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MOLECULAR MECHANISMS BY WHICH c-ABL AND ARG MEDIATE MELANOMA INVASION AND METASTASIS

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MOLECULAR MECHANISMS BY WHICH c-ABL AND ARG MEDIATE MELANOMA INVASION AND METASTASIS

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

Sourik S. Ganguly

Lexington, Kentucky

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2013

ABSTRACT OF DISSERTATION

MOLECULAR MECHANISMS BY WHICH c-ABL AND ARG MEDIATE MELANOMA INVASION AND METASTASIS

Metastasis is one of the main causes of death in cancer patients. Metastatic melanoma is a death sentence, as chemotherapeutic agents have a 5% success rate or do not extend survival beyond 10 months**.** The lack of effective chemotherapeutic agents for treating metastatic melanoma indicates a dire need to identify new drug targets and develop new therapies. Our lab has previously shown that the kinase activity of Abelson family of non-receptor tyrosine kinases (c-Abl and Arg) is elevated in invasive breast cancer cell lines as compared to non-invasive cell lines. Previous studies from our lab have shown that Abl kinases are convergent point of ErbB2 and Src Kinases in melanoma cells and Abl kinases promote invasion by an undefined mechanism. Although Abl kinases promote invasion, it is not known whether they are important for metastastic potential. For the first time, we report that Abl kinases promote melanoma cell proliferation, survival, matrigel-invasion and single-cell 3D invasion. To investigate the mechanism by which Abl kinases promote invasion, we found out that active c-Abl transcriptionally upregulates MMP-1, and using rescue approaches we show that c-Abl promotes invasion via a STAT3→MMP-1 pathway. In contrast, active Arg drives invasion in a STAT3-independent manner, and upregulates the expression of MMP-3 and MT1-MMP, in addition to MMP-1. We also found that Abl kinases promote invasion via lysosomal degradation of a metastasis suppressor, NM23-H1 by activating lysosomal cathepsins B and L, which directly cleave and degrade NM23-H1. Furthermore, c-Abl and Arg are activated in primary melanomas and cAbl/Arg activity is inversely correlated with NM23-H1 expression both in primary melanoma and human melanoma cells. We also demonstrate, for the first time that active Abl kinases promote metastasis *in vivo*, as inhibition of c-Abl/Arg with nilotinib, dramatically inhibits lung colonization/metastasis in a mouse model using two different melanoma cell lines. In summary, we identify Abl kinases as critical, novel, drug targets in metastatic melanoma, and our data indicate that nilotinib may be useful in preventing metastasis in a select group of patients, harboring active Abl kinases.

Keywords: c-Abl, Arg, invasion, metastasis, melanoma

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I dedicate this dissertation to my father

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CHAPTER 1: Introduction

1.1 Melanoma

1.1.1 Melanoma incidence and challenges in treatment

More than 1 million cases of skin cancer are diagnosed in the United States of America. Melanoma a malignant tumor of the melanocytes, the pigment producing cell in the epidermal layer of the skin, represents less than 10% of all skin cancer. Melanoma accounts for 75% of all skin cancer related deaths with a mean survival rate of 6-9 months. The incidence of melanoma has increased to more than 600% in the last 50 years and is considered to be a significant health problem in western world countries [1, 2]. Metastatic melanoma is uncurable and chemotherapeutic drugs have a 5% success rate and do not extend life expectancy beyond 10 months [1, 3].

Chemotherapy initially was considered an accepted first-line of therapy to treat melanoma until most of the chemotherapeutic drugs showed poor response in patients. Various chemotherapies like dacarbazine, temozolomide (oral analogue of dacarbazine), cisplatin, vinblastine and f1otemustine all showed poor response rates. Combination chemotherapies such as Dartmouth regimen (cisplatin/vinblastine/dacarbazine/tamoxifen) have failed to show any better survival benefit than dacarbazine alone, and many new chemotherapeutic drugs (temozolomide, carboplatin, vinblastine) failed to elicit any better response [1, 3, 4] Two immunotherapies, high-dose of interleukin-2 and ipilimumab, are currently recommended for unresectable or metastatic melanoma patients, but these drugs also have low clinical response rate (10-15%) [1,3]. As an adjuvant therapy

some patients are treated with interferon-alpha2b [5]. Even though all the above agents show poor responses, they are still used in the clinic due to the lack of better agents that can increase the survival rate in melanoma. In the past decade, there has been a focus on identifying various new targets in metastatic melanoma. Mutations in BRAF, a serine-threonine kinase in RAS-RAF-MEK-ERK signaling pathway occur in more than 50% of patients with cutaneous melanoma [1]. Vemurafenib, selective inhibitor of V600E activating mutation in BRAF, showed promise in a phase III clinical trial, however, a large number of patients relapsed in less than one year [1, 6]. Molecular studies led to the identification of some resistance pathways and thus dampened the initial success seen with targeting BRAF in melanoma treatment. The pathways that are often mutated in vemurafenib resistant patient tumors will be discussed later.

1.1.2 Clinical classification and melanoma progression

Melanoma is anatomically classified into the following groups [2]:

1. Superficial spreading melanoma- affects the trunk of men and legs of women. It is the most common form of cutaneous melanoma, occurring due to sun exposure and generally evolves from a precursor lesion [2, 7].

2. Nodular melanoma- affects the same sites as superficial spreading but the frequency of incidence is much lower. Nodular melanomas do not invade the epidermis in a radial fashion but more vertical growth is observed [2, 7].

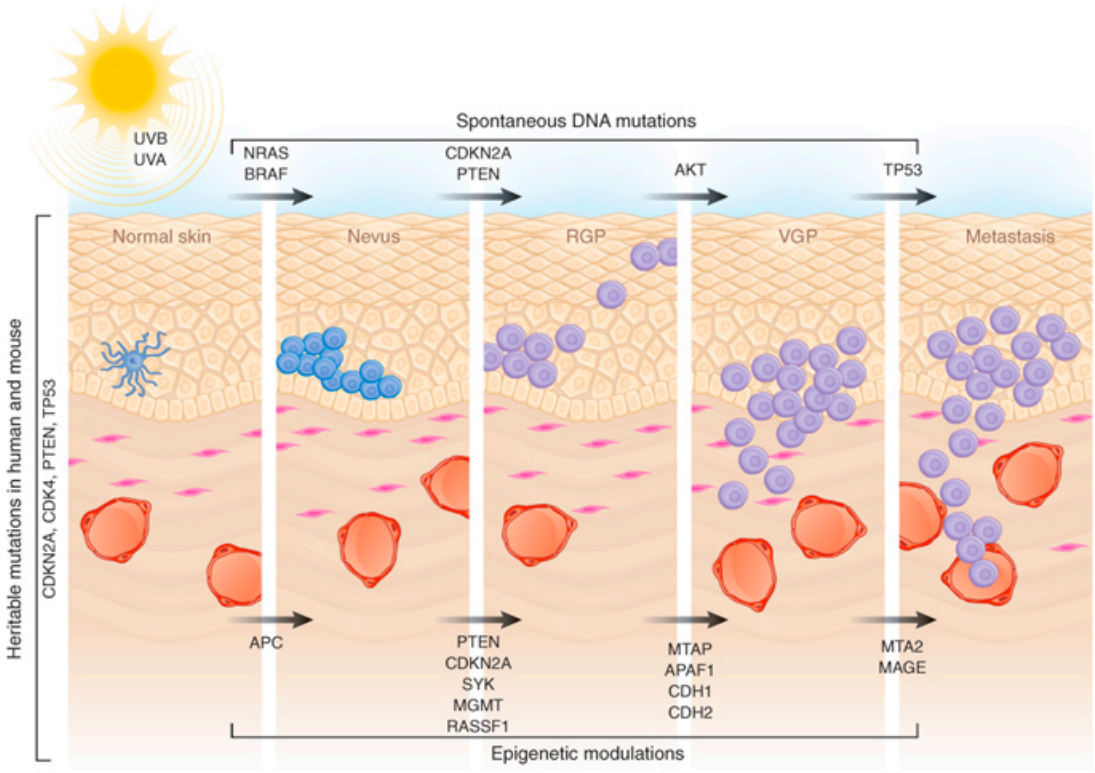


Figure 1.1 Proposed molecular changes in the progression of melanoma. Aberrant proliferation of normal melanocytes due to UV radiation or genetic mutations, results in benign nevi. Further mutations lead to the formation of RGP, where cells invade within the epidermis and epigenetic changes induce progression to VGP. Many spontaneous mutations and epigenetic modifications lead to metastatic progression. Figure adapted from Zaidi M R *et al*; Journal of Investigative Dermatology (2008) 128, 2381-2391.

3. Acral lentiginous melanoma- affects non-hair containing palms, soles and nails.

4. Lentigo maligna melanoma, arising from melanoma in situ, is commonly seen in the elderly, and is associated with chronic sun exposures but the prevalence is very low.

5. Noncutaneous melanoma is observed in the ocular and mucosal areas of patients. Exposure to sun is not a risk factor for non-cutaneous melanoma.

The Clark model (Figure 1.1) describes how melanocytes undergo various stages of malignant transformation to form a metastatic melanoma. The first noticeable change is the formation of benign nevus (atypical mole), which have uncontrolled yet limited growth of the melanocytes [8]. Most nevi remain dormant for decades; they have to free themselves from growth restraints to become malignant [8]. Dysplastic nevi having aberrant growth arise within and from the pre-existing nevus or in a completely new location. During the next step in progression, Radial Growth Phase (RGP), the cells proliferate in the epidermis without invading the dermis. RGP melanoma is metastatic incompetent and can be removed surgically [2, 8]. Some lesions can invade the epidermal layer to form nodules in the dermis, called as Vertical Growth Phase (RGP). In contrast to the RGP cells, VGP cells can form colonies in soft agar and can also from tumor nodules when implanted in nude mice [8]. VGP cells can also invade the local blood vessels, lymphatics and can also successfully proliferate in the secondary sites and form metastatic nodules, called as metastatic melanoma [2, 8]. As

melanoma progresses from RGP to VGP, treatment options and survival rates decrease [2, 8, 9]. Melanoma staging performed via histo-pathological analysis of the depth of local invasion, called Breslow index, also serves as a principal prognostic factor in melanoma progression.

Metastatic spread of cancer cells from the primary tumor is responsible for cancer patient morbidity and mortality. In cancer metastasis, a small percentage of cells that are released from the primary tumor ultimately form metastasis in distant sites. To metastasize to ectopic sites melanoma cell must have the ability to invade the extracellular matrix (ECM), one of the most important steps in cancer metastasis. Tumor cells have to breach the ECM in order to metastasize. As opposed to invasion as a single cell, in the absence of cell-cell junction melanoma cells invade as a group where cell-cell junctional proteins are retained [10, 11]. From the primary tumor site the cancer cells also have invade the collagen layers of the blood vessels, a process called intrasavation and also invade out from the blood vessels to form metastasis (extravasation) in the secondary site. In the blood vessels the cells have to survive is the absence of growth factors, another hallmark of cancer cells. Tumors cells must extravasate out of the blood stream, form metastasis in a favorable ectopic site. The choice of the secondary site is in accordance to the “seed soil hypothesis”, which proposes that the tumor cells colonize in the tissue which releases specific chemokines favorable for the tumor cells to form a niche. After favorable extravasation, the cancer cells form micrometastasis and proliferate further to form micrometastasis [12-14].

1.1.3 Environmental and genetic interactions

Sun ray sensitivity and exposure to ultraviolet radiation are risk factors in melanoma formation. Ultraviolet radiation, known to cause genetic changes in the skin architecture, elevates the risk of melanoma. Due to exposure of ultraviolet light, the cutaneous immune response is compromised, which generates reactive oxygen species that affect melanocytes. As a defensive mechanism, melanocytes synthesize melanin and transfer it to the keratinocytes, which absorb and dissipate the ultraviolet energy. Normally, after exposure to sun light, skin pigmentation increases because of the production of melanin by alpha melanocyte-stimulating hormone (alpha-MSH). MSH stimulates the melanocortin receptor1 present on the melanocytes resulting in the production of excess melanin and thus as a protective mechanism against ultraviolet light, the skin undergoes tanning. In the light-skinned population there is a germ-line polymorphism in the MC1R gene that impairs the activity of the receptor [15, 16]. All these factors lead to a genetic predisposition, which promotes the origin of melanoma.

Previous melanoma or family history of melanoma also are risk factors for melanoma. BRAF mutation is rare (7%) in all cancers but 50-60% of cutaneous melanoma patients have a V600E activating mutation patients. In the benign nevi stage (limited growth stage) BRAF is frequently mutated (80%) (Figure 1.1). Mutation in the BRAF gene results in the aberrant activation of the mitogen activated protein kinase (MAPK) pathway [2, 8, 17, 18]. Other genes that are commonly mutated in melanoma are the metastasis suppressor gene, cyclin-

dependent kinase inhibitor 2A (CDKN2A), activated NRAS mutations and microphthalima-associated transcription factor (MIFT) amplification/alterations, predisposing patients to the risk of melanoma. Tumor-suppressor gene, phosphatase and tensin homologue (PTEN) also is often inactivated in melanoma leading to phosphatidylinositol-3- kinase (PI3K)/AKT activation [2, 8]. In mucosal melanoma patients, c-Kit is frequently mutated [28]. In zebra-fish models, mutation of BRAF in combination with inactivation of tumor-suppressor gene p53 caused melanocytes to become malignant, where as in mouse models mutant BRAF cooperates with activation to PI3K/Akt and drive melanoma formation [2, 8, 21]. These sorts of mutations predispose certain populations to the risk of melanoma. Signaling pathways that are important in melanoma progression will be discussed in more detail in Chapter 1.1.4.

1.1.4 Growth factor signaling in melanoma progression

Activated growth factor signaling is critical in driving the process of oncogenesis in melanoma. RAS/RAF/MAPK and RAS/PI3K/AKT, signaling cascades in the Ras pathway is activated in melanoma. As discussed earlier, V600E BRAF is the driver mutation in over 50% of melanoma patients, while almost 20-30% of the melanomas have activating NRAS mutation [2, 8]. The RAS/PI3K/AKT pathway is regulated by PTEN, a negative regulator of PI3K signaling [2, 8, 21]. Almost 85% of melanoma cell lines have mutations in NRAS, BRAF and loss of PTEN is also detected in approximately 60% of primary melanoma as compared

to only 10% of nevi indicating that loss of PTEN can be a driving factor in the progression of melanoma [19, 20]. Mutant BRAF alone cannot drive the formation of melanoma in mouse models; loss of PTEN cooperates with mutant BRAF in melanocytes and drive melanoma formation [21]. The authors also show that selective inhibition of MEK1/2 by PD325901 and mTorc1 by Rapamycin respectively, prevented induction of melanoma by mutant BRAF [21], indicating that there is a cross-talk between signaling pathways that govern melanomagenesis.

In pursuit of new drugs to treat melanoma patients, research has been focused on identifying signaling pathways that are activated in melanoma. Melanoma cells resistant to BRAF inhibitors show upregulated EGFR-Src Family Kinase (SFK)-STAT3 signaling which promoted invasion, and metastasis in these resistant cells [22]. Vemurafenib resistant tumors showed increased EGFR and SFK activity and blocking SFK with dasatinib suppress the growth and metastasis of vemurafenib-resistant tumors [22]. Some of the resistance pathways include reactivated ERK signaling which are either Ras/Raf dependent, activating mutation in MEK, activation of MAPK signaling pathway by receptor tyrosine kinase and activated Fibroblast growth factor receptor -3 (FGFR-3)/Ras signaling pathway [23]. Identification of new targets and also combinatorial approaches are now on-going in clinical trials.

Targeting IGF1R in melanoma is also being considered as a therapeutic option. Insulin-like Growth Factor 1-Receptor (IGF1-R), which activates both the MAPK and PI3K pathway, is often overexpressed in many tumors including

melanoma. Activation of IGF-1R promotes growth of human melanoma cells in mice [24, 25]. Yeh *et al*., reported that inhibition of IGF1R signaling inhibits melanoma cell survival, induces apoptosis in both the wild type and mutant B-RAF cells, indicating that mutation of BRAF does not make melanoma cells resistant to IGF1-R targeting [26]. IGF-R/PI3K activity is upregulated in melanoma cells which are resistant to selective BRAF inhibitor and relapse patient samples have increased IGF-1R expression [27], indicating that inhibiting of both these pathways might be of therapeutic benefit.

Unresectable melanoma, known for being resistant to all known chemotherapies, metastasizes rapidly to ectopic sites. Targeting BRAF was considered a key discovery in treating melanoma patients but the quick emergence of BRAF-resistant patient population has dampened the possibility of treating metastatic melanoma with B-Raf inhibitors alone. There is an urgent need to identify signaling molecules that are activated in primary melanoma and cell lines and investigate how these proteins promote melanoma progression. This study will identify different mechanisms in melanoma progression in order to identify new drug targets.

