prognostic classification in the molecular era: the role of histological grade.* Breast Cancer Res, 2010. **12**(4): p. 207.

20. Rakha, E.A., et al., *Prognostic significance of Nottingham histologic grade in invasive breast carcinoma.* J Clin Oncol, 2008. **26**(19): p. 3153-8.
21. Society, A.C. *Breast Cancer*. 2013; Available from: http://www.cancer.org/cancer/breastcancer/detailedguide/breast-cancer-staging.
22. Cancer.org. *Stages of Breast Cancer*. 2013; Available from: http://breastcancer.org/symptoms/diagnosis/staging.
23. Huotari, J. and A. Helenius, *Endosome maturation.* EMBO J, 2011. **30**(17): p. 3481-500.
24. Gould, G.W. and J. Lippincott-Schwartz, *New roles for endosomes: from vesicular carriers to multi-purpose platforms.* Nat Rev Mol Cell Biol, 2009. **10**(4): p. 287-92.
25. Mellman, I. and Y. Yarden, *Endocytosis and cancer.* Cold Spring Harb Perspect Biol, 2013. **5**(12).
26. Babst, M., *MVB vesicle formation: ESCRT-dependent, ESCRT-independent and everything in between.* Curr Opin Cell Biol, 2011. **23**(4): p. 452-7.
27. Saftig, P. and J. Klumperman, *Lysosome biogenesis and lysosomal membrane proteins: trafficking meets function.* Nat Rev Mol Cell Biol, 2009. **10**(9): p. 623-35.
28. Kirkegaard, T. and M. Jaattela, *Lysosomal involvement in cell death and cancer.* Biochim Biophys Acta, 2009. **1793**(4): p. 746-54.
29. Cuervo, A.M. and J.F. Dice, *Lysosomes, a meeting point of proteins, chaperones, and proteases.* J Mol Med (Berl), 1998. **76**(1): p. 6-12.
30. Pfeffer, S.R., *Rab GTPase regulation of membrane identity.* Curr Opin Cell Biol, 2013. **25**(4): p. 414-9.
31. Barr, F.A., *Review series: Rab GTPases and membrane identity: causal or inconsequential?* J Cell Biol, 2013. **202**(2): p. 191-9.
32. Saftig, P., *Physiology of the lysosome*, in *Fabry Disease: Perspectives from 5 Years of FOS*, A. Mehta, M. Beck, and G. Sunder-Plassmann, Editors. 2006: Oxford.
33. Schwake, M., B. Schroder, and P. Saftig, *Lysosomal membrane proteins and their central role in physiology.* Traffic, 2013. **14**(7): p. 739-48.
34. Huynh, K.K., et al., *LAMP proteins are required for fusion of lysosomes with phagosomes.* EMBO J, 2007. **26**(2): p. 313-24.
35. Cassimeris, L., et al., *Lewin's cells*. 2nd ed. 2011, Sudbury, Mass.: Jones and Bartlett Publishers. xxiv, 1053 p.
36. Rotty, J.D., C. Wu, and J.E. Bear, *New insights into the regulation and cellular functions of the ARP2/3 complex.* Nat Rev Mol Cell Biol, 2013. **14**(1): p. 7-12.
37. Turk, V., et al., *Cysteine cathepsins: from structure, function and regulation to new frontiers.* Biochim Biophys Acta, 2012. **1824**(1): p. 68-88.
38. Reiser, J., B. Adair, and T. Reinheckel, *Specialized roles for cysteine cathepsins in health and disease.* J Clin Invest, 2010. **120**(10): p. 3421-31.