1.2 Abelson kinases

1.2.1 Abl kinases: Structure and regulation

c-Abl and Arg (Abl-Related-Gene) are the two structurally homologous protein members of Abelson non-receptor tyrosine kinase family. c-Abl is essential for development, as c-Abl knockout mice are embryonic lethal [29,30], but Arg- deficient mice are born normal [31]. c-Abl and Arg double knockout mice are embryonic lethal and double knock out embryos have defects in neurulation [31]. The N-termini of c-Abl and Arg are highly homologous and consist of the kinase domain and Src homology (SH2 &SH3) domains (Figure 1.2). c-Abl and Arg both have F- and G-actin binding domains which assist in cytoskeletal reorganization and regulate membrane ruffling and motility [33]. Unlike Arg, c-Abl has 3 nuclear localization signals and DNA binding domains at the C-terminus [33, 34]. c-Abl has nuclear export signals, which helps it to shuttle between the nucleus and cytoplasm [32, 33], and acetylation and binding to 14-3-3 promotes its cytoplasmic retention. In response to DNA damage, 14-3-3 is phosphorylated by c-Jun N-terminal kinase (JNK), resulting in dissociation of the c-Abl/14-3-3 complex, and nuclear localization of c-Abl [32, 35].

Nuclear c-Abl promotes DNA damage-induced-apoptosis via a p53-mediated pathway [36]. In cancer cells, c-Abl binds and phosphorylates the oncoprotein, MUC-1. Phosphorylation of MUC1 inhibits the interaction of c-Abl with 14-3-3 and thus MUC1 permanently sequesters c-Abl in the cytoplasm, preventing the shuttling of c-Abl to nucleus following DNA damage. Unlike Arg, c-

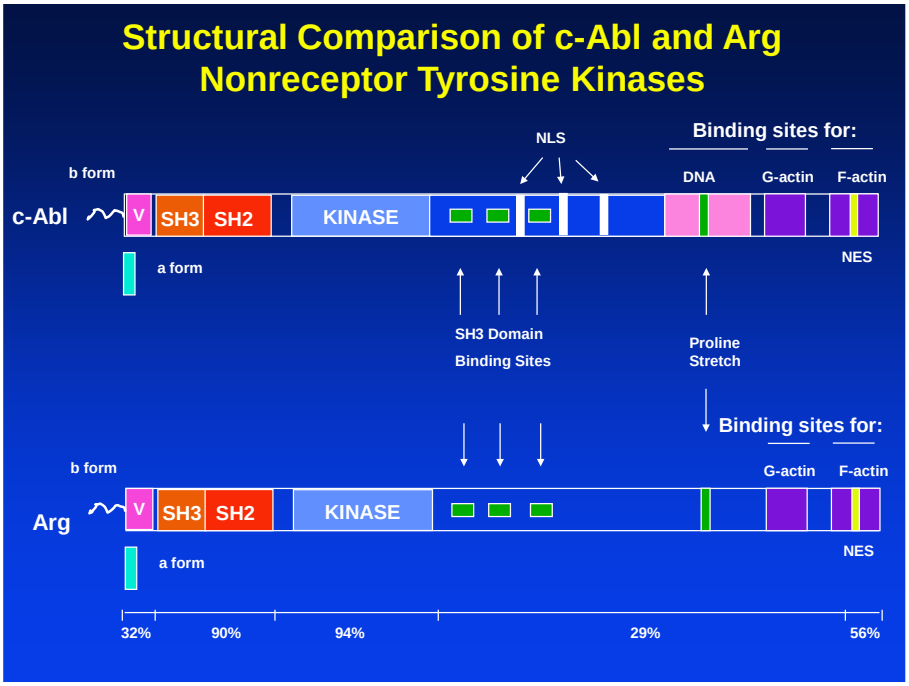


Figure 1.2: **Domain structure of Abl Kinases**. c-Abl and Arg contain highly conserved N- termini while the carboxy termini are more divergent. Unlike Arg, c-Abl have three nuclear localization signals (NLS), a nuclear export signal and a DNA-binding domain. c-Abl and Arg both have binding sites for F-actin and G-actin binding domains.

Abl is also found in the endoplasmic reticulum (ER) and mitochondria [33]. Ito Y *et al.,* have demonstrated that during ER stress, ER-localized c-Abl is targeted to the mitochondria. ER stress induces c-Abl dependent mitochondrial cytochrome c release and apoptosis [37].

Alternative splicing of the first exon generates Abl1a and 1b, the two isoforms of c-Abl in mammalian cells [38]. The isoform c-Abl1b is present ubiquitously in all cell types and carries a myristolate group at its N-terminus. Although this form is myristoylated, there is no evidence of palmitoylation or additional polybasic amino acids that can interact with the negatively charged phospholipids on the cytoplasmic side of plasma membrane. The absence of such sites confers weak binding of c-Abl1b to the plasma membrane and thus only a minor fraction of c-Abl 1b is seen in the membrane-proximal site [38]. The presence of the N-terminal myristoylated cap plays an important role in the downregulation of the kinase activity of c-Abl. The N-terminal cap binds to the C-lobe in the kinase domain of Abl, inducing a bend in the C-lobe, resulting in the docking of the SH2 domain onto the C-lobe and inducing an auto-inhibitory state [39]. Activation of c-Abl in the nucleus in response to ionizing radiation and genotoxic stress, in various cell types leads to apoptosis [30], while activated c-Abl in the cytoplasm induces proliferation and survival [33, 40].

1.2.2 Abl kinases: Activation and inhibition

Tyrosine phosphorylation of c-Abl in the kinase domain (Y412) and interlinker region (Y245) is required for the activation of c-Abl and both these sites are

phosphorylated by Src [33,40]. In fibroblasts, platelet derived growth factor (PDGF) activates Abl kinases in a Src-dependent manner, and once activated c-Abl induces membrane ruffling [40]. c-Abl is regulated in an autoinhibitory state by the interaction of its N-terminal cap, SH2 and SH3 domain to the kinase domain. In hematopoietic cells, the *Abl1* gene undergoes chromosomal translocation [t(9;22)] fusing with the BCR gene to form a mutant fusion protein, BCR-Abl. Fusion of BCR to c-Abl disrupts the N-terminal auto-inhibition, which leads to activation and oncogenic signaling[33,41-43]. BCR-Abl promotes the development of three different types of leukemia: chronic myelogenous leukemia (CML), acute myelogenous leukemia (AML) and chronic neutrophilic leukemia (CNL) depending of the break point of BCR. In another translocation event, *Abl1* and *Abl2* (Arg) forms fusion with the Tel gene (ETS family of transcription factor) to form Tel-Abl and Tel-Arg, which drive the development of myoproliferative diseases. A viral, oncogenic form of Abl, v-Abl, is constitutive active and transforms lymphoid cells [33]. These fusion proteins promote proliferation and inhibit apoptosis of the leukemic cells. Bcr-Abl also induces membrane ruffling and invasion of hematopoietic cells and its expression can solely lead to the development of leukemia in an animal model [33, 44].

Controlling Abl kinase signaling is important for inhibiting its oncogenic function. PAG/Msp2 and Abl interactor proteins (Abi-1 & 2) are potent Abl kinases inhibitors [33]. Pag binds to the c-Abl SH3 domain and inhibits the kinase activity of Abl (45). Abi-1 and Abi-2 activate the inhibitory function of the SH3

domain, a negative regulatory domain, and activated Abl kinases in-turn induce the proteosome-mediated degradation of Abi proteins [46, 47].

Imatinib mesylate (Gleevec), an ATP competitor inhibitor, targets the ATP binding domain in Abl kinases rendering them inactive [48, 49]. Imatinib also inhibits c-Kit and PDGF receptors [50]. Nilotinib, a second generation Abl kinase inhibitor with the same mechanism of action as imatinib, is more sensitive towards Abl kinases compared to its other targets (c-kit, PDGFR, DDR and CSF-1R) and thus shows greater promise in inducing remission of patients with CML [51]. Some drugs targeting Src family kinases (SFKs) (SKI-606/bosutinib, dasatinib) also inhibit Abl kinases [52, 53]. Some CML patients develop resistance to Abl inhibitors by developing a T315I mutation (gatekeeper), which renders BCR-Abl resistant to imatinib and nilotinib. Ponatinib, and DCC-2036 inhibit both the wild type and T315I gatekeeper mutation in mouse models and Phase 2 clinical trial with ponatinib show promising results against imatinib-resistant CML [38].

Many proteins are activated by Bcr-Abl and Abl kinases, among which the Crk family of adapter proteins are the most studied. Phosphorylation of CrkL is used as a read-out of c-Abl and Arg activities and CrkL binding to Bcr-Abl is necessary for oncogenic transformation. Another studied Bcr-Abl substrate is the transcription factor STAT5. Bcr-Abl phosphorylation of STAT5 is independent of the canonical upstream JAK2 kinase [38].

1.2.3 Activation of Abl kinases in solid tumors

To investigate the activation status and role of Abelson kinases in solid tumors, many researchers have examined whether Abl kinases are highly expressed in solid tumors. c-Abl and Arg expression was significantly increased in breast, infiltrating lobular and ductal carcinoma, ovarian serous carcinoma, gastric adenocarcinoma, colon, lung, brain, colon, rectal, ovarian, oral squamous carcinoma as compared to normal tissue or benign tumors [54]. However these studies utilized immunohistochemical methods, using c-Abl antibodies, without the presence of Abl knockout cells as proper control. c-Abl antibodies cross-react with a lot of other proteins; thus experiments were needed to show antibody specificity. Also, expression of Abl kinases does not necessarily correlate with their activation status due to tight regulation of the proteins [54]. Forced high levels of c-Abl induce its constitutive activation, but such mode of action has not been demonstrated in solid tumors. Immunohistochemical analyses using c-Abl and Arg antibodies does not allow for the examination of activation status of Abl kinases in solid tumors since specific phospho-Abl antibodies are not commercially available, as all the phospho- specific c-Abl antibodies cross react with phospho-EGFR and/or PDGFR [54]. These shortfalls have been overcome by our laboratory, which suggests that pCrk/CrkL can be used as a read out of Abl kinase activity as inhibition of Abl kinases with imatinib mesylate in many breast cancer and melanoma cell lines suppress pCrk/CrkL levels in a dose-dependent manner similar to silencing c-Abl and Arg [55].

Our laboratory was the first to show that Abl kinases are activated in solid tumors. We showed that Abl kinases are activated in breast cancer cell lines relative to human mammary epithelial cells by performing *in vitro* kinase assay, using GST-Crk as a substrate [55]. Using *in vitro* kinase assay, other labs have also shown that Abl kinases are activated in cancer cell lines [56,57]. Using kinase assays we also showed that activation of c-Abl/Arg did not correlate to the expression of Abl kinases in breast cancer cells, indicating that expression does not correlate with activation [55].

Using pharmacological and siRNA approaches our lab has previously showed that constitutive active IGF-1R, Her-2, EGFR and Src activate Abl kinases in melanoma and breast cancer cell lines, providing evidence that mutation or chromosomal translocation is not the mode of activation of Abl in solid tumors [55]. Human breast cancers have aberrant constitutively active EFGR family members [54, 55]. Abl kinases are activated by EGFR in breast cancer cells, the SH-2 domain of c-Abl and Arg bind to EGFR directly and the binding is EGF inducible [55]. Constitutively active SFKs also induce the activation of c-Abl in breast cancer cell lines [55]. Inhibition of RTK/SFK did not completely abolish c-Abl/Arg activities indicating that some other signaling events are also necessary for c-Abl/Arg activation [55]. Results from our lab also showed that PDGFR activates Abl kinases in glioblastoma cells and supporting studies by Furlan *et al.,* demonstrated that Abl kinases are activated downstream of c-Met in gastric and hepatocarcinoma cells [58].

1.2.4 Role of Abl kinases in solid tumor development and progression

Results from our laboratory provide evidence that c-Abl/ Arg activities were highest in triple-negative [Estrogen receptor negative (ER-), Progesterone receptor negative (PR-) and Her-2 negative] breast cancer cells (BT-549, MDA-MB-231 and MDA-MB-468), and Her-2^{+} breast cancer cell lines as compared to MCF-7 a non invasive, $ER^{+}/PR^{+}/Her\text{-}2^{-}$ cell line [55]. In the breast cancer panel cell lines tested (MDA-MB-468 and MDA-MB-231) Abl kinases were predominantly cytoplasmic [59], which is consistent with them having tumor promoting functions rather than suppressive functions. Triple-negative breast cancers are the most aggressive as compared to other breast cancers and patients harboring these tumors have the worst prognosis [60, 61]. Some triple-negative breast cancer cells, which are highly invasive and metastatic, have activated c-Abl/Arg. Activation of Abl/Arg promotes matrigel invasion, endothelial cell proliferation, angiogenesis and invadopodia formation of MDA-MB-231 cells [62]. Src was shown to activate Arg kinase activity, promote degradation of the extracellular matrix, and mediate invasion of MDA-MB-231 breast cancer cells [63]. Abl kinases also mediate anchorage-independent growth and proliferation of triple-negative, MDA-MB-468, MDA-MB-231 cells and proliferation in BT-549 cells [64]. Abl kinases promote matrix degradation, survival in response to serum deprivation and motility in triple negative breast cancer cells but no animal studies have been conducted to determine whether Abl kinases promote breast cancer metastasis. There also have been no studies directed to determine

whether Abl kinase activation is associated with grade or stage of breast cancer progression.

In human non-small lung cancer cells, FUS1, a tumor suppressor, inhibits c-Abl kinase activity. FUS1 expression is lost in primary lung cancers and the majority of small cell lung cancer and NSCLC tumor samples have been shown to have loss of FUS1 expression [57]. However, it is not clear whether Abl kinases might be activated via this mechanism in other type of cancers. In prostate cancer cells aberrant activation of Abl kinases by PDGFR induced cells survival by the inducing expression of the anti-apoptotic protein MCL-1 in a p68/β-catenin dependent manner [65]. This evidence points towards a role for Abl kinases in lung and prostate cancer progression.

Although the role of Abl kinases in many solid cancers is established, there is little information regarding whether Abl kinases are important in melanoma progression. MDA-MB-435s cells, originally thought to be of breast cancer origin, were recently identified as melanoma M14 cells [66]. Prior to its identification as a melanoma cell line, our lab utilized this cell line as a breast cancel model and showed that Abl kinases are activated downstream of receptor tyrosine kinases and promote matrigel-invasion and proliferation [55,64]. c-Abl/Arg also are activated in metastatic murine melanoma cells (B16F10) relative to its non-metastatic counterpart (B16F0), and likewise c-Abl/Arg promoted invasion and matrix degradation [62]. Inhibition of Abl kinases by imatinib repressed B16F10 tumor growth; and imatinib cooperated with dacarbazine to restrict B16F10 metastatic progression [67, 68]. Imatinib also inhibited

proliferation of melanoma cell lines expressing Abl kinases, c-Kit and/or PDGFR, but the authors did not examine the activities of c-Abl and Arg in these cells [69]. The above studies utilized murine melanoma cells not human melanoma cells and also none of the above studies other than those performed by our laboratory, addressed the dependence of these effects on c-Abl/Arg.

Inhibition of SFK by dasatinib, a drug that targets c-Abl and Arg and SFKs, in human melanoma cells, decreased migration, invasion and matrix metalloproteinase-9 expression; however the authors did not address whether the effects on invasion and migration were mediated by inhibition of Abl kinases by dasatinib [70]. Imatinib mesylate showed no effect on tumor growth of melanoma cells expressing PDGFRα and β injected subcutaneously in nude mice [71]. These results do not negate the hypothesis that active Abl kinases may promote melanoma progression and metastasis as none of these studies probed the activation status of Abl kinases nor did they investigate the effect of imatinib mesylate or nilotinib on metastasis.

To investigate the effects of Abl kinase inhibitors on metastasis, metastatic melanoma patients treated with imatinib mesylate for 2 weeks showed decreased expression of Abl, Arg,c-Kit and PDGFR in their tumor biopsies as compared to the starting time of enrollment in the study however the authors did not address the activity status of these molecules [72]. In a human uveal melanoma model, rabbits were injected with human uveal melanoma cells, treated with imatinib mesylate, primary tumor cells extracted and re-cultured. Cells from imatinib-treated animals had a lower proliferation rate and were less invasive as

compared to the controls. The imatinib mesylate-treated cells also had upregulated expression of SERPINB5, aa tumor suppressor gene, and KISS1, a metastasis suppressor gene as compared to the vehicle-treated cells. Although a direct correlation between Abl kinases and the metastasis suppressor gene was not investigated, it could be a plausible mechanism by which Abl kinases promote melanoma metastasis [73].

Although our lab previously described that Abl kinases promote melanoma cell proliferation and invasion, there are no reports that elucidate the mechanism by which Abl kinases promote melanoma cell invasion. In addition, although Abl kinases upregulate all the *in vitro* processes of cancer progression but there are no studies that test whether Abl kinases promote cancer metastasis. To validate that Abl kinase inhibitors can be used to treat melanoma patients, the mechanisms by which Abl kinases promote melanoma progression need to be identified. The current study will elucidate several mechanisms by which Abl kinases promote melanoma cell invasion and metastasis.

1.3 STAT3

1.3.1 Structure, activation and role in cancer progression

The signal transducers and activators of transcription factor (STAT) family of transcription factors are composed of seven members STAT1-4, STAT5a, 5b and STAT6. All the family members have six distinct structural domains, which are phosphorylated during activation of STATs by growth factor receptor tyrosine kinases (EGFR), cytokine receptor-associated Janus kinases (JAKs), and Src kinases. Generally STATs exist as monomers in the cytoplasm and upon phosphorylation form a STAT: STAT dimer via reciprocal phospho-Tyr (pTyr)-SH2 domain interactions. The dimer translocates to the nucleus and acts as a transcription factor inducing expression of various genes involved in proliferation, survival, invasion, differentiation, development and inflammation (Figure 1.3) [74,75].

STAT family member proteins do not all share the same function in the progression of cancer. STAT1 has been shown to have tumor suppressor functions [76] and STAT1 deficient mice are susceptible to infection with microbial pathogens and lack interferon responsiveness [76]. In contrast, one study showed that STAT1 has tumor promoting functions as silencing STAT1 in B16F1 cells led to a less aggressive tumor phenotype and decreased lung colonization of tumors cells [76]. These results indicate that the role of STAT1 in cancer progression might be tumor cell specific and more studies are needed to decipher the role of STAT1 in cancer progression. STAT5 another member of

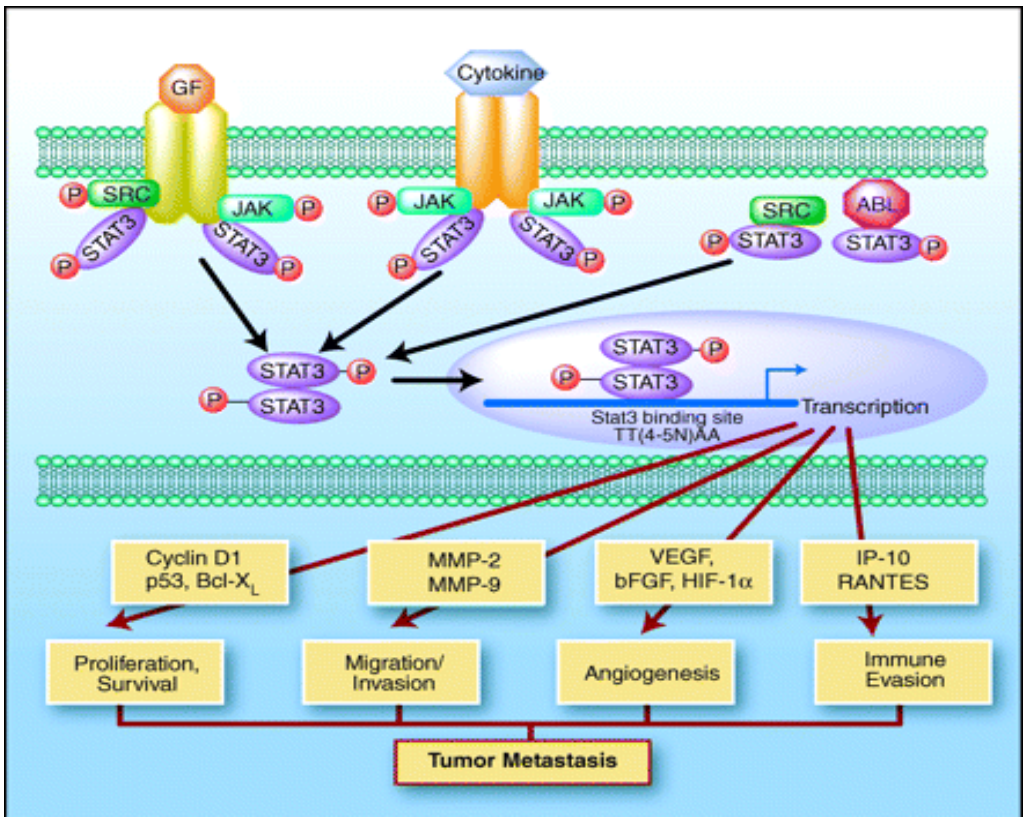


Figure 1.3 Activated STAT3 signaling promotes cancer metastasis. STAT3 is activated by growth factors, cytokines, Src and Abl kinases by phosphorylation of a tyrosine residue on STAT3. Phosphorylation of STAT3 results in its dimerization and translocation to the nucleus and binding to DNA in a sequence specific manner to upregulate genes involved in proliferation, survival, invasion and angiogenesis. Figure adapted from Suyun Huang, Clin Cancer Res 2007; 13:1362-1366 [75].

this family is reported to regulate the transcription of bcl-xl, mcl-1 and cyclin D1 in similar fashion as STAT3. STAT5 has been reported to promote the progression of CML, and myoproliferative diseases induced by TEL-JAK2, and BCR-Abl activates STAT5 [77]. Constitutive STAT5 signaling enhances squamous cell carcinoma invasion and tumor growth [78], indicating that STAT5 promotes cancer progression. A seminal study from Bromberg *et al.,* has showed that constitutively active STAT3 alone can transform immortalized fibroblasts and induce tumor formation in nude mice, establishing STAT3 as an oncogene [79]. In contrast to other STAT isoforms, loss of STAT3 from mice is embryonic lethal due to a defect in visceral endoderm function [77, 80].

STAT3 is activated by the cytokine, interleukin-6 and tyrosine kinases, like BCR-Abl, EGFR, PDGFR, FGFR and Src [77]. Unlike normal cells, many human cancers have constitutively active STAT3, which promotes invasion and metastasis of cancer cells [74, 76, 77, 80, 81]. Phosphorylation of tyrosine 705 and serine 727, in response to various cytokines and growth factors activates STAT3. Constitutive activation of STAT3 is associated with poor prognosis in many cancers [76, 81]. Activated STAT3 regulates the expression of genes which mediate proliferation (c-myc, Cyclin D1), inhibit apotosis (Bcl-xL, survivin), and promote invasion and metastasis (matrix metalloproteinases) [75, 76]. Constitutive activation of STAT3 also promotes Cyclin D1 upregulation in many forms of cancer [75, 76]

In the majority of human melanoma cells and in primary tumors, constitutive DNA-binding of STAT3 is reported whereas matched normal skin

specimens from the same patient lacked this activity indicating the presence of activated STAT3 in melanoma [82]. Using poorly immunogenic B16 melanoma tumor cell line, Niu G *et al.,* showed that injection of B16 cells harboring a dominant negative STAT3 in mice, lead to significant inhibition of tumor growth followed by tumor cell apoptosis. Dominant negative STAT3 cells are arrested in the G_0-G_1 phase of the cell cycle [86]. Inhibition of STAT3 signaling in melanoma cells also induces the release of TRAIL, a soluble factor which can promote apoptosis and cell cycle arrest [83]. p53, a commonly studied tumor suppressor, inhibits cell proliferation and induces apoptosis in cancer cells. p53 is often mutated in cancer and in large number of cancer cell lines, including melanoma [85]. Some cancers can progress without p53 mutation and some tumors have been detected with no p53 mutations but have lower expression of p53. This phenomenon was explained by the fact that STAT3 directly interacts with the p53 promoter and inhibits the transcriptional activity of p53. Blocking STAT3 activity in human melanoma cells induced p53-mediated cancer cells apoptosis [84]. These results indicate that activated STAT3 signaling is a negative prognostic factor in human cutaneous melanoma.

Accumulating evidence demonstrates that STAT3 has role in cancer cell invasion and metastasis [74, 75, 79, 81]. High STAT3 activation was reported in human brain melanoma specimens and constitutively activated STAT3 is required to form brain metastasis because of increased invasive potential of these cells conferred by STAT3 activation [87]. In melanoma cells, STAT3 promotes invasion by transcriptionally activating Matrix Metalloproteinases -2

(MMP-2). Blocking STAT3 activity in melanoma cells suppressed MMP-2 expression and downregulated invasion, and inhibited tumor growth and metastasis in nude mice [88]. STAT3 has also been reported to promote the expression of MMP-1 and 9 in human cancer cells [89, 90]. In pancreatic cancer, cells harbouring STAT3 shRNA had reduced tumor growth cell invasiveness, microvessel density and had reduced expression of collagen degrading enzyme, MMP-7 [91]. Furthermore, silencing STAT3 activity with a specific Janus Kinase inhibitor, AG490, repressed invasion and also led to decreased MMP-2 expression [92].

Proliferation and resistance to apoptosis in an intrinsic ability of cancer cells, and the ability to survive in close proximity of a capillary and to produce new blood vessels for survival is a must for cancer cells to metastasize. Cancer cells stimulate the formation of new blood vessels via a process called angiogenesis. STAT3 has been shown to directly promote transcription of vascular endothelial growth factor (VEGF), the most potent angiogenesis-inducing signal [93, 94]. Forced expression of a constitutively active form of STAT3 into B16 murine melanoma cells, upregulated the expression of VEGF and increased tumor vascularization when cell were injected *in vivo* [93]. STAT3 also is required for VEGF upregulation by Src, Her2/Neu and IL-6 [77, 93, 95]. To summarize these findings, constitutively active STAT3 promotes cancer cell survival, proliferation and invasion, which gives mechanistic insight as to how cancer cells can bypass programmed cell death, and promote invasion and metastasis.

Suppressor of cytokine signaling 3 (SOCS-3), a natural inhibitor of STAT3 signaling is negatively correlated with phospho-STAT3 levels in melanoma patents, also suggesting that deregulated STAT3 may play a significant role in the development of human cutaneous melanoma [96]. SHP-2 and PIAS-3, function as negative regulators of STAT3 signaling and are expressed at high levels in normal cells as compared to cancer cells [77, 97]. Curcumin, a proven anti-cancer agent, has been shown to inhibit STAT3 activity in ovarian cancer cells by upregulating the expression of PIAS-3 [98], and PIAS inhibits the DNA binding ability of STAT3. These results indicate that upregulating negative regulators of STAT3 signaling can be used to as approach to block activated STAT3 to prevent cancer progression.

1.3.2 Targeting STAT3 in solid tumors

Various approaches have been utilized to inhibit STAT3 signaling in cancer treatment. Inhibitors of STAT3, which target SH2 domain sequences and prevent dimerization of STAT3, inhibit malignant cell growth, increase apoptosis and decrease invasion in a wide variety of cancers such as breast, prostate, lung cancer and glioblastoma cell lines [74]. In addition, novel platinum inhibitors (IV) designed to target the DNA-binding domain of STAT3, have been shown to inhibit STAT3 dependent transcription of Cyclin D and Bcl-Xl and thus inhibit cell cycle progression and proliferation in breast cancer, prostate cancer and lung cancer cell lines [76]. A variety of plant polyphenols like resveratrol and circumin

have shown promise in blocking STAT3 signaling and inhibit cancer progression [76]. Moreover, FLLL32, a structural analogue of the natural product circumin, interacts with the SH2 domain of STAT3 and inhibits its phosphorylation and dimerization, inducing apoptosis and inhibiting VEGF production in human renal cell carcinoma cells [101]. Some small molecule STAT3 inhibitors (Static, S-3I-201) also have shown promise in inducing apoptosis in cancer cells. S-3I-201 inhibits the formation of STAT3:STAT3 complexes and STAT3s DNA binding and transcriptional activities, and inhibits anchorage independent growth, induces apoptosis, and xenograft regression of human breast cancer cells containing, activated STAT3 [102].

All these data establish STAT3 as a potential drug target in cancer patients. Our lab has shown that Abl kinases activate STAT3 in melanoma cells independent of Jak [64] however the mechanisms by which Abl kinases mediate STAT3 activation is unknown. This study will identify the mode of STAT3 activation by Abl kinases and also identify downstream genes affected by Abl kinase-mediated activation of STAT3 during melanoma invasion and metastasis.

1.4 Matrix Metalloproteinases

1.4.1 Structure and function

Matrix metalloproteinases (MMPs) aid in the degradation of ECM promote invasion and metastasis [103-105]. MMPs play a major role in ECM remodeling in a variety physiological progress including development, rheumatoid arthritis, pulmonary emphysema, and tumor invasion and metastasis [103-105]. Active MMPs are secreted from the stromal compartment surrounding the tumor (such as connective tissue, fibroblasts, endothelial cell, osteoblasts, macrophages and neutrophils), and cancer cells utilize these stromal-secreted MMPs to invade the matrix [104, 106]. MMP secretion by the tumor cells also promotes invasion and metastasis [104].

The MMP family consists of 26 different members which are zinc-dependent endopeptidases. Most MMPs have four distinct domains, consisting of the N-terminal pro-domain, catalytic domain, hinge region and the C-terminal hemopexin-like domain (Figure 1.4). Among the 26 MMPs, 6 are membrane-type MMP (MT-MMPs) which have additional transmembrane domain which anchors them to the cell surface [104,107,108]. MMP-7 is the smallest member of the MMP family, as it lacks a C-terminal domain. The propeptide domain contains a highly conserved sequence PRCGxPD, and the cysteine in this sequence forms a covalent bond with a catalytic zinc ion, maintaining the pro-form in a latent form [112]. A proline rich hinge region links the catalytic domain to the C-terminal hemopexin domain [104, 107,108] as depicted in Figure 1.4. MMPs are secreted

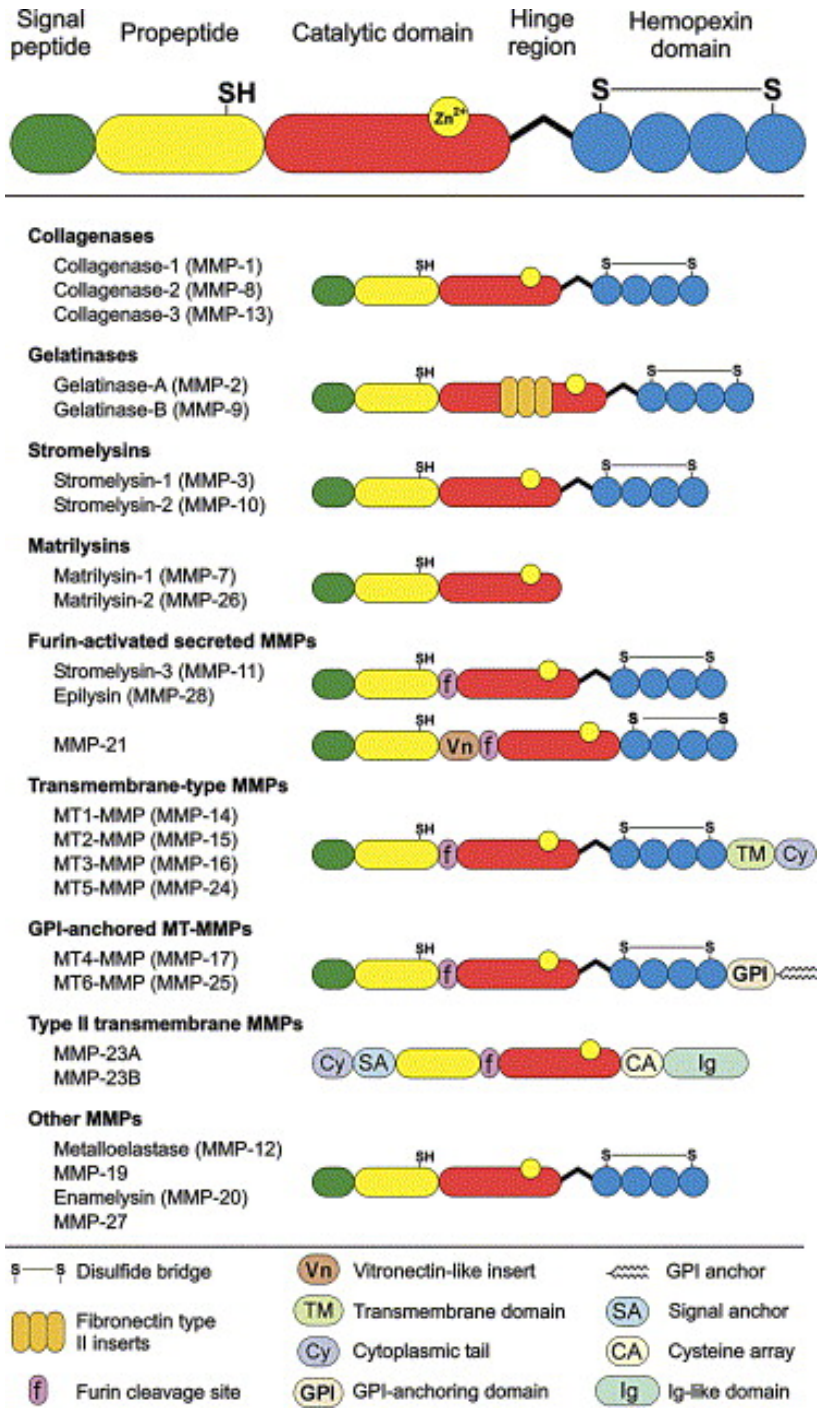


Figure1.4: Domain structures of human MMPs (Adapted from Risto Al-aho and Veli-Matti Kahari, Biochemi 87(3-4) 2005

in inactive forms and are activated by serine proteases like plasmin as well as by other MMPs in the extracellular environment [104,110,111]. Under normal physiological conditions, MMP activity is controlled either by endogenous MMP inhibitors (TIMPs) or via transcriptional regulation [108,110,112].MMPs have substrate selectivity for various matrix proteins. Collagenases (MMP-1, -3 and -13) cleave fibrillar collagen of type I, II, III, V and lamin. Stromelysins (MMP-3 and 10) degrade collagen II, III, IV, IX, fibronectin, laminin and elastin and also activate MMP-1 and 7 [104, 105, 107, 112]. Gelatinases, consisting of MMP-2 and 9 can degrade components of the matrix membrane like gelatin and collagen IV, and thus, are widely studied for their role in tumor progression. Melanoma cells injected in mice deficient in gelatinase-A (MMP-2) showed reduced tumor progression [113].