39. Collette, J., et al., *Biosynthesis and alternate targeting of the lysosomal cysteine protease cathepsin L.* Int Rev Cytol, 2004. **241**: p. 1-51.
40. Tan, G.J., et al., *Cathepsins mediate tumor metastasis.* World J Biol Chem, 2013. **4**(4): p. 91-101.
41. Gocheva, V. and J.A. Joyce, *Cysteine cathepsins and the cutting edge of cancer invasion.* Cell Cycle, 2007. **6**(1): p. 60-4.
42. Ganguly, S.S. and R. Plattner, *Activation of abl family kinases in solid tumors.* Genes Cancer, 2012. **3**(5-6): p. 414-25.
43. Greuber, E.K., et al., *Role of ABL family kinases in cancer: from leukaemia to solid tumours.* Nat Rev Cancer, 2013. **13**(8): p. 559-71.
44. Sirvent, A., C. Benistant, and S. Roche, *Cytoplasmic signalling by the c-Abl tyrosine kinase in normal and cancer cells.* Biol Cell, 2008. **100**(11): p. 617-31.
45. Nagar, B., et al., *Structural basis for the autoinhibition of c-Abl tyrosine kinase.* Cell, 2003. **112**(6): p. 859-71.
46. Bradley, W.D. and A.J. Koleske, *Regulation of cell migration and morphogenesis by Abl-family kinases: emerging mechanisms and physiological contexts.* J Cell Sci, 2009. **122**(Pt 19): p. 3441-54.
47. Plattner, R., et al., *c-Abl is activated by growth factors and Src family kinases and has a role in the cellular response to PDGF.* Genes Dev, 1999. **13**(18): p. 2400-11.
48. Plattner, R., et al., *A new link between the c-Abl tyrosine kinase and phosphoinositide signalling through PLC-gamma1.* Nat Cell Biol, 2003. **5**(4): p. 309-19.
49. Lin, J. and R. Arlinghaus, *Activated c-Abl tyrosine kinase in malignant solid tumors.* Oncogene, 2008. **27**(32): p. 4385-91.
50. Wang, J.Y., *Regulation of cell death by the Abl tyrosine kinase.* Oncogene, 2000. **19**(49): p. 5643-50.
51. Woodring, P.J., T. Hunter, and J.Y. Wang, *Regulation of F-actin-dependent processes by the Abl family of tyrosine kinases.* J Cell Sci, 2003. **116**(Pt 13): p. 2613-26.
52. Druker, B.J., et al., *Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia.* N Engl J Med, 2001. **344**(14): p. 1031-7.
53. Deininger, M.W., *Nilotinib.* Clin Cancer Res, 2008. **14**(13): p. 4027-31.
54. Manley, P.W., et al., *Extended kinase profile and properties of the protein kinase inhibitor nilotinib.* Biochim Biophys Acta, 2010. **1804**(3): p. 445-53.
55. Eiring, A.M., et al., *Advances in the treatment of chronic myeloid leukemia.* BMC Med, 2011. **9**: p. 99.
56. Srinivasan, D. and R. Plattner, *Activation of Abl tyrosine kinases promotes invasion of aggressive breast cancer cells.* Cancer Res, 2006. **66**(11): p. 5648-55.
57. Ganguly, S.S., et al., *c-Abl and Arg are activated in human primary melanomas, promote melanoma cell invasion via distinct pathways, and drive metastatic progression.* Oncogene, 2012. **31**(14): p. 1804-16.

58. Srinivasan, D., D.M. Kaetzel, and R. Plattner, *Reciprocal regulation of Abl and receptor tyrosine kinases.* Cell Signal, 2009. **21**(7): p. 1143-50.
59. Lin, J., et al., *Oncogenic activation of c-Abl in non-small cell lung cancer cells lacking FUS1 expression: inhibition of c-Abl by the tumor suppressor gene product Fus1.* Oncogene, 2007. **26**(49): p. 6989-96.
60. Furlan, A., et al., *Abl interconnects oncogenic Met and p53 core pathways in cancer cells.* Cell Death Differ, 2011. **18**(10): p. 1608-16.
61. Srinivasan, D., J.T. Sims, and R. Plattner, *Aggressive breast cancer cells are dependent on activated Abl kinases for proliferation, anchorage-independent growth and survival.* Oncogene, 2008. **27**(8): p. 1095-105.
62. Dai, Z., et al., *Oncogenic Abl and Src tyrosine kinases elicit the ubiquitin-dependent degradation of target proteins through a Ras-independent pathway.* Genes Dev, 1998. **12**(10): p. 1415-24.
63. Chen, X., et al., *A kinase-independent function of c-Abl in promoting proteolytic destruction of damaged DNA binding proteins.* Mol Cell, 2006. **22**(4): p. 489-99.
64. Jacob, M., et al., *Endogenous cAbl regulates receptor endocytosis.* Cell Signal, 2009. **21**(8): p. 1308-16.
65. Yogalingam, G. and A.M. Pendergast, *Abl kinases regulate autophagy by promoting the trafficking and function of lysosomal components.* J Biol Chem, 2008. **283**(51): p. 35941-53.
66. Tanos, B.E. and A.M. Pendergast, *Abi-1 forms an epidermal growth factor-inducible complex with Cbl: role in receptor endocytosis.* Cell Signal, 2007. **19**(7): p. 1602-9.
67. Li, X., et al., *c-Abl and Arg tyrosine kinases regulate lysosomal degradation of the oncoprotein Galectin-3.* Cell Death Differ, 2010. **17**(8): p. 1277-87.
68. Marino, N., et al., *Insights into the biology and prevention of tumor metastasis provided by the Nm23 metastasis suppressor gene.* Cancer Metastasis Rev, 2012. **31**(3-4): p. 593-603.
69. Steeg, P.S., et al., *Evidence for a novel gene associated with low tumor metastatic potential.* J Natl Cancer Inst, 1988. **80**(3): p. 200-4.
70. Leone, A., et al., *Reduced tumor incidence, metastatic potential, and cytokine responsiveness of nm23-transfected melanoma cells.* Cell, 1991. **65**(1): p. 25-35.
71. Leone, A., et al., *Transfection of human nm23-H1 into the human MDA-MB-435 breast carcinoma cell line: effects on tumor metastatic potential, colonization and enzymatic activity.* Oncogene, 1993. **8**(9): p. 2325-33.
72. Kantor, J.D., et al., *Inhibition of cell motility after nm23 transfection of human and murine tumor cells.* Cancer Res, 1993. **53**(9): p. 1971-3.
73. Ma, D., J.R. McCorkle, and D.M. Kaetzel, *The metastasis suppressor NM23-H1 possesses 3'-5' exonuclease activity.* J Biol Chem, 2004. **279**(17): p. 18073-84.
74. Steeg, P.S., C.E. Horak, and K.D. Miller, *Clinical-translational approaches to the Nm23-H1 metastasis suppressor.* Clin Cancer Res, 2008. **14**(16): p. 5006-12.