1.4.2 Role of MMPs in cancer invasion and metastasis

The presence or elevated protein levels and transcript of MMP-1, 2, 3, 7,9,14, in both primary tumors and/or metastasis, positively correlate with tumor progression and poor disease prognosis in pancreatic, breast, cervical, colorectal and melanoma [105, 108,100,111,114]. There also is a significant elevation of MMP levels in metastatic nodules as compared to primary tumors or tumor cells in cultures [105]. Naglich J *et al.,* reported inhibition of tumor growth in a murine, B16BL6 model of experimental metastasis by a MMP-1, 2, 7 9 and 14 potent inhibitor BMS-255291, indicating that MMP activities promote melanoma invasion and metastasis. Increased expression of MMP-2 and 9 also has been positively

correlated with increased melanoma invasion and progression, [115] however MMP-1 has also been identified as one of the most highly upregulated MMPs in a variety of cancers [105,110]. MMP-1 degrades collagen I, the main component of ECM and is regarded a prognostic marker for melanoma and breast cancer [116,117].

Previously, it was widely accepted that MMP-1 was secreted by the stromal cells surrounding the tumor cells but in the last decade experiments have shown expression of MMP-1 by the cancer cells themselves as well, indicating a role for stromal and tumor-secreted MMP-1 in tumor progression [105,118]. Expression of MMP-1 in cancer cells as detected by immunohistochemistical methods has been associated with a poor prognosis of colorectal carcinoma patients [119]. Patients with MMP-1 -positive metastases had significantly shorter disease-free survival than patients with MMP-1 negative metastasis and patients with MMP-3-positive metastasis also had the same out come as MMP-1 positive metastasis patients [117]. Furthermore, MMP-1 levels increase during colon cancer progression and higher levels are associated with shorter disease free survival [120]. Boire *et al.,* showed that fibroblast-produced MMP-1 activated the G-protein coupled Protease Activated Receptor (PAR-1) on breast cancer cells to promote invasion and metastasis [121]. These results indicate that MMP-1 play an important role in cancer progression. Elevated expression of MMP-1 and MMP-13 is associated with increased invasion of primary melanomas [122]. shRNA mediated inhibition of MMP-1 reduced metastasis of melanoma cells to the lungs, but did not affect primary tumor growth. Tumor cells expressing MMP-

1 shRNA also showed marked reduction in collagenase activity and angiogenesis [123]. MMP-1 is only expressed in VGP melanoma, its activity promotes melanoma invasion and metastasis and expression of MMP-1 can convert RGP melanoma to VGP [123-125]. PAR-1 is expressed in VGP melanoma and MMP-1 activates PAR-1 in an autocrine signaling manner in melanoma cells and upregulates tumor cell invasion [124]. Summarizing these results, MMP-1 plays an important role in melanoma, breast and colon cancer progression.

MMP-14, also known as MT1-MMP, another extensively studied MMP, is over expressed in many tumor types and is a marker for poor patient prognosis [105, 126,127] MMP-14 degrades interstitial collagens, such as type I, which is highly resistant to proteolysis due to its complex helical structure [129-131]. Mice deficient in MMP-14 show severe growth defects like dwarfism, and connective tissue disease due to the inability to degrade and process intestinal collagen required in bone and soft tissue formation [132]. A selective MMP-14 peptide-inhibitor effectively inhibits migration and invasion of melanoma, or tongue carcinoma cells and also increases the survival of tumor bearing mice [133]. Along these same lines Devy L *et al*. showed that DX-2400, a selective antibody inhibitor to MMP-14, inhibited angiogenesis, slowed breast cancer tumor progression and metastasis by inhibiting proteolysis and also blocked proMMP-2 processing [134]. MMP-14 cleaves pro-MMP-2 to activate MMP-2 [105,107]. Moreover Src kinases phosphorylate MMP-14 on Tyr573, and this phosphorylation event is essential for tumor cell invasion of 3D collagen matrices

and impaired tyrosine phosphorylation completely inhibits tumor growth in mice [128]. These results indicate a role for MMP-14 in cancer progression.

In summary, MMPs play an important role in cancer progression, and specifically in melanoma progression. Prior to our studies no one has linked activation of Abl kinases to MMP expression. This study will give mechanistic insights on how Abl kinases promotes melanoma invasion by demonstrating that they increase MMP expression.

1.4.3 TIMPs

The TIMP family (TIMP-1-4), which initially were thought primarily to be only endogenous inhibitors of MMPs, bind to MMPs in a 1:1 stiochiometric ratio to inhibit the matrix degradative function of MMPs [106]. However, in recent years it has become clear that TIMP-1 has a pro-metastatic function as it is over-expressed in lung, ovarian, breast cancer and this is correlated with decreased survival and a poor clinical outcome [135]. These findings suggest that TIMP-1 may play a positive role in tumor progression depending on the tumor environment or the TIMP-1 expression level in the tumor cell. TIMP-1 levels positively correlate with melanoma cell migration rate [136] and TIMP-1 levels are higher in unresected stage IV melanoma patients as compared to patients with resected stage I/II disease [137]. Moreover, TIMP-1 overexpression in murine melanoma cells decreased tumor latency and increased tumor volume and metastatic potential [138]. Furthermore, TIMP-1 expression is elevated in metastatic melanoma as compared to primary melanoma samples, [138]

indicating that TIMP-1 may exert a metastasis stimulatory function rather than a metastasis inhibitory function.

The TIMP-1 literature is very contradictory as TIMP-1 has been shown to induce proliferation in lung and breast carcinoma cells, [135,139] whereas in MCF-10A, breast epithelial cells, TIMP-1 inhibits cell growth by stabilizing the cells cycle regulatory protein, p27 [140]. TIMP-1 also has anti-apoptotic effects independent of its ability to inhibit MMPs [135]. The growth stimulatory function of TIMP-1 is dependent on TIMP-1- mediated stimulation of the MEK/ERK and p38 kinase pathway [135,141] .TIMP-1 induces the phosphorylation of Akt in breast carcinoma T47D and MDA-MB-231 cell lines [141,142], indicating that it can induce activation of the Akt/Bad/Bcl-2 cell-survival pathway and thereby prevent activation of the caspase cascade. TIMP-1 protects MCF-10A cells from apoptosis induced by TRAIL, radiation, cell detachment, serum starvation and chemotherapy treatment by upregulating the FAK/PI3K pathway [143,144].

Not much is known whether or not TIMP-1 has a metastasis-promoting role in melanoma and the mechanism by which TIMP-1 transcription and expression is upregulated. This study will shed light on how TIMP-1 expression is regulated in melanoma cells.

1.5 NM23

1.5.1 Metastasis suppressor genes

A classical metastasis suppressor gene is one that can suppress the cascade of metastasis without effecting tumor growth rate. In 1988 seminal experiments by Steeg *et al* identified NM23-H1 as a novel metastasis suppressor gene by colony hybridization studies. They injected seven cell lines derived from a K-1735 murine melanoma cell line in syngenic and nude mice and noticed that all formed primary tumors but some cell lines had less noticeable metastasis as an end point. Using differential gene expression studies, they found that NM23 was highly expressed in the cell lines with lesser metastasis as compared to the ones which had more metastatic nodules. Re-expression of NM23-H1 suppressed metastasis without effecting tumor growth in mice [145-149]. Moreover hepatocarcinoma cells injected in murine homologue of NM23-H1, NM23-M1 knockout mice, did not show any change in primary tumor size but had a significant increase in metastatic burden [150].

Even though a negative correlation is observed with NM23-H1 expression and metastatic capacity in melanoma, breast, gastric carcinoma and colon cells [147-149, 151], there is a positive correlation in testicular, thyroid, prostate and renal carcinoma, indicating that NM23-H1 might play different roles in different tissue types [151]. Also, NM23-H1 is dramatically overexpressed in primary tumors as compared to the late metastatic stages, indicating a dual role of NM23 in cancer progression [151]. More studies are needed to understand this dual behavior of NM23-H1 in cancer.

More recently, studies have focused on identifying other metastasis suppressors, leading to the isolation of MMK4, KAI1, KISS1, CRSP3 and VDUP1. Metastasis suppressors affect many aspects of the metastasis cascade including invasion, cell-cell communication, growth-factor-receptor signaling and transcription [152]. KAI1, a metastasis suppressor found in prostate and breast carcinoma attenuates EGF-induced migration [153-155]. MKK4, another example of metastasis suppressor in prostate and ovarian cancer, suppressed invasion of ovarian cancer cells by inactivating EMT via the downregulation of phosphorylated NF-κB, Twist and upregulation of E-cadherin [156].

1.5.2 NM23-H1 inhibits cancer progression

To date, ten different human NM23 members (NM23-H1-10) have been reported but among them NM23-H1 and H2 are abundantly expressed and widely studied in terms of their roles in attenuating cancer progression [148], NM23-H1 and H2 are highly homologus and share 88% amino acid identity, but have distinct functions . NM23-H1 possesses three enzymatic activities *in vitro*: 1). Nucleotide diphosphate kinase (NDPK) activity; 2). Histidine protein kinase activity and 3) 3'-5' exonuclease activity [157]. In breast carcinoma histidine kinase activity was shown to be essential for suppressing metastasis and also 3'-5' exonucleases confer proofreading function in DNA replication and repair. Recently Zhang *et al* established that disrupting the exonuclease activity of NM23-H1 inhibits the metastasis suppressor function of NM23-H1 in melanoma cells [158].

NM23-H1 silencing disrupted the normal epithelioid clustering of human liver cancer cells by altering E-Cadherin/ catenin- mediated intracellular adhesion and inhibition of NM23-H1 promotes migration and invasion in cancer cells [159]. NM23-H1 expression is inversely correlated with LPA receptor EDG2 expression, and overexpression of EDG2 restored motility in NM23-H1-overexpressing breast cancer cells [160]. Overexpression of NM23-H1 also partially abolished cell motility of MDA-MB-435 breast cancer cells towards chemoattractants such as serum, IGF and PDGF and also attenuated anchorage-independent growth [159-163. Similar effects of NM23-H1 have been reported in breast cancer, colon and prostate cancer cells [149,160]. NM23-H1 also plays a significant role in suppressing *in vitro* cancer cell invasion through Matrigel components. Silencing NM23-H1 increased the ability of human liver cancer cells to degrade the type I collagen matrix and invade through gelatin and matrigel [159,160].

ER-α-positive breast cancer cells express more NM23-H1 as compared to ER-α-negative cell lines, [164,165] indicating that NM23-H1 could be activated by Estrogen or its receptor. NM23-H1 interacted directly with ER-α and altered estrogen-induced gene transcription [164]. NM23-H1 has been reported to transcriptionally repress PDGF-A promoter activity in lung carcinoma cells [166], indicating that NM23-H1 can alternatively bind to promoters of oncogenes and repress their metastatic activity. These results indicate that silencing NM23-H1 promotes invasive capacity of cancer cells by downregulating several metastasis-associated genes.

Since the discovery of NM23-H1 and its anti-metastatic capacity research has focused on determining out how this gene could be re-expressed to block the metastasis cascade. Many compounds like estradiol and idomethacin elevated NM23-H1 expression in non-metastatic to low metastatic breast cancer cells but failed to increase NM23-H1 expression in metastatic cells [165,167]. Medroxyprogesterone acetate (MPA), a glucocorticoid receptor agonist increased NM23-H1 expression, by elevating the NM23-H1 promoter activity, in progesterone receptor-negative and glucocorticoid receptor-positive, MDA-MB-231 and MDA-MB-435 breast cancer cells [168]. Treatment with MPA suppressed anchorage independent growth of breast cancer cells and also reduced the metastatic colonization of breast cancer cells in mice [168,169]. As an alternate approach, gene therapy was tested to elevate NM23-H1 levels. Using an adeno-associated virus in an ovarian cancer mouse model, Li *et al.,* demonstrated that efficient (95%) delivery of the NM23-H1 gene resulted in the reduction of the number of animals having detectable lung colonization and a 35 day increase in the median survival time as compared to the control group [170]. Clinical trials based on the hormonal activation of NM23-H1 are also in progress [171]. In a recent study, Lim *et al*., demonstrated that the NM23-H1 protein coupled to a macromolecule transduction domain (MTD), which promotes protein uptake by cultured cells, inhibits the metastatic phenotype of breast, melanoma and lung cancer cells lines. Furthermore, systemic delivery of NM23-MTD inhibited lung metastasis and also eliminated previously established pulmonary

metastasis in mice [172]. Summarizing these results, NM23-H1 inhibits metastatic capability of various cancers.

1.5.3 NM23-H1 and endocytosis

In MDCK cells, NM23-H1 plays an important role in endocytosis of different proteins. NM23-H1 is recruited to the adherens junction by ARF6 GTPase, resulting in endocytosis of E-Cadherin, adherens junction disassembly and downregulation of Rac-1 GTP activity [173]. Drosophila NM23, *abnormal wing disc* (awd) promotes synaptic vesicle internalization by activating dynamin [174]. In another study Dammai V *et al*., showed that awd and dynamin are recruited to the cell membrane, endocytose FGFR, and modulate RTK signaling to regulate programmed epithelial cell migration in Drosophila trachea [175]. Additionally in Dictyostelium, NM23-H1 reduces both macropinocytosis and exocytosis, thus increasing the longer residence time of these vesicles and thus more complete digestion of the nutrients present in the vesicles [176]. These data demonstrate that NM23-H1 aids endocytosis of various oncoproteins and thus provide one plausible mechanism for its metastasis suppressor function.

Although, it is well established that NM23-H1 acts a metastasis suppressor in melanoma and many other cancers, but there is no explanation for how this protein is lost during in late stages of cancer progression. Mechanistic studies are needed to demonstrate the mechanism by which this protein is degraded in highly aggressive and metastatic human cancer cells.

1.6 Cathepsin Lysosomal Proteases

1.6.1 Activation and processing of cathepsins

The class of cysteine lysosomal proteases is composed of eleven family members, all of which share a conserved active site formed by cysteine and histidine residues. These proteases called cathepsins are secreted into the extracellular milieu by tumor cells and aid in cancer progression [177]. The majority of cathepsins are ubiquitously expressed in human tissue and play important roles in protein degradation and turn over [178]. Cathepsins are synthesized as inactive precursors. The signal peptide is cleaved in the endoplasmic reticulum, the protein transported to the Golgi, and in the Golgi the mannose residues are phosphorylated. Cathepsins enter the endocytic pathway by binding to the mannose-6-phosphate (M6P) receptors on the surface of endosomes. In endosomes, cathepsins are cleaved into a single chain form and then further cleaved into an active double chain form in the acidic lysosomes (Figure 1.5) [178-181]. The lysosomal localization of cathepsins requires it to be active in a slightly acidic pH. Cathepsins are inactivated at neutral pH, which protects the neutral pH cytosol from accidental release of cathepsins [182]. Excess cathepsins that do not bind to the M6P receptor are exocytosed outside the cell.

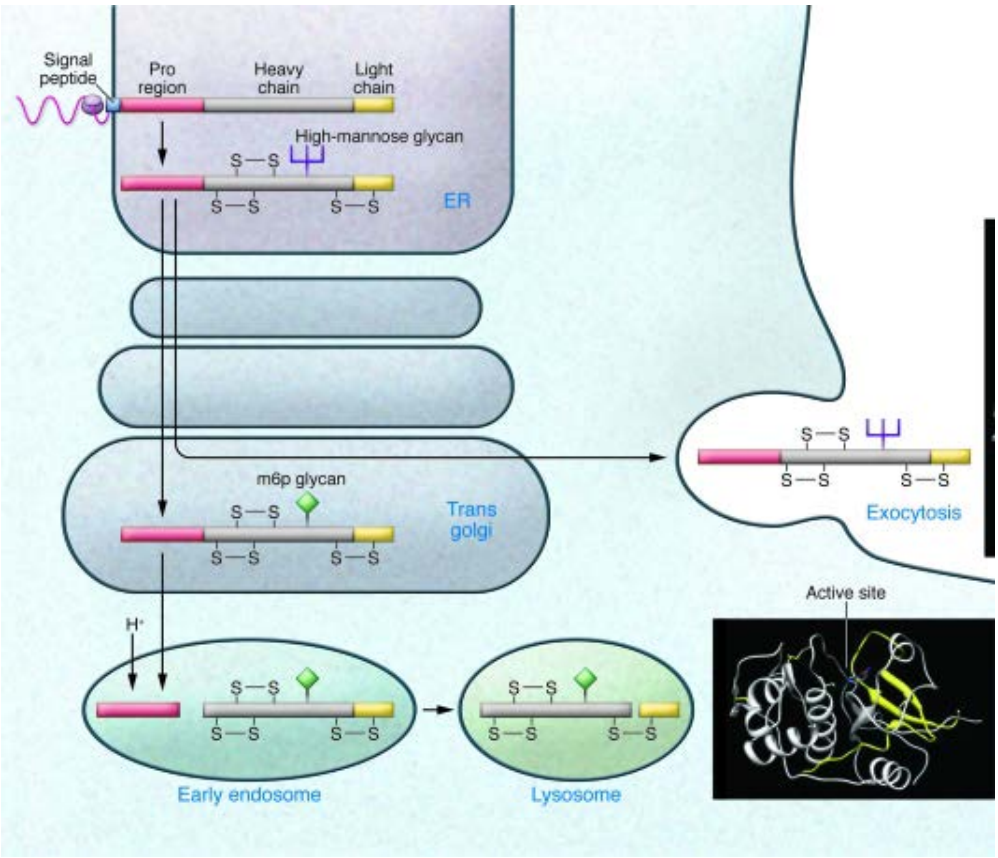


Figure 1.5: Cathepsin structure and trafficking. The signal peptide in blue is cleaved in the endoplasmic reticulum (ER), resulting in protein folding. In the Golgi, the mannose residues are phosphorylated and form M6P, a rate-limiting step. By binding to the M6P receptors on the surface of endosomes, cathepsins enter the endocytic pathway and proteins that are not converted to the M6P form are exocytosed. These extracellular cathepsins play an important role in invasion and metastasis during cancer progression. The cathepsins in the endocytic pathway are cleaved into a single-chain form in the late endosome, and further cleaved in the acidic lysosomes to form heavy and light chain forms. Figure adapted from Reisier J *et al.,* J Clin Invest 2010; 3421-3431

1.6.2 Physiological and cellular function of cathepsins

All cathepsin family members aid in protein degradation in the lysosomes. One biological/physiological function of cathepsins is to promote apoptosis by promoting protein degradation [179,180,182, 183]. Cathepsin B has been shown to induce hepatocyte apoptosis via a caspase-8-dependent process and cathepsin B knockout mice have reduced hepatocyte apoptosis [184]. In addition, cathepsin B and L double knockout mice display a significant increase in apoptotic neuronal cells in the brain [185], indicating that these two proteases may compensate for each other *in vivo.* Cathepsin B has been shown to activate apoptosis in hepatocytes, neuronal cells, and immune cells. During apoptosis cathepsins are released from the lysosomes into the cytoplasm, where they activate apoptotic pathways by cleaving the anti-apoptotic proteins Bid and Bcl-2, thereby increasing the release of cytochrome C and activating caspases-8, caspases-9 and caspases-3 [183, 186, 187]. Although there is a pleothora of data, indicating that cathepsins can mediate caspase-dependent apoptosis, other evidence suggests that cathepsins have anti-apoptotic functions. For example, forced expression of cathepsin B has shown to protect cells from serum deprivation-induced apoptotic death [188]. Furthermore, treatment with an inhibitor of cysteine cathepsins was shown to promote apoptosis in cancer cells [189]. Thus in some instances (perhaps in normal cells), cathepsin activation promote apoptosis, while in other instances they prevent apoptosis. This rest of this chapter will focus on the role of cathepsins during cancer invasion and metastasis.

Increased cathepsin expression correlates with a poor prognosis in breast, lung, ovarian, melanoma, and many other forms of cancer, and is associated with a shorter survival in breast, colorectal, and head and neck carcinoma patients [180,182]. Cathepsin upregulation has documented in many forms of cancer such as breast, lung, gastrointestinal, colorectal, and melanoma, indicating they have a role in invasion and metastasis [180,182]. Cathepsin upregulation can be attributed to various mechanisms such as like gene amplification, presence of transcript variants due to the presence of alternative promoters, and alternative splicing. Transcriptional regulation of cathepsins, such as via increased expression of transcription factors like Ets, Sp1, and Sp3, also promotes cathepsin overexpression in tumor cells. Post-translation modifications due to changes in mRNA stability also can increase the half-life of cathepsins, leading to overexpression [190]. Another potential mode of increased cathepsin expression in tumors is via downregulation of cystatin and serpins, endogenous inhibitors of cathepsins [190]. In malignant cells, cathepsin expression can also be upregulated by preferential overexpression of a splice variant with high efficiency of translation [190]. Cancer cells upregulate cathepsins via any of these aforementioned processes, and cathepsin overexpression promotes cancer progression.

Excess cathepsins, which are secreted by tumor cells, aid in degradation of extracellular matrix proteins, activate proteases (MMP), and inactivate TIMPs, thereby promoting angiogenesis, invasion, and metastasis [178, 182, 190]. Extracellular cathepsins also promote invasion by degrading collagen. In the

extracellular media the process that leads to activation of cathepsins are not well understood. It is reported that procathepsin L is activated by heparan sulphate, although activated cathepsins are also secreted by tumor cells [182,190,191]. Like MMPs, cathepsins can also activate each other in the extracellular milieu. Inactive precursor forms of cathepsins also aid in the invasion of cancer cells [190]. Expression of cathepsin L is elevated in the sera of metastatic melanoma patients as compared to normal healthy population [192]. Secreted cathepsin L promotes invasion, melanoma tumor growth and metastasis in nude mice [193]. Inhibition of cathepsin L reduces migration and invasion of melanoma cells, and forced expression of cathepsin L in low tumorigenic and non-metastatic melanoma cells confers metastatic abilities, indicating that cathepsin L activity is required for melanoma metastasis [194,195]. These studies assume that the invasion and metastasis-promoting role of cathepsin L is due to its secretion; however, these studies did not exclude possibility that intracellular cathepsins also are involved.

Cathepsin B also promotes invasion and metastasis of cancer cells. Inhibition of cathepsin B delays lung metastasis in a MMTV- PyMT-induced transgenic mammary carcinoma model reduces collagen I degradation and inhibits bone metastasis of breast cancer cells [196-198]. Extracellular cathepsin B also has been shown to increase breast cancer cell invasion by promoting extracellular matrix degradation [199]. Although, the role of extracellular cathepsins in promoting cancer cell invasion is widely studied, less is known regarding how intracellular cathepsins promote invasion

Lysosomal cysteine cathepsins have been shown to cleave proteins such as collagen, laminin, a type IV collagen intracellularly, indicating a role for intracellular cathepsin in cancer cell invasion [200-203, 207]. Invasive Ras transformed MCF-10A, engulf collagen-IV, in vesicle-like structures and degrade collagen intracellularly, in a cathepsin-dependent manner, thereby promoting invasion [202]. Endothelial cells also endocytose and degrade collagen IV intracelluarly in a cathepsin B-dependent manner, demonstrating its role in neovascularization process [203]. Intracellular cathepsins also may promote invasion and metastasis by cleaving the cell adhesion protein E-cadherin and dynamin, which has a role in endocytosis [179,204]. Like cathepsin B, inhibition of intracellular cathepsin L has been shown to repress carcinoma cell invasion and metastasis (Lewis Lung cells). Furthermore, inhibition of intracellular cathepsin L activation has shown to sequester IGF-1R in a sub-cellular compartment and reduce its expression at the plasma membrane, resulting in reduced metastatic propensity of Lewis Lung carcinoma cells, indicating that IGF-1R signaling is mediated by the expression of cathepsin L [205, 206].

In Ras-transformed MCF-10A cells, inhibition of intracellular cathepsin B also was shown to repress matrigel invasion. Inhibition of extracellular cathepsins in these cells blocked invasion to same levels as blocking intracellular invasion, indicating that, like extracellular cathepsins, intracellular cathepsins plays an equally important role in invasion [207].

Several studies have shown that both the intracellular and extracellular cathepsins promote invasion, indicating that the secretion of active enzyme and

cathepsin-mediated intracellular degradation pathways are important [182]. However, while the role of extracellular cathepsins in promoting cancer cell invasion is widely studied, less is known regarding how intracellular cathepsins promote invasion. This project will determine the mechanism by which Abl kinases upregulate intracellular cathepsin B and L to promote invasion and metastasis in melanoma.

1.7 Project Objectives and Aims

Previous studies from our lab and others have established that Abl kinases play an important role in breast cancer invasion; however, there are no studies to determine whether Abl kinases have a role in melanoma progression. Although Abl kinases are shown to promote melanoma invasion, the mechanism of action has not been unexplored. This study will identify mechanisms by which Abl kinases promote melanoma invasion and investigate whether Abl kinases promote melanoma metastasis. Two aims are intended to determine the role of Abl kinases in melanoma progression:

1. **Specific Aim 1**: Determine whether c-Abl and Arg are activated in melanoma cell lines and in primary melanomas and whether they promote proliferation and/or survival under nutrient deprivation conditions. We will ascertain downstream effector proteins, which mediate c-Abl and Arg effects on melanoma survival, invasion and metastasis (Chapter Two).

2. **Specific Aim 2:** In parallel with the protein pathways investigated in Specific Aim 1, we will also evaluate whether c-Abl and Arg mediate melanoma invasion and metastasis via the downregulation of a metastasis suppressor protein. We will also investigate the mechanism of c-Abl and Arg-mediated degradation of the metastasis suppressor (Chapter Three).

CHAPTER 2: c-Abl and Arg Are Activated In Human Primary Melanomas, Promote Melanoma Cell Invasion, Single-Cell 3D Invasion, Proliferation, Survival and Drive Metastatic Progression

2.1 Introduction

Metastatic melanoma is an uncurable disease as known chemotherapeutic agents have a 5% success rate or do not extend survival beyond 10 months indicating a dire need for development for new therapies [1-3]. For the development of new therapies, identification of new targets are necessary. Data from our lab and others have shown that Abl kinases are activated in breast cancer cells and also in lung cancer cells [55]. In solid tumors, Abl kinases are activated by receptor tyrosine kinases like PDGFR and EGFR in contrast to the chromosomal translocation event found in leukemia, which renders constitutive activation of Abl kinases; however the role of Abl kinases in melanoma progression has yet to be identified [55].

MMP's are known to play an important role in invasion of cancer cells by degrading the extracellular matrix and several transcription factors have known MMP promoter binding sites [215-216]. These transcription factors modulate the function of several MMP's and in-turn promotes invasion and metastasis of cancer cells. MMP-1, 3 and 14 activities are elevated in several human tumors and their expression is negatively correlated with patient survival [103-105, 114]. It is known that MMP-1 expression induces VGP expression in melanoma cells and acquisition of the VGP phenotype is essential for melanoma metastasis

[123-125].v-Src upregulates MMP-1 transcription in rabbit synovial fibroblasts [208], and Src is also known to activate Abl kinases [55] indicating that Abl kinases might also induce the transcription of MMPs.

Recent studies from our lab have shown that Abl kinases promote invasion and also phosphorylate STAT3 in a Jak-independent manner in melanoma cells [64]. STAT3 is known to induce VGP to RGP transition in melanoma cells and STAT3 is constitutively activated in melanoma patients [81, 82, 86]. In colon cancer cells STAT3 activates MMP-1 by binding to the promoter region of MMP-1 [209]. These results indicate that Abl kinases might promote invasion in a STAT3-dependent manner. Herein, we will evaluate the molecular mechanism of Abl kinase mediated invasion and also identify whether activated Abl kinases promote invasion in melanoma cells.

Cancer cells have the unique ability to survive and proliferate in the absence of serum [12, 13, 210] and survival in the absence of serum is an important determinant for cancer cells to grow and proliferate in distant metastasis sites and form macrometastasis. Excessive proliferation and survival in the absence of serum are important for neoplastic development. Deregulated proliferation is required for primary tumor growth and also for growth of micrometastasis to macrometastasis [12, 13, 210]. When cells undergo apoptosis molecular changes like cleavage of poly (ADP-ribose) polymerase (PARP) occur. Prior to PARP cleavage, the cells undergoing stress activate caspases-3, which mediates the cleavage of PARP into a 89 KDa fragment, resulting in PARP inactivation and apoptosis [211, 212]. For cancer cells to survive in nutrient

deprived conditions there has to be self-sufficiency of growth and also an ability to escape apoptosis. BCR-ABL and v-Abl inhibit apoptosis of leukemic cells, and Abl kinases promote survival of breast cancer cells in serum-free conditions [33, 64]; however, no one has tested whether Abl kinases-mediate melanoma survival in serum-free conditions. Based on these evidences, we hypothesize that Abl kinases promote melanoma cell survival. STAT3 is also known to promote proliferation and survival in the absence of serum in many cancers and Abl kinases drive proliferation and survival in absence of serum in breast cancers. Here, we will test whether active Abl kinases promote proliferation or survival in a wide range of melanoma cells and we will identify the mechanism of Abl kinase-mediated mitogenesis.

Several pieces of evidence point towards a possible role for Abl kinases in melanoma progression; however, prior to our studies there was no direct evidence. Using 435s/M14 cells, our lab has shown that Abl kinases promote proliferation, anchorage-independent growth and invasion of melanoma cells; however, the mechanism remains to be explored. Along the same lines, treatment of several melanoma cell lines with imatinib mesylate (STI571, Gleevec) inhibited proliferation; however, since imatinib also inhibits c-Kit and PDGFR in addition to Abl kinases, the imatinib target that promotes proliferation was not determined [69]. Furthermore, imatinib also was shown to inhibit murine melanoma tumor growth in a model that lacked c-kit and PDGFR [67, 78]. These data provided the rationale for us to study whether Abl kinases have a role in human melanoma progression. Here, in this study we demonstrate that Abl

kinase expression is increased in primary melanoma and in melanoma cell lines as compared to primary melanocytes. We show that activated Abl kinases promote melanoma proliferation, invasion, survival and single-cell 3D invasion; they promote invasion via distinct molecular pathways; and they potently drive metastatic progression.

2.2 Materials and Methods

Cell Lines:

435S/M14 cells were obtained from University of North Carolina Tissue culture Facility (Chapel Hill, NC), who purchased them from ATCC. WM239, WM278, WM164, WM3248, SBCL2 were obtained from the Herlyn Lab (Wistar Institute, Philadelphia). A375 cells were from Dr Suyan Huang (M.D Anderson Cancer Centre, Houston, TX. Primary melanocytes were a kind gift of Dr John D'Orazio (University of Kentucky, Lexington, KY). 435s/M14-GFP-Luciferase cells were created by Lipofectamine 2000-mediated transfection with pcDNA-EGFP-N1 (Clontech, Mountain view, CA) and selecting with G418 (800µg/ml). Later these 435s-GFP cells were transfected with PGL3-Luciferase and selected with 600 µg/ml of Zeocin. pGL3-Luciferase (Promega, Madison,WI) was cloned into pcDNA 3.1 Zeo using XbaI and HindIII, by sticky end ligation. Clones that were Zeocin-resistant were picked and clones that were positive for GFP and expressing high levels of luciferase activity were pooled to make the cell line. WM3248-GFP cells were created by transfecting cells with pcDNA-EFGP-N1 followed by 200ug/ml G418 selection. The clones were pooled to make a

polyclonal population and cells were sorted to make a stable GFP-positive cell population. 435s/M14-STAT3CA cells were made by transfecting pRc-CMV-STAT3C (Addgene, Cambridge, MA) followed by G418 selection. Clones expressing similar levels of Flag were pooled to make a stable polyclonal cell line. Dr Divyamani Srinivasan, a previous graduate student of our lab, established this cell line. 435s/MMP-1-GFP cells were obtained by stably transfecting a MMP-1 plasmid (Origene, Rockville, MD), followed by selection with G418 and sorted to obtain a high GFP positive population.

Reagents:

Antibodies directed against beta-actin, IGF-1R(C-20), Arg (9H5), alpha-tubulin, PARP and c-Abl (K12; immunoprecpitation) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); c-Abl (8E9; western blotting), and STAT3 was procured from BD Biosciences (Chicago, IL). pSTAT3 (pY702), caspase-3 and pCrkL/CrkL antibodies were purchased from Cell Signaling Technology (Danvers, MA). MMP-1 and TIMP-1 antibodies were purchased from Millipore (Billercia, MA).c-Kit antibody and recombinant MMP-1 was purchased from Upstate Biotechnology (Lake Placid, NY). Antibodies to PDGFR were provided by Dr Andrius Kazlauskas (Harvard University; Cambridge, MA). The Arg antibody (16-04) used for immunoprecipitation was prepared by immunizing rabbits using a sequence in the Arg C-terminus, DKDRPRRVKPK. MMP-3 and MMP-14 antibodies and recombinant MMP-3 was obtained from Abcam.

Pharmacological inhibitors to Abl kinases (imatinib a.k.a STI571, Gleevec and nilotinib) were kind gifts from Novartis Pharmaceuticals, Basel Switzerland. Imatinib was dissolved in water at a concentration of 10mM and frozen in aliquots in -80^{0}C, while nilotinib was dissolved in DMSO at 10mM and stored in aliquots in 4^{0}C. Matrigel invasion assay chambers and collagen I was obtained from BD Biosciences (Chicago IL) and IGF-1 was purchased from Upstate Biotechnology (Charlottesville, VA).

Transfection and siRNA:

Cells were transfected with DNA or siRNA using Lipofectamine 2000, according to the manufacturer's protocol (Invitrogen, Grand Island, NY). Pre-validated siRNA against Abl#1 (1336), Abl#2 (s866), Arg#1(1478), Arg #2(s872), MMP-1 (s8848), MMP-3 (s8853), MMP-14 (s8877), STAT3 (s742) and scrambled control #1 were purchased from Applied Biosystems/ Ambion; Carlsbad, CA. Abl 1336 and Arg 1478 was used at 20nM concentrations and transfected on two consecutive days to increase silencing efficiency. Abl s866 and Arg s872 and STAT3 were used at 5nM and transfected only once. MMP-1, 3 and 14 were use at 10nM and were transfected once.