75. Otsuki, Y., et al., *Tumor metastasis suppressor nm23H1 regulates Rac1 GTPase by interaction with Tiam1.* Proc Natl Acad Sci U S A, 2001. **98**(8): p. 4385-90.
76. Fournier, H.N., C. Albiges-Rizo, and M.R. Block, *New insights into Nm23 control of cell adhesion and migration.* J Bioenerg Biomembr, 2003. **35**(1): p. 81-7.
77. Palacios, F., et al., *ARF6-GTP recruits Nm23-H1 to facilitate dynamin-mediated endocytosis during adherens junctions disassembly.* Nat Cell Biol, 2002. **4**(12): p. 929-36.
78. Curtis, C.D., et al., *Interaction of the tumor metastasis suppressor nonmetastatic protein 23 homologue H1 and estrogen receptor alpha alters estrogen-responsive gene expression.* Cancer Res, 2007. **67**(21): p. 10600-7.
79. Jarrett, S.G., et al., *Metastasis suppressor NM23-H1 promotes repair of UV-induced DNA damage and suppresses UV-induced melanomagenesis.* Cancer Res, 2012. **72**(1): p. 133-43.
80. Jarrett, S.G., et al., *NM23 deficiency promotes metastasis in a UV radiation-induced mouse model of human melanoma.* Clin Exp Metastasis, 2013. **30**(1): p. 25-36.
81. Kim, H.D., et al., *Regulators affecting the metastasis suppressor activity of Nm23-H1.* Mol Cell Biochem, 2009. **329**(1-2): p. 167-73.
82. Marino, N., J.C. Marshall, and P.S. Steeg, *Protein-protein interactions: a mechanism regulating the anti-metastatic properties of Nm23-H1.* Naunyn Schmiedebergs Arch Pharmacol, 2011. **384**(4-5): p. 351-62.
83. Aktary, Z. and M. Pasdar, *Plakoglobin represses SATB1 expression and decreases in vitro proliferation, migration and invasion.* PLoS One, 2013. **8**(11): p. e78388.
84. Aktary, Z., et al., *Plakoglobin interacts with and increases the protein levels of metastasis suppressor Nm23-H2 and regulates the expression of Nm23-H1.* Oncogene, 2010. **29**(14): p. 2118-29.
85. Lin, K.H., et al., *Activation of antimetastatic Nm23-H1 gene expression by estrogen and its alpha-receptor.* Endocrinology, 2002. **143**(2): p. 467-75.
86. Ouatas, T., D. Halverson, and P.S. Steeg, *Dexamethasone and medroxyprogesterone acetate elevate Nm23-H1 metastasis suppressor gene expression in metastatic human breast carcinoma cells: new uses for old compounds.* Clin Cancer Res, 2003. **9**(10 Pt 1): p. 3763-72.
87. Palmieri, D., et al., *Medroxyprogesterone acetate elevation of Nm23-H1 metastasis suppressor expression in hormone receptor-negative breast cancer.* J Natl Cancer Inst, 2005. **97**(9): p. 632-42.
88. Lim, J., et al., *Cell-permeable NM23 blocks the maintenance and progression of established pulmonary metastasis.* Cancer Res, 2011. **71**(23): p. 7216-25.
89. Fiore, L.S., et al., *c-Abl and Arg induce cathepsin-mediated lysosomal degradation of the NM23-H1 metastasis suppressor in invasive cancer.* Oncogene, 2013.

90. Singer, C.F., et al., *Expression of tyrosine kinases in human malignancies as potential targets for kinase-specific inhibitors.* Endocr Relat Cancer, 2004. **11**(4): p. 861-9.
91. Simpson, L., et al., *Renal medullary carcinoma and ABL gene amplification.* J Urol, 2005. **173**(6): p. 1883-8.
92. Niyazi, M., et al., *Expression of p73 and c-Abl proteins in human ovarian carcinomas.* J Nippon Med Sch, 2003. **70**(3): p. 234-42.
93. O'Neill, A.J., et al., *Abl expression in human fetal and adult tissues, tumours, and tumour microvessels.* J Pathol, 1997. **183**(3): p. 325-9.
94. Koos, B., et al., *The tyrosine kinase c-Abl promotes proliferation and is expressed in atypical teratoid and malignant rhabdoid tumors.* Cancer, 2010. **116**(21): p. 5075-81.
95. Slomovitz, B.M., et al., *Expression of imatinib mesylate-targeted kinases in endometrial carcinoma.* Gynecol Oncol, 2004. **95**(1): p. 32-6.
96. Birge, R.B., et al., *Crk and CrkL adaptor proteins: networks for physiological and pathological signaling.* Cell Commun Signal, 2009. **7**: p. 13.
97. Singer, C.F., et al., *Active (p)CrkL is overexpressed in human malignancies: potential role as a surrogate parameter for therapeutic tyrosine kinase inhibition.* Oncol Rep, 2006. **15**(2): p. 353-9.
98. Youn, B.S., et al., *NM23 as a prognostic biomarker in ovarian serous carcinoma.* Mod Pathol, 2008. **21**(7): p. 885-92.
99. Hsu, N.Y., et al., *Expression of nm23 in the primary tumor and the metastatic regional lymph nodes of patients with gastric cardiac cancer.* Clin Cancer Res, 1999. **5**(7): p. 1752-7.
100. McDermott, N.C., et al., *Immunohistochemical expression of nm23 in primary invasive malignant melanoma is predictive of survival outcome.* J Pathol, 2000. **190**(2): p. 157-62.
101. Ferrari, D., et al., *Dermatopathological indicators of poor melanoma prognosis are significantly inversely correlated with the expression of NM23 protein in primary cutaneous melanoma.* J Cutan Pathol, 2007. **34**(9): p. 705-12.
102. Dome, B., B. Somlai, and J. Timar, *The loss of NM23 protein in malignant melanoma predicts lymphatic spread without affecting survival.* Anticancer Res, 2000. **20**(5C): p. 3971-4.
103. Florenes, V.A., et al., *Levels of nm23 messenger RNA in metastatic malignant melanomas: inverse correlation to disease progression.* Cancer Res, 1992. **52**(21): p. 6088-91.
104. Lee, C.S., A. Pirdas, and M.W. Lee, *Immunohistochemical demonstration of the nm23-H1 gene product in human malignant melanoma and Spitz nevi.* Pathology, 1996. **28**(3): p. 220-4.
105. Pacifico, M.D., et al., *nm23 as a prognostic marker in primary cutaneous melanoma: evaluation using tissue microarray in a patient group with long-term follow-up.* Melanoma Res, 2005. **15**(5): p. 435-40.
106. Easty, D.J., et al., *Expression of NM23 in human melanoma progression and metastasis.* Br J Cancer, 1996. **74**(1): p. 109-14.