Cell culture: MDA-MB-435S, originally thought to be of breast cancer origin, is recently been identified as melanoma M14 [66]. 435s/M14 was grown in DMEM supplemented with 10%Fetal Bovine Serum (FBS), 1mM Glutamine and 1%

Insulin (1mg/ml). Melanoma cell lines were cultured in Tu 2% media (80:20 MCDB153:L-15 Media+ 2% FBS+ Insulin 5µg/ml and 1.68mM calcium chloride). A375 cells were cultured in DMEM supplemented with 10% FBS and 1mM Glutamine.

Serum starvation: Cells were washed three times in basal media, and maintained in basal media containing glutamine deprived of insulin and FBS.

Cell lysis and western blotting: Cell lysates were prepared by lysing in RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.5, 1% triton-X 100, 0.1% SDS, 1% sodium deoxycholate, 1mM pefabloc,10ug/ml leupeptin, 10ug/ml aprotinin, 1mM sodium orthovanadate, 25 mM sodium fluoride). Whole cell lysates were taken and protein concentration was estimated using BCA method (Pierce) and protein fractions were run on SDS-PAGE gels. Immunoblotting was performed according to protocols from antibody manufacturers. Bands were quantified using ImageQuant (GE Healthcare, Piscataway, NJ) on scanned blots.

Kinase assays [55, 64]: Two days after plating, the cells were serum starved and lysed 20-24 hrs after starvation in a Triton-X-100 based lysis buffer(150mM Nacl, 50mM Hepes pH7.0, 1% triton-X, 10% Glycerol, 1.5mM $Mgcl_2$, sodium deoxycholate, 1mM pefabloc,10ug/ml leupeptin, 10ug/ml aprotinin, 1mM sodium

orthovanadate, 25 mM sodium fluoride). 30µg of cell lysates were used to immunoprecipitate c-Abl and Arg using K12 and 16-04 antibodies respectively. Antibodies bound to protein A-sepharose beads were washed in four different stringent buffers: two times with RIPA buffer (above) followed by two washes in buffer#2 (10mM Tris pH7.4, 5mM EDTA, 1% triton-X-100 and 100mM Nacl) with inhibitors. The complexes were again washed twice in buffer#2 without Nacl followed by washing twice in kinase assay reaction buffer (20mM Tris pH7.4. 10mM $Mgcl_2$ and 1mM DTT). The immunoprecipitates were incubated with the Abl kinase substrate GST-Crk 0.5µg, 1 µM cold ATP in kinase assay reaction buffer having 5µCi 32P-ɣ-ATP for 40 mins at room temperature. At the end of the reaction equal volume of 2X sample buffer was added, samples were boiled, loaded onto a 10% SDS PAGE gel and run overnight. The gel was dried and then exposed to a radiosensitive film and phosphoscreen. The phosphoscreen was scanned on a Storm phosphoimager (GE Health care) and bands were quantified before.

Invasion assays [55]: Cells were transfected with siRNAs, and serum-starved overnight for 16-20hrs. Trypsinized cells were washed with invasion assay media (basal media containing 1% BSA) thrice and $5X10^5$cells resuspended in invasion assay medium (serum starvation media) were placed in the top well of matrigel invasion chambers. The lower chamber contained 10nM of chemoattractant, IGF-1, resuspended in invasion assay medium. The cells were allowed to invade/migrate for 48 hours at 37^oC. Cells and matrigel on the upper surface of

the membrane were removed, and cells on the undersurface were fixed with methanol for 2 minutes, stained for 2 minutes (1% borax and 1% toluidine blue in water) and all the cells on the membranes were counted. For some experiments recombinant MMP-1 or MMP-3 (25ng/ml) were added to the top well of chambers, along with the cell suspension. Nilotinib was added to the top and bottom chambers for experiments involving the drug.

MMP expression/activation assays: MMP levels were assessed in serum starved, similar density subconfluent cells. Cells were serum starved as serum contains growth factors like EGF, which alters the expression of MMPs [213, 214]. For 8h imatinib treatment, cells were serum-starved overnight prior to treatment and for 48h treatment, cells were serum-starved and treated simultaneously. For siRNA experiments, cells were transfected with c-Abl and Arg siRNA for 2 consecutive days and then replated to obtain equal confluency and cells were serum starved for 48h. Transcript levels were determined by semi-quantitative PCR as described below. Activation/ secretion of MMPs was determined by concentrating the serum-starvation conditioned media using Ultracel-10K; Millipore; Temecula, CA, USA. As a loading control for activation experiments media amounts were normalized to β-actin levels from the whole cell lysates.

3D Single-cell Invasion assay [217]: Four volumes of Collagen I (Invitrogen, Grand Island, NY) solution with a final concentration of 1mg/ml was mixed with 1 volume of pre-cooled Basal Media (10x), 2.65 volumes of normal growth growth media, 5 volumes of HBSS, 1 volume of 0.25M $NaHCO_3$, and 0.3 volumes of 1M NaOH, to create a pH>9. The collagen gel solution was mixed properly by pipetting and kept on melting ice. One mililiter of the prepared collagen solution was placed in 6-well dishes and allowed to harden at 37^oC and 10% Co_2 for 1h. Melanoma cells transfected with siRNAs, were trypsinized and plated on the collagen matrix at 37^oC for indicated times, after which time invasive cells (lack refractile halos and contain invasive extensions) and non-invasive cells were counted from 20 random fields.

Tritiated Thymidine Assays [64]: Cells were plated in 12-well dishes to obtain 50% confluency one day after plating and treated with imatinib or nilotinib for 24h, or siRNA transfected cells were plated to have equal density a day after plating. Cells were labeled with 5µCi of tritiated thymidine for two hours, washed with cold PBS, fixed in 10% tricholoroacetic acid for 40 mins in 4^0C harvested with 0.2N NaOH, mixed with 4mls of scintillation fluid, and read on a scintillation counter.

Semi-Quantitative RT-PCR [55, 64]: RNA was isolated from serum-starved cells transfected with siRNAs or treated with imatinib using an RNAeasy kit (Qiagen,

Valencia, CA), and traces of contaminating DNA was removed by DNAase digestion with DNase I (Applied Biosystems, Carlesbad, CA). c-DNA was synthesized by incubating five micrograms of DNase-treated RNA with Superscript Reverse Transcriptase and random primers (Invitrogen, Carlesbad, CA). Target genes were amplified by PCR using cDNA and specific primers with internal β-actin control primers, 10x PCR reaction mixture (Invitrogen), $MgCl_2$ (1.5mM), dNTPs, and Taq DNA polymerase (Genscript, Piscataway, NJ). PCR reactions conditions were optimized for linearity and cycling parameters involved 35 cycles of 95^{o}C-1', 55^{o}C-1', 72^{o}C-1'. The PCR products were run on 2% agarose gels. Scanned photographs were quantified with ImageQuant (Molecular Dynamics) and specific bands normalized to β-actin internal control bands as described in the western blotting section.

c-Abl forward primer, 5'CCTTCATCCCTCTCATATCAACC3',

c-Abl reverse primer, 5'TGGACCACTGCCTGCTGTCGC3'

Arg forward primer, 5'CATCCGTCCATCTGCTCAGAC3'

Arg reverse primer, 5'GGACAGTAGGTCAGCACATTC3'

MMP-1 forward primer, 5'AGCGTGTGACAGTAAGCTAAC3'

MMP-1 reverse primer, 5'TCCTCAGAAAGAGCAGCATCG3'

MMP-3 forward primer, 5'GTTCCGCCTGTCTCAAGATGA3'

MMP-3 reverse primer, 5'ATCCAGCTCGTACCTCATTTCC3'

MT1-MMP forward primer, 5'GCAGGCCGACATCATGATCTTC3'

MT1-MMP reverse primer, 5'TCCTCTCGTAGGCAGTGTTG3'

TIMP-1 forward primer, 5'TCCTGTTGTTGCTGTGGCTGA3'

TIMP-1 reverse primer, 5'GGACTGGAAGCCCTTTTCAGA3'

β-actin forward primer, 5'CCTTCCTGGGCATGGAGTCCT3',

β-actin reverse primer 5'GGAGCAATGTCTTTGATCTTC3'

Metastasis Assays: 435s/M14-GFP-Luciferase cells were injected ($2X10^6$ cells/100ul of Hanks Balanced Salt Solution, (HBSS) into the tail vein of 7-8 week old SCID Beige mice (Harlan Laboratories, Indianapolis, IN). Following the day of injection, mice were treated with nilotinib (30mg/kg b.i.d) or vehicle (0.5% hydroxymethylcelluose/0.05% Tween-80) by oral gavage. On days, 17, 21 and 24 after initial injection of cells, mice were intraperitoneally injected with Luciferin –D (100/mg/kg) and fluorescence was measured by IVIS Xenogen Spectrum (Caliper Life Sciences, Hopkinton, MA). The flux values were normalized with Living Image 3.1 software in order to observe differences between time points and also for quantification. On Day 24 the mice were euthanatized, lungs were removed, washed in cold PBS, and fixed in 100% formalin. In the WM3248 metastasis assay, cells ($2x10^6$ cells/100ul HBSS) were injected into the tail vein of 7-8 week-old nude (*nu/nu*) mice (Harlan Laboratories). Mice were treated with 33mg/kg of nilotinib, same way as described above and on day 34, the mice

were euthanatized, lungs removed and fixed in 100% formalin. GFP-fluorescent metastatic nodules were counted and photographs were taken using a 0.63X objective and 2X-Zoom on an Olympus MVX-10 stereomicroscope with fluorescence illumination.

Paraffin embedding: Lungs pre-fixed in formalin and stored at 4^0C were dehydrated in 70% ethanol, 100% ethanol, 1:1 ethanol/xylene, followed by 100% xylene. Lungs were placed in melted paraplast X-tra: xylene (1:1) for 1hr at 54^oC with constant shaking followed by overnight room temperature incubation. Warm paraplast (100%) was then added to the tissue for 1h at 54^oC with constant shaking, incubated at room temperature for 2hr, paraffin was melted, and lungs were mounted at room temperature. The mounted lungs were stored at room temperature overnight followed by desiccation for long-term storage. Lungs were sectioned on a ThermoShandon Finesse microtome.

Statistics: Student t-tests were used for comparisons using a SAS software, sigma stat or online-website (Vassar website). One-way Anova with Tukey post hoc tests were performed in experiments, involving multiple groups. $p<0.05$ was considered significant (*, $0.01\leq p<0.05$; **, $0.001\leq p<0.01$; ***, $p<0.001$).

2.3 Results

2.3.1 c-Abl and Arg are activated in invasive melanoma cell lines and in primary melanomas

Our lab has previously shown that the activities of Abl kinases are dramatically elevated in highly invasive breast cancer cell lines as compared to non- invasive breast cancer cell line (MCF-7) [55], and activation of c-Abl and Arg did not correlate to their expression levels in these cells. To investigate whether Abl kinases also are activated in invasive melanoma cell lines, serum-starved lysates of a panel of human melanoma cell lines and primary melanocytes were immunoblotted for c-Abl and Arg. c-Abl and Arg expression were dramatically elevated in all the melanoma cell lines as compared to primary melanocytes (Figure 2.1). Since Abl kinases are tightly regulated by intermolecular interactions and activation does always correlate with their activity [54], we investigated whether Abl kinases are activated in these cell lines. Using *in vitro* kinase assay, we found that several melanoma cell lines had increased c-Abl and/or Arg kinase activities as compared to the human primary melanocytes (Figure 2.1). Immunoblotting for phosphorylation of known Abl kinase targets, Crk and CrkL on Abl tyrosine phosphorylation sites (Y221/Y207), showed elevated levels in all the melanoma cell lines as compared to primary human melanocytes, and the level Crk/CrkL phosphorylation, paralleled c-Abl/ Arg kinases activities with the exception of WM278. Unlike in breast cancers, we observed that c-Abl/Arg expression correlated with c-Abl/Arg activities in melanoma cells, in that lines with higher c-Abl and Arg expression had higher activities. To test whether Abl

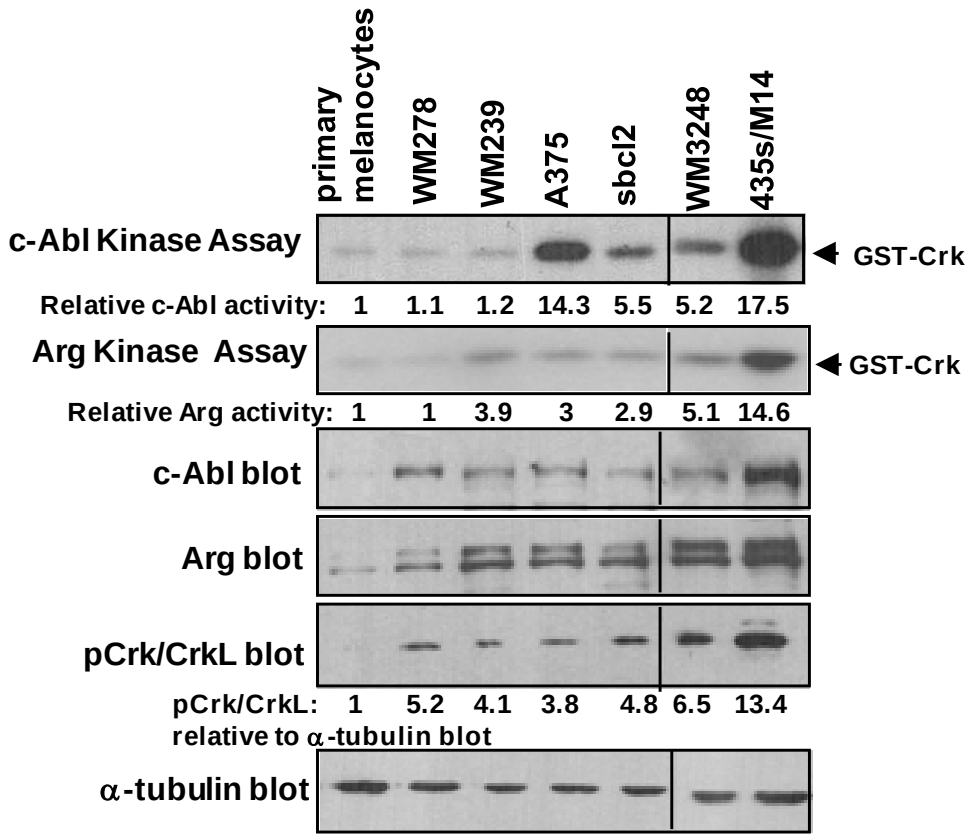


Figure 2.1: c-Abl and Arg highly activated in melanoma cell lines. Melanoma cells were serum-starved overnight, lysed, c-Abl and Arg were immunoprecipitated and incubated with GST-Crk in the presence of radioactive gamma-ATP. Kinase activities were assayed by *invitro* kinase assay using GST-Crk as a substrate. Lysates were blotted for indicated antibodies (bottom 4 panels). Performed in collaboration with J.W Friend.

kinases are activated in primary human melanoma samples, we performed immunohistochemistry on melanoma tissue mircoarrays (TMA). Phospho-specific antibodies to c-Abl (Y412 and Y245) cross-react with phospho-EGFR and phospho-PDGF and phospho-specific Arg antibodies are not commercially available [54]. Therefore we stained the TMAs with an antibody to the Abl phosphorylation sites in targets, Crk/CrkL, since inhibition of Abl kinase activities with imatinib or knockdown of c-Abl and Arg expression reduced pCrk/CrkL levels in a dose response manner [55,64], indicating that Crk/CrkL phosphorylation at Y221/Y207 correlates with c-Abl/Arg activity in cancer cell lines. In normal skin samples, pCrk/CrkL staining was observed predominantly in the cytoplasm and nuclei of keratinocytes and the nuclei of lymphocytes (Figure 2.2). Sixty-seven percent of benign nevi demonstrated weak to no nuclear staining, whereas 33% had moderate-to strong staining (Score >1.4; Score=Intensity x Proportion of positively stained tumor cells). pCrk/CrkL staining (red) was nuclear in primary melanomas whereas melanin, which was mainly cytoplasmic was brown. Cores with strong melanin staining were excluded from the study owing to difficulty in scoring. Twenty-nine out of forty-eight melanomas (60%) in contrast to 33% (6/18) of benign nevi had moderate-strong pCrk/CrkL staining. Chronically and intermittently sun exposed skin samples had more positive cases of pCrk/CrkL staining in contrast to minimal sun-exposed samples. These results indicate that c-Abl/Arg is activated in primary melanomas and as well as in human melanoma cell lines.