107. Hennessy, C., et al., *Expression of the antimetastatic gene nm23 in human breast cancer: an association with good prognosis.* J Natl Cancer Inst, 1991. **83**(4): p. 281-5.
108. Bevilacqua, G., et al., *Association of low nm23 RNA levels in human primary infiltrating ductal breast carcinomas with lymph node involvement and other histopathological indicators of high metastatic potential.* Cancer Res, 1989. **49**(18): p. 5185-90.
109. Tokunaga, Y., et al., *Reduced expression of nm23-H1, but not of nm23-H2, is concordant with the frequency of lymph-node metastasis of human breast cancer.* Int J Cancer, 1993. **55**(1): p. 66-71.
110. Bal, A., et al., *Expression of nm23 in the spectrum of pre-invasive, invasive and metastatic breast lesions.* Diagn Pathol, 2008. **3**: p. 23.
111. Heimann, R., D.J. Ferguson, and S. Hellman, *The relationship between nm23, angiogenesis, and the metastatic proclivity of node-negative breast cancer.* Cancer Res, 1998. **58**(13): p. 2766-71.
112. Han, S., et al., *Abnormal expression of four novel molecular markers represents a highly aggressive phenotype in breast cancer. Immunohistochemical assay of p53, nm23, erbB-2, and cathepsin D protein.* J Surg Oncol, 1997. **65**(1): p. 22-7.
113. Toulas, C., et al., *Potential prognostic value in human breast cancer of cytosolic Nme1 protein detection using an original hen specific antibody.* Br J Cancer, 1996. **73**(5): p. 630-5.
114. Dong, S.W., et al., *Expression patterns of ER, HER2, and NM23-H1 in breast cancer patients with different menopausal status: correlations with metastasis.* Mol Diagn Ther, 2011. **15**(4): p. 211-9.
115. Sgouros, J., et al., *Correlation of nm23-H1 gene expression with clinical outcome in patients with advanced breast cancer.* In Vivo, 2007. **21**(3): p. 519-22.
116. Martinez, J.A., et al., *Overexpression of nm23-H1 and nm23-H2 genes in colorectal carcinomas and loss of nm23-H1 expression in advanced tumour stages.* Gut, 1995. **37**(5): p. 712-20.
117. Cipollini, G., et al., *Down-regulation of the nm23.h1 gene inhibits cell proliferation.* Int J Cancer, 1997. **73**(2): p. 297-302.
118. Caligo, M.A., et al., *NM23 gene expression in human breast carcinomas: loss of correlation with cell proliferation in the advanced phase of tumor progression.* Int J Cancer, 1997. **74**(1): p. 102-11.
119. Garrido, M.C. and B.C. Bastian, *KIT as a therapeutic target in melanoma.* J Invest Dermatol, 2010. **130**(1): p. 20-7.
120. Subramanian, C. and E.S. Robertson, *The metastatic suppressor Nm23-H1 interacts with EBNA3C at sequences located between the glutamine- and proline-rich domains and can cooperate in activation of transcription.* J Virol, 2002. **76**(17): p. 8702-9.
121. Postel, E.H., *NM23/Nucleoside diphosphate kinase as a transcriptional activator of c-myc.* Curr Top Microbiol Immunol, 1996. **213 ( Pt 2)**: p. 233-52.