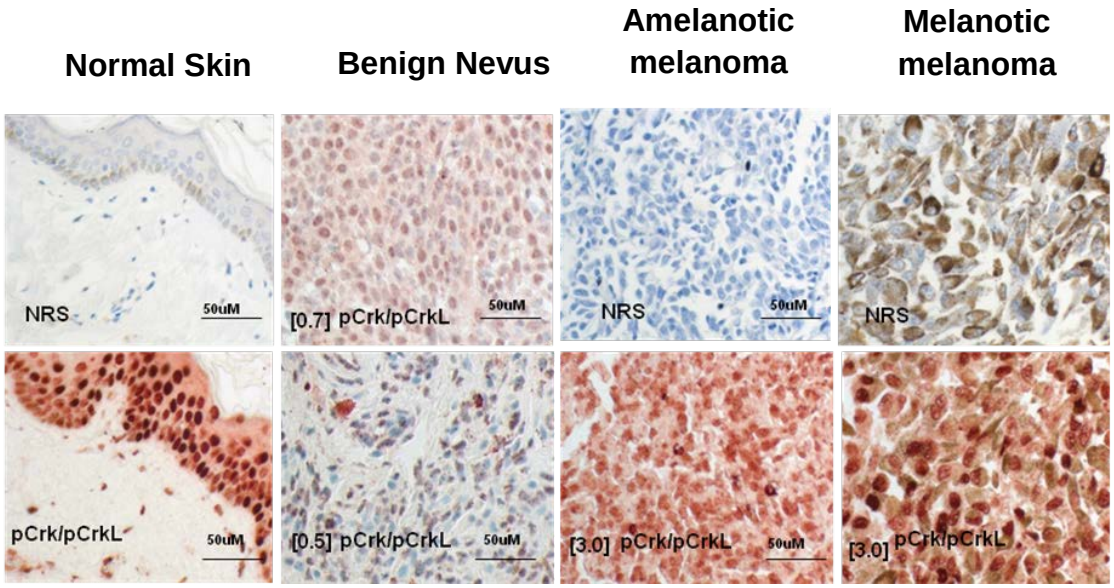


Figure 2.2 Abl kinases are activated in primary melanoma. Melanoma TMAs were incubated with normal rabbit serum (NRS) or pCrk/CrkL antibody, hemotoxylin-stained and visualized with Dako Red. Cores having a score > 1.4 was considered moderate-strongly positive (Score=Intensity x Proportion of positively stained tumor cells). Scores for each core is indicated in brackets. Photographs were taken under 400Xmagnifications. The staining was performed by Leann Fiore and scored by Dr. Michael Cibull MD (Director of Surgical Pathology).

2.3.2 Active Abl kinases promote matrigel-invasion, single-cell 3D invasion, proliferation and survival of melanoma cells

Our lab has previously shown that c-Abl and Arg promotes invasion in 435s/M14 cells [55].To determine whether Abl kinases promoted invasion in other melanoma cell lines, we used WM3248 cells, which have highly active c-Abl and Arg (Figure 2.1). Consistent with our previous findings with 435s/M14 cells, silencing c-Abl or Arg inhibited matrigel invasion of WM3248 (Figure 2.3A). These results were confirmed using a second siRNA directed against c-Abl and Arg (Figure 2.3A). To confirm that active Abl kinases promote invasion in melanoma cells, we treated WM278, which have low c-Abl and Arg kinase activities (Figure 2.1) with nilotinib, a second-generation Abl kinase inhibitor and compared the effects in a variety of melanoma cell lines. Nilotinib did not inhibit invasion in WM278, which does not contain active Abl kinases (Figure 2.1), whereas it significantly inhibited invasion in 435s/M14 and WM3248 cells(Figure 2.3B) containing highly active c-Abl and Arg. These results indicate that active Abl kinases are required to promote invasion in melanoma cells and also suggests that nilotinib specifically blocks invasion by inhibiting Abl kinases. The process of invasion involves degradation of the collagen bed in the extracellular matrix. Collagen I is a prime constituent of the extracellular matrix [104, 105, 217,]. To investigate whether Abl kinases can degrade the collagen bed we performed a single-cell 3D invasion assay.

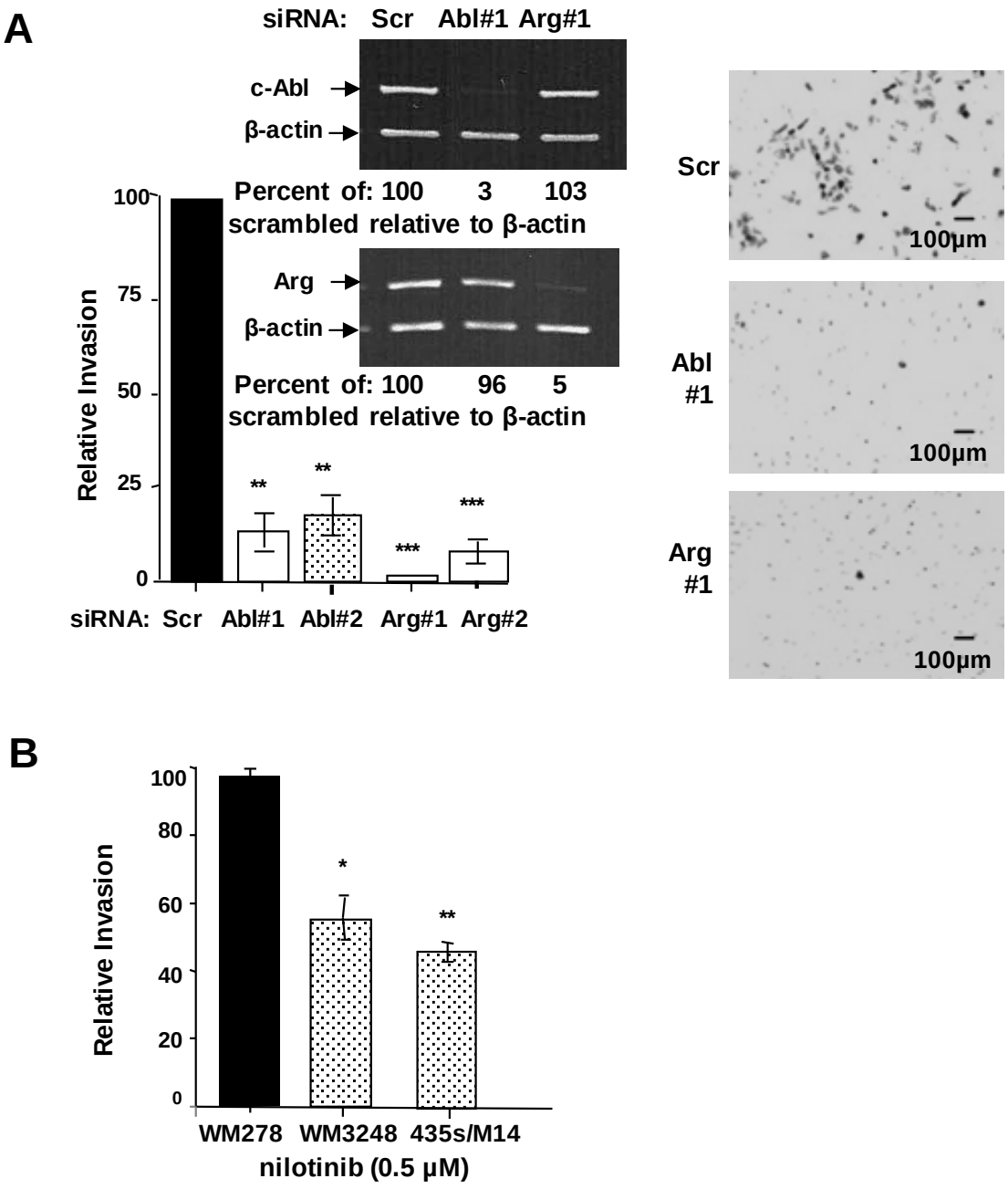


Figure 2.3: Abl kinases promote invasion in melanoma cells. (A) WM3248 cells transfected with two individual siRNA were serum for 16-20 hrs and incubated in matrigel invasion chambers for 48h with IGF-1 as chemoattractant. The lower surface of the membranes was stained, and the total number of invaded cells on the undersurface was scored and compared to Scr control. The graphs are mean± s.e.m (n=3 independent experiments)*0.01 ≤ p <0.05; **0.001 ≤p<0.01. Photographs are in 100x magnification. Knockdown of cells were analyzed by semi-quantitative RT-PCR using specific c-Abl and Arg primers (Right). **(B).** Melanoma cells were serum-starved, treated with 0.5μM nilotinib, and matrigel invasion assays were performed with nilotinib in bottom and upper chambers. Invasion assays were performed using 10nM of IGF-1 as a chemoattractant. Cells were stained and scored as in (**A**). Graphs were normalized to vehicle treated controls and plotted as mean±s.e.m (n=3, normalized to untreated) *0.01 ≤ p <0.05; **0.001 ≤p<0.01.

In this assay, siRNA-transfected 435s/M14 and WM3248 cells were placed on collagen I-coated beds and incubated for a period of 5h and 24h, respectively. Cells that degrade the matrix form extensions and invade the collagen bed, where as non-invasive cells fail to form extensions [217]. Inhibition of c-Abl or Arg significantly reduced single-cell 3D invasion in WM3248 cells and 435s/M14 (Figure 2.4), indicating that c-Abl and Arg promote single-cell 3D invasion by degrading the collagen matrix and thus, likely promote collagenase activity. This assay also lacks the migratory component that is generally present in Boyden chamber invasion assays, indicating that Abl kinases promote invasion cannot be solely explained by an effect on cell motility.

Another key process in cancer metastasis is deregulated proliferation. Our lab previously showed that Arg but not c-Abl promotes proliferation of 435s/M14 cells. To test whether Abl kinases promote proliferation of other melanoma cell lines, we examined WM3248 cells. As opposed to 435s/M14, in WM3248 cells both c-Abl and Arg promoted proliferation/S-phase entry (Figure 2.5 A). To investigate whether active Abl kinases regulate proliferation in other melanoma cells lines, we treated a panel of lines with nilotinib for 24h (Figure 2.1) and performed tritiated thymidine proliferation assays. Nilotinib inhibits Abl kinases and other targets such as c-Kit, and PDGFR; thus we examined these cell lines for the expression of these receptors (Figure 2.6 and Table 2.1). 435s/M14 and WM3248 did not express any of the other nilotinib and imatinib targets. Adding the targets together gave the total number of targets that nilotinib can inhibit in these cell lines. Taking the amount of tritiated thymidine

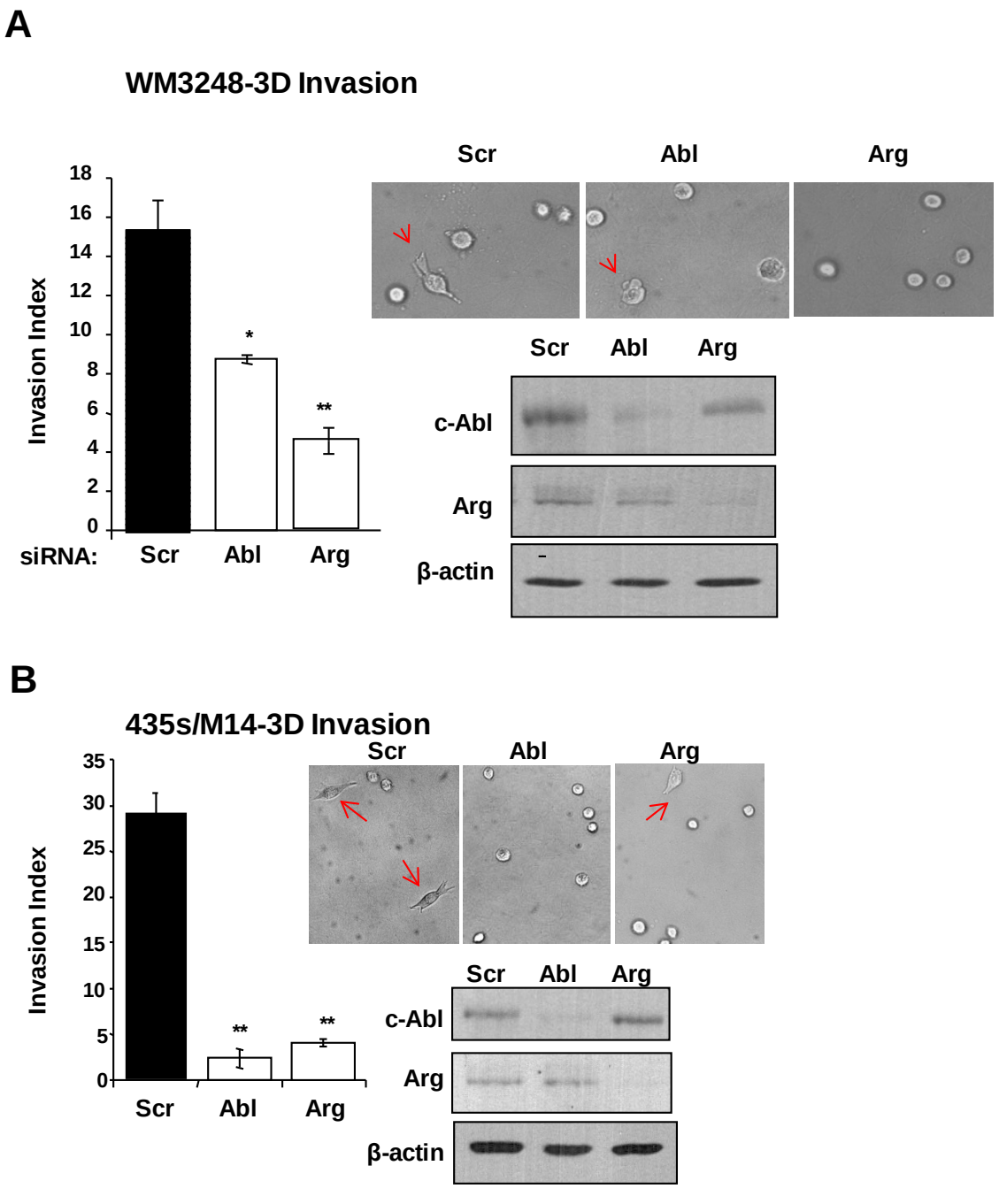


Figure 2.4: Active Abl kinases drive single-cell 3D invasion. Melanoma cells, transfected with siRNAs, were incubated in a single-cell 3D invasion assay (deWever et al. 2010) for 20h (**A**- WM3248) or 5h (**B**-435s/M14); cells that lose their refractile appearance, gain invasive potential and form extensions on collagen bed. The number of invasive cells (containing extensions) and non-invasive cells were counted in 20 random fields obtain an invasion index (cells with invasive extensions/total cells*100). Representative fields were photographed. Mean± s.e.m, n=3. *p<0.05, **p≤0.01, ***p<0.001. Knockdown of cells was analyzed by western blotting using specific c-Abl and Arg antibodies (Right).

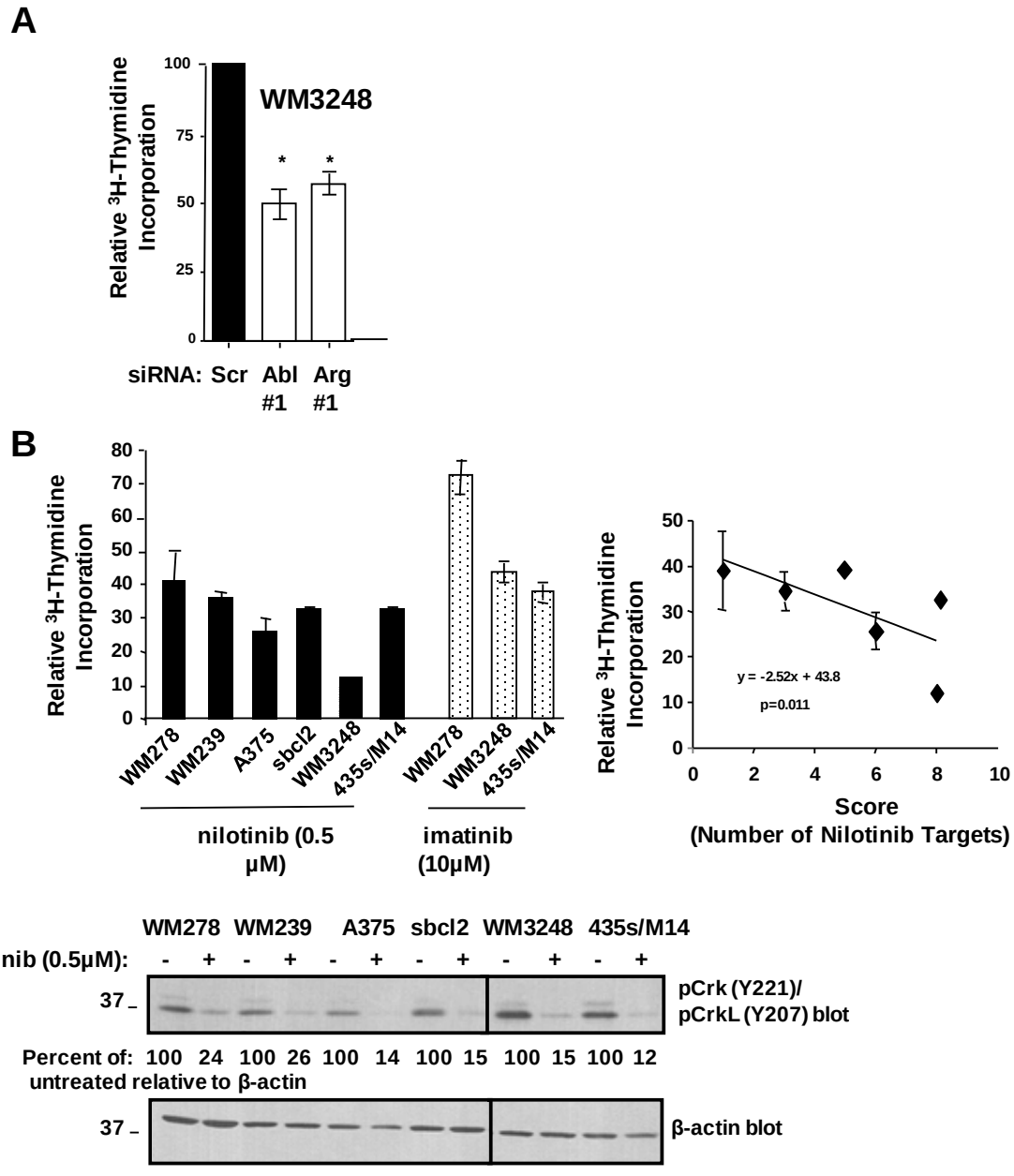


Figure 2.5: Active c-Abl and Arg promote proliferation in melanoma cells. (**A**) WM3248 cells, transfected with c-Abl or Arg siRNA were subjected to tritiated thymidine assays, CPMs were normalized to those obtained in scrambled control cells. Mean± s.e.m, n=3. *p<0.05. (Performed in collaboration with J.Sims). (**B**, Left) Tritiated thymidine incorporation was assessed in melanoma cells treated with 0.5μM nilotinib or 10μM imatinib for 24h. CPM was normalized to vehicle treated cells. Mean± s.e.m, n=3. *p<0.05 (**B**, right) The level of c-Abl and Arg activation from Figure 1 was added with the number of nilotinib targets from Figure 2.6 to get a Score. An inverse correlation comparing sensitivity to nilotinib against the Score was observed (R^2=0.45 p=0.011). (**B**, bottom) Cells treated with nilotinib under the same conditions as **B** left bottom, were lysed and blotted with pCrK/CrkL antibody to demonstrate efficacy of the drugs in inhibiting c-Abl/Arg.