122. Thakur, R.K., et al., *Metastases suppressor NM23-H2 interaction with G-quadruplex DNA within c-MYC promoter nuclease hypersensitive element induces c-MYC expression.* Nucleic Acids Res, 2009. **37**(1): p. 172-83.
123. Rankin, S., et al., *Putative DNA quadruplex formation within the human c-kit oncogene.* J Am Chem Soc, 2005. **127**(30): p. 10584-9.
124. Anderson, W.F., et al., *Divergent cancer pathways for early-onset and late-onset cutaneous malignant melanoma.* Cancer, 2009. **115**(18): p. 4176-85.
125. Cust, A.E., et al., *Early-life sun exposure and risk of melanoma before age 40 years.* Cancer Causes Control, 2011. **22**(6): p. 885-97.
126. Fidler, I.J., et al., *The seed and soil hypothesis: vascularisation and brain metastases.* Lancet Oncol, 2002. **3**(1): p. 53-7.
127. Sims, J.T., et al., *Imatinib reverses doxorubicin resistance by affecting activation of STAT3-dependent NF-kappaB and HSP27/p38/AKT pathways and by inhibiting ABCB1.* PLoS One, 2013. **8**(1): p. e55509.
128. Ma, D., et al., *Association between NM23-H1 gene expression and metastasis of human uveal melanoma in an animal model.* Invest Ophthalmol Vis Sci, 1996. **37**(11): p. 2293-301.
129. Goodall, R.J., et al., *Evaluation of the expression levels of nm23-H1 mRNA in primary breast cancer, benign breast disease, axillary lymph nodes and normal breast tissue.* Pathology, 1994. **26**(4): p. 423-8.
130. Lin, L.I., et al., *Significance of nm23 mRNA expression in human hepatocellular carcinoma.* Anticancer Res, 1998. **18**(1B): p. 541-6.
131. Shore, S.K., R.V. Tantravahi, and E.P. Reddy, *Transforming pathways activated by the v-Abl tyrosine kinase.* Oncogene, 2002. **21**(56): p. 8568-76.
132. Varticovski, L., et al., *Activation of phosphatidylinositol 3-kinase in cells expressing abl oncogene variants.* Mol Cell Biol, 1991. **11**(2): p. 1107-13.
133. Leevers, S.J., B. Vanhaesebroeck, and M.D. Waterfield, *Signalling through phosphoinositide 3-kinases: the lipids take centre stage.* Curr Opin Cell Biol, 1999. **11**(2): p. 219-25.
134. Raiborg, C., K.O. Schink, and H. Stenmark, *Class III phosphatidylinositol 3-kinase and its catalytic product PtdIns3P in regulation of endocytic membrane traffic.* FEBS J, 2013. **280**(12): p. 2730-42.
135. Plattner, R., et al., *Bidirectional signaling links the Abelson kinases to the platelet-derived growth factor receptor.* Mol Cell Biol, 2004. **24**(6): p. 2573-83.
136. Burton, E.A., T.N. Oliver, and A.M. Pendergast, *Abl kinases regulate actin comet tail elongation via an N-WASP-dependent pathway.* Mol Cell Biol, 2005. **25**(20): p. 8834-43.
137. Stuart, J.R., et al., *c-Abl interacts with the WAVE2 signaling complex to induce membrane ruffling and cell spreading.* J Biol Chem, 2006. **281**(42): p. 31290-7.
138. Sossey-Alaoui, K., X. Li, and J.K. Cowell, *c-Abl-mediated phosphorylation of WAVE3 is required for lamellipodia formation and cell migration.* J Biol Chem, 2007. **282**(36): p. 26257-65.

139. Boyle, S.N., et al., *A critical role for cortactin phosphorylation by Abl-family kinases in PDGF-induced dorsal-wave formation.* Curr Biol, 2007. **17**(5): p. 445-51.
140. Jean, D., N. Rousselet, and R. Frade, *Expression of cathepsin L in human tumor cells is under the control of distinct regulatory mechanisms.* Oncogene, 2006. **25**(10): p. 1474-84.
141. Kreuzaler, P.A., et al., *Stat3 controls lysosomal-mediated cell death in vivo.* Nat Cell Biol, 2011. **13**(3): p. 303-9.
142. Goldberg, Z., et al., *Tyrosine phosphorylation of Mdm2 by c-Abl: implications for p53 regulation.* EMBO J, 2002. **21**(14): p. 3715-27.
143. Cao, C., Y. Leng, and D. Kufe, *Catalase activity is regulated by c-Abl and Arg in the oxidative stress response.* J Biol Chem, 2003. **278**(32): p. 29667-75.
144. Dabir, S., A. Kluge, and A. Dowlati, *The association and nuclear translocation of the PIAS3-STAT3 complex is ligand and time dependent.* Mol Cancer Res, 2009. **7**(11): p. 1854-60.
145. Murphy, J.E., et al., *Endosomes: a legitimate platform for the signaling train.* Proc Natl Acad Sci U S A, 2009. **106**(42): p. 17615-22.
146. Krishnan, K.S., et al., *Nucleoside diphosphate kinase, a source of GTP, is required for dynamin-dependent synaptic vesicle recycling.* Neuron, 2001. **30**(1): p. 197-210.
147. Radhakrishna, H., et al., *ARF6 requirement for Rac ruffling suggests a role for membrane trafficking in cortical actin rearrangements.* J Cell Sci, 1999. **112 ( Pt 6)**: p. 855-66.
148. Sirvent, A., et al., *The tyrosine kinase Abl is required for Src-transforming activity in mouse fibroblasts and human breast cancer cells.* Oncogene, 2007. **26**(52): p. 7313-23.
149. Akhurst, R.J. and A. Hata, *Targeting the TGFbeta signalling pathway in disease.* Nat Rev Drug Discov, 2012. **11**(10): p. 790-811.
150. Seong, H.A., H. Jung, and H. Ha, *NM23-H1 tumor suppressor physically interacts with serine-threonine kinase receptor-associated protein, a transforming growth factor-beta (TGF-beta) receptor-interacting protein, and negatively regulates TGF-beta signaling.* J Biol Chem, 2007. **282**(16): p. 12075-96.
151. Hartsough, M.T., et al., *Nm23-H1 metastasis suppressor phosphorylation of kinase suppressor of Ras via a histidine protein kinase pathway.* J Biol Chem, 2002. **277**(35): p. 32389-99.
152. Robertson, S.E., et al., *Extracellular signal-regulated kinase regulates clathrin-independent endosomal trafficking.* Mol Biol Cell, 2006. **17**(2): p. 645-57.
153. Sims, J.T., et al., *STI571 sensitizes breast cancer cells to 5-fluorouracil, cisplatin and camptothecin in a cell type-specific manner.* Biochem Pharmacol, 2009. **78**(3): p. 249-60.
154. Fabian, M.R., N. Sonenberg, and W. Filipowicz, *Regulation of mRNA translation and stability by microRNAs.* Annu Rev Biochem, 2010. **79**: p. 351-79.

155. Nickel, W. and C. Rabouille, *Mechanisms of regulated unconventional protein secretion.* Nat Rev Mol Cell Biol, 2009. **10**(2): p. 148-55.
156. Caserman, S., et al., *Cathepsin L splice variants in human breast cell lines.* Biol Chem, 2006. **387**(5): p. 629-34.
157. Salesse, S., S.J. Dylla, and C.M. Verfaillie, *p210BCR/ABL-induced alteration of pre-mRNA splicing in primary human CD34+ hematopoietic progenitor cells.* Leukemia, 2004. **18**(4): p. 727-33.
158. Wagner, M., et al., *Brefeldin A causes structural and functional alterations of the trans-Golgi network of MDCK cells.* J Cell Sci, 1994. **107 ( Pt 4)**: p. 933-43.
159. Liljedahl, M., et al., *Protein kinase D regulates the fission of cell surface destined transport carriers from the trans-Golgi network.* Cell, 2001. **104**(3): p. 409-20.
160. Storz, P., et al., *Tyrosine phosphorylation of protein kinase D in the pleckstrin homology domain leads to activation.* J Biol Chem, 2003. **278**(20): p. 17969-76.
161. Okabe-Kado, J., T. Kasukabe, and Y. Kaneko, *Extracellular NM23 Protein as a Therapeutic Target for Hematologic Malignancies.* Adv Hematol, 2012. **2012**: p. 879368.
162. Manjithaya, R. and S. Subramani, *Autophagy: a broad role in unconventional protein secretion?* Trends Cell Biol, 2011. **21**(2): p. 67-73.
163. Blott, E.J. and G.M. Griffiths, *Secretory lysosomes.* Nat Rev Mol Cell Biol, 2002. **3**(2): p. 122-31.
164. Zhang, M. and R. Schekman, *Cell biology. Unconventional secretion, unconventional solutions.* Science, 2013. **340**(6132): p. 559-61.
165. Razi, M., E.Y. Chan, and S.A. Tooze, *Early endosomes and endosomal coatomer are required for autophagy.* J Cell Biol, 2009. **185**(2): p. 305-21.
166. Liou, W., et al., *The autophagic and endocytic pathways converge at the nascent autophagic vacuoles.* J Cell Biol, 1997. **136**(1): p. 61-70.
167. Muppirala, M., V. Gupta, and G. Swarup, *Tyrosine phosphorylation of a SNARE protein, syntaxin 17: implications for membrane trafficking in the early secretory pathway.* Biochim Biophys Acta, 2012. **1823**(12): p. 2109-19.
168. Itakura, E. and N. Mizushima, *Syntaxin 17: the autophagosomal SNARE.* Autophagy, 2013. **9**(6): p. 917-9.
169. Kramer, H., *Route to destruction: autophagosomes SNARE lysosomes.* J Cell Biol, 2013. **201**(4): p. 495-7.
170. Beaujouin, M., et al., *Pro-cathepsin D interacts with the extracellular domain of the beta chain of LRP1 and promotes LRP1-dependent fibroblast outgrowth.* J Cell Sci, 2010. **123**(Pt 19): p. 3336-46.
171. Mai, J., et al., *Human procathepsin B interacts with the annexin II tetramer on the surface of tumor cells.* J Biol Chem, 2000. **275**(17): p. 12806-12.
172. Lorusso, A., et al., *Annexin2 coating the surface of enlargeosomes is needed for their regulated exocytosis.* EMBO J, 2006. **25**(23): p. 5443-56.
173. Zhang, W., et al., *Annexin A2 promotes the migration and invasion of human hepatocellular carcinoma cells in vitro by regulating the shedding*

*of CD147-harboring microvesicles from tumor cells.* PLoS One, 2013. **8**(8): p. e67268.
174. Dupont, N., et al., *Autophagy-based unconventional secretory pathway for extracellular delivery of IL-1beta.* EMBO J, 2011. **30**(23): p. 4701-11.
175. Buxton, I.L. and N. Yokdang, *Extracellular NM23 Signaling in Breast Cancer: Incommodus Verum.* Cancers (Basel), 2011. **3**(3): p. 2844-57.
176. Niitsu, N., et al., *Prognostic implications of the differentiation inhibitory factor nm23-H1 protein in the plasma of aggressive non-Hodgkin's lymphoma.* Blood, 1999. **94**(10): p. 3541-50.
177. Gogas, H., et al., *Biomarkers in melanoma.* Ann Oncol, 2009. **20 Suppl 6**: p. vi8-13.
178. Sheffield, M.V., et al., *Comparison of five antibodies as markers in the diagnosis of melanoma in cytologic preparations.* Am J Clin Pathol, 2002. **118**(6): p. 930-6.
179. Villanueva, J., et al., *Acquired resistance to BRAF inhibitors mediated by a RAF kinase switch in melanoma can be overcome by cotargeting MEK and IGF-1R/PI3K.* Cancer Cell, 2010. **18**(6): p. 683-95.
180. Sabbatino, F., et al., *PDGFRalpha up-regulation mediated by sonic hedgehog pathway activation leads to BRAF inhibitor resistance in melanoma cells with BRAF mutation.* Oncotarget, 2014.

# Vita

**Name:** Leann S. Fiore

**Place of Birth**: Tulsa, Oklahoma

EDUCATION

August 2002-July 2006 — B.S. (Physiology)
Department of Zoology
Oklahoma State University
Stillwater, Oklahoma

PROFESSIONAL POSITIONS

August 2006-May 2007 — Oklahoma State University
Department of Zoology
Biology Instructor

POSTER PRESENTATIONS

**L. Fiore**, S. Ganguly, M. Cibull, C. Wand, D. Richards, D. Kaetzel, and R. Plattner. "c-Abl and Arg Induce Cathepsin-Mediated Lysosomal Degradation of the NM23-H1 Metastasis Suppressor in Invasive Cancer" Tumor Progression and Therapeutic Resistance, Boston, Massachusetts, March 2014

**L. Fiore**, S. Ganguly, J. Sims, D. Srinivasan, M. Cibull, D. Kaetzel and R. Plattner "c-Abl and Arg Promote Invasion via Lysosomal Degradation of the Metastasis Suppressor, NM23-H1, by Activating Cathepsins L and B." Markey Cancer Day, University of Kentucky, Lexington KY, May 2012

PEER-REVIEWED PUBLICATIONS

**Fiore LS**, Ganguly SS, Sledziona J, Cibull ML, Wang C, Richards DL, Neltner JM, Beach C, McCorkle JR, Kaetzel DM, Plattner R. c-Abl and Arg induce cathepsin-mediated lysosomal degradation of the NM23-H1 metastasis suppressor in invasive cancer. Oncogene. 2013 Oct 7.

Ganguly SS, **Fiore LS**, Sims JT, Friend JW, Srinivasan D, Thacker MA, Cibull ML, Wang C, Novak M, Kaetzel DM, Plattner R. c-Abl and Arg are activated in human primary

melanomas, promote melanoma cell invasion via distinct pathways, and drive metastatic progression. Oncogene. 2012 Apr 5;31(14):1804-16.

Sims JT, Ganguly S, **Fiore LS**, Holler CJ, Park ES, Plattner R. STI571 sensitizes breast cancer cells to 5-fluorouracil, cisplatin and camptothecin in a cell type-specific manner. Biochem Pharmacol. 2009 Aug 1;78(3):249-60