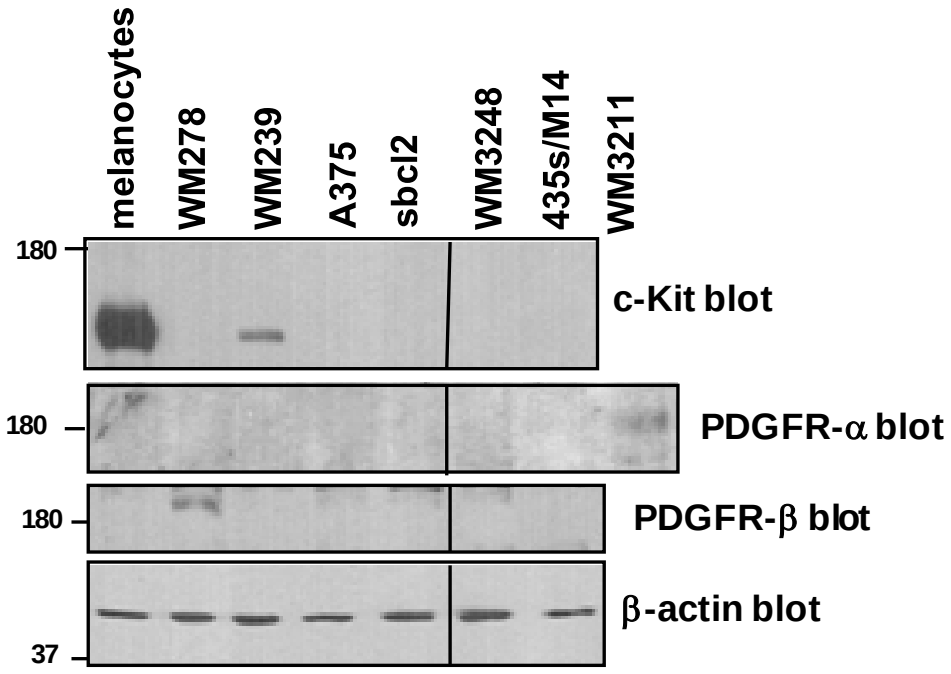


Figure 2.6: c-kit and PDGFR expression in melanoma cells. Melanoma cells were plated, serum-starved for 24h, lysed, and blotted with indicated antibodies. WM3211 cells were used as a control for the PDGFR α- blot.

Table 2.1 Sensitivity of melanoma cells to Abl kinase inhibitor, nilotinib. c-Abl and Arg activities from Figure 2.1 were added with other nilotinib targets in Figure 2.6 and a Score was achieved for each cell line indicating the total number of nilotinib targets.

Cell line	WM278	WM239	A375	sbcl2	WM3248	435s/M14
Abl activity	-	-	++++	+++	++++	++++
Arg activity	-	++	++	++	++++	++++
c-Kit	-	+	-	-	-	-
PDGFR-α	+	-	-	-	-	-
PDGFR-β	-	-	-	-	-	-
# of nilotinib targets	1	3	6	5	8	8
^{3}H-Thymidine	41±8.4	36±2.07	26±2.4	39.5±1.5	12±0.38	32.7±.0.48

incorporation in each cell line versus the total number of targets we found an inverse correlation, indicating that activation ok known imatinib or nilotinib targets (including c-Abl and Arg) promotes proliferation of melanoma cells (Figure 2.5B). However, nilotinib-mediated inhibition of proliferation in WM278 cells is efficient (Table 2.1), indicating that pCrk/CrkL levels might be more predictive of nilotinib's anti-proliferative effects than c-Abl/Arg activities. The anti-proliferative effects of nilotinib might also be due to the expression of PDGFR-β receptor in WM278 cells. Imatinib, also efficiently inhibited proliferation/S-phase entry of 435s/M14 and WM3248 cells (both harboring highly active c-Abl and Arg activities). Using imatinib, we also found that the degree of inhibition of proliferation was much higher in 435s/M14 and WM3248 cells (both harboring highly active c-Abl and Arg activities) as compared to WM278, which have low Abl kinase activities (Fig 2.1). However, in 435s/M14, WM3248, and WM278 the anti-proliferative effect of nilotinib is greater than imatinib (Figure 2.1) indicating that nilotinib might be more effective than imatinib in inhibiting proliferation of melanoma cells.

Cancer cells have the ability to survive in serum-free conditions. This property helps them survive in nutrient-free conditions, which is essential during the process of metastasis and resistance to apoptosis also contributes to chemotherapeutic resistance of cancer cells [12, 13, 210]. Results from our laboratory have shown that inhibition of Abl kinases promotes apoptosis of breast cancer cells [64]. Thus we sought to investigate whether Abl kinases promote survival in melanoma cells in serum-free conditions. As shown in Figure 2.7, inhibition of Abl kinases in 435s/M14 and WM3248 cells, via imatinib, induced

apoptosis in serum-starved conditions, as evidenced by PARP cleavage and caspase-3 activation. Since these cells lines do not express any other known imatinib targets, these results indicate a role for Abl kinases in melanoma cell survival.

2.3.3 c-Abl and Arg promote the transcription of matrix metalloproteinases and induce their activation in melanoma cells

An initial step in cancer invasion involves degradation of the ECM. MMPs aid in the degradation of ECM, and thus promote invasion and metastasis [103, 104]. Regulation of MMP-1 transcript and activity is important in cancer cell invasion and metastasis. MMP-1 mRNA and activity is upregulated in breast cancer tissues as compared to normal breast tissues [218].Transcriptional regulation of MMP-1 has been shown to be dependent on the MAPK pathway via AP-1 and Ets family of transcription factors [219, 220]. The MMP-1 promoter has an Ets binding domain [219, 221]. To evaluate whether MMP's are essential for invasion of 435s/M14 cells, we first screened for the presence of MMP expression in 435s/M14 cells. We found that MMP-1, -3 and 14 mRNAs are expressed whereas MMP-2 was not detected by semi-quantitative reverse transcriptase PCR (RT-PCR). To investigate whether these detected MMPs are involved in regulating invasion in 435s/M14 cells, we silenced MMP-1, -3 or 14 and performed matrigel invasion assays. Inhibition of MMP 1, 3 or 14 independently decreased invasion of 435s/M14 cells (Figure 2.8), indicating that these MMPs

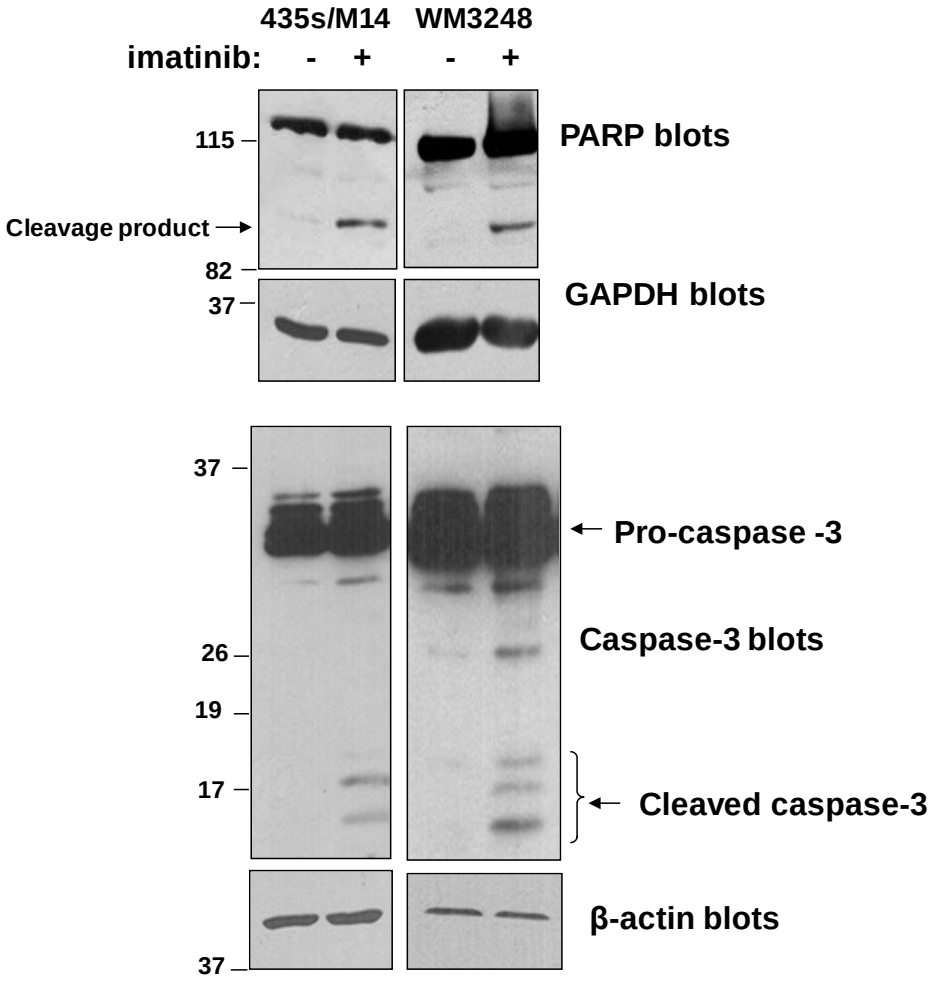


Figure 2.7 Active Abl kinases promote survival of melanoma cells in response to serum deprivation. 435s/M14 cells and WM3248 cells were treated with vehicle or imatinib and deprived of serum for 96h and 7 h respectively. Lysates from detached and attached cells were probed with indicated antibodies. Representative blot shown from one of the three independent experiments.

are important in melanoma invasion. Silencing of MMP- 14 had a smaller effect on invasion as compared to silencing MMP-1 or MMP-3, indicating that MMP-14 probably plays a less significant role in regulating invasion in these cells, perhaps due the fact that one of its functions is to activate MMP-2 and MMP-2 is not expressed in these cells.

To determine whether Abl kinases promote MMP transcription, we treated serum-starved, 435s/M14 cells with imatinib (8h, 48h) and performed semi-quantitative RT-PCR on the extracted RNA samples. Imatinib treatment significantly inhibited MMP-1, MMP-3 and MMP-14 transcription (Figure 2.9). Silencing c-Abl and Arg also significantly decreased m-RNA levels of MMP-1 in 435s/M14 cells, and silencing Arg significantly decreased the m-RNA levels of MMP- 3 and 14, respectively (Figure 2.9 A-C,right), whereas silencing c-Abl had no significant effect on the transcription of MMP-3 and 14. These results indicate that c-Abl and Arg promote MMP-1 transcription in melanoma cells, whereas Arg increases MMP-3 and 14 transcription.

Under normal physiological conditions, MMP activity is controlled either by transcriptional regulation, or by via endogenous MMP inhibitors (TIMPs). Our previous results (Figure 2.9) indicate that Abl kinases promote MMP transcription, so we wanted to investigate whether Abl kinases also promote MMP activation. To investigate whether Abl kinases induce the activation and secretion of MMPs conditioned media from serum-starved cells expressing c-Abl or Arg siRNAs were blotted with antibodies that recognize both the inactive and

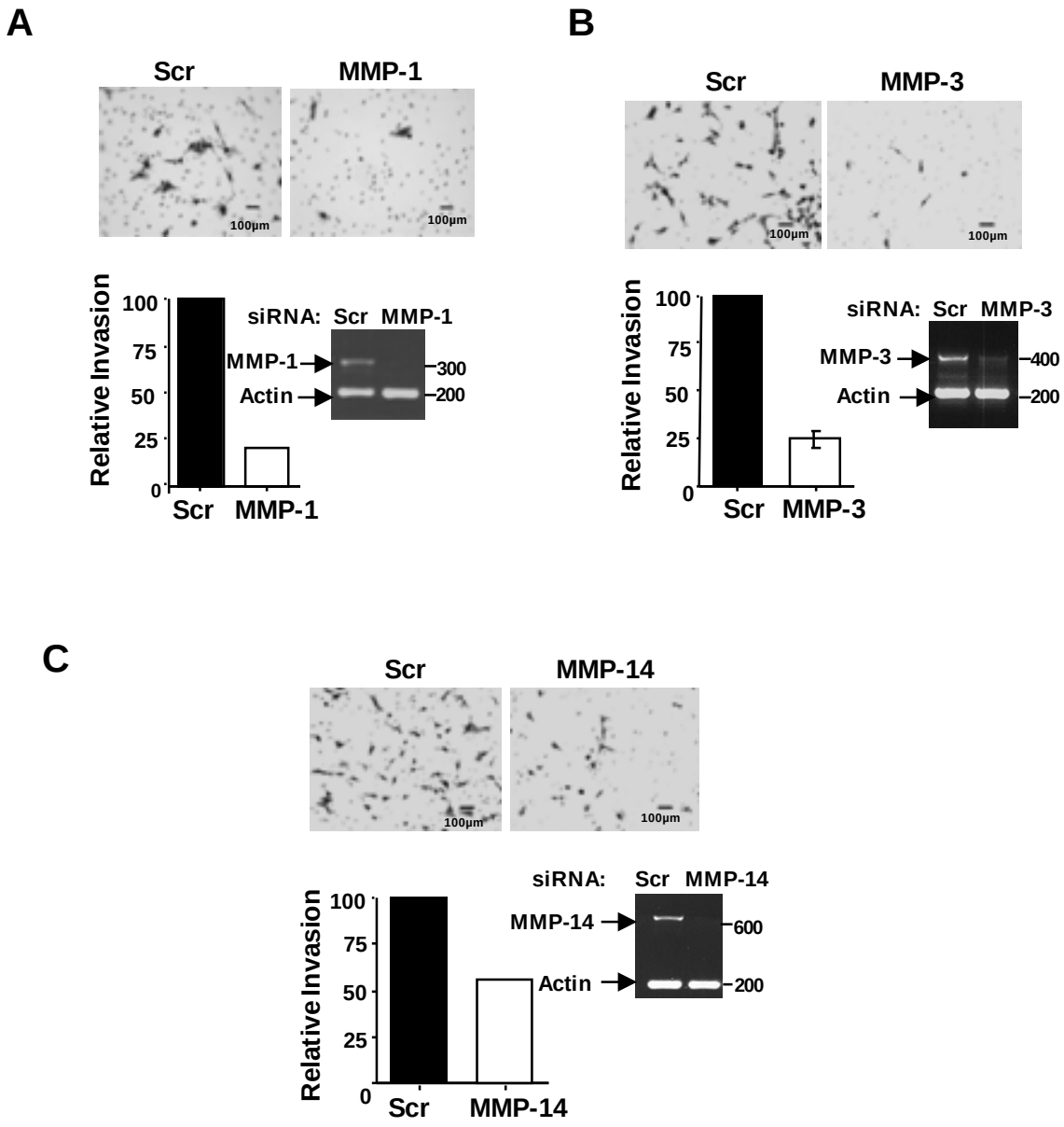


Figure 2.8 MMP-1, 3 and 14 promote invasion of 435s/M14 cells. 435s/M14 cells were transfected with siRNAs against MMP-1, 3, 14 or a scrambled control, and invasion assays were performed. Mean ± s.e.m.for two experiments.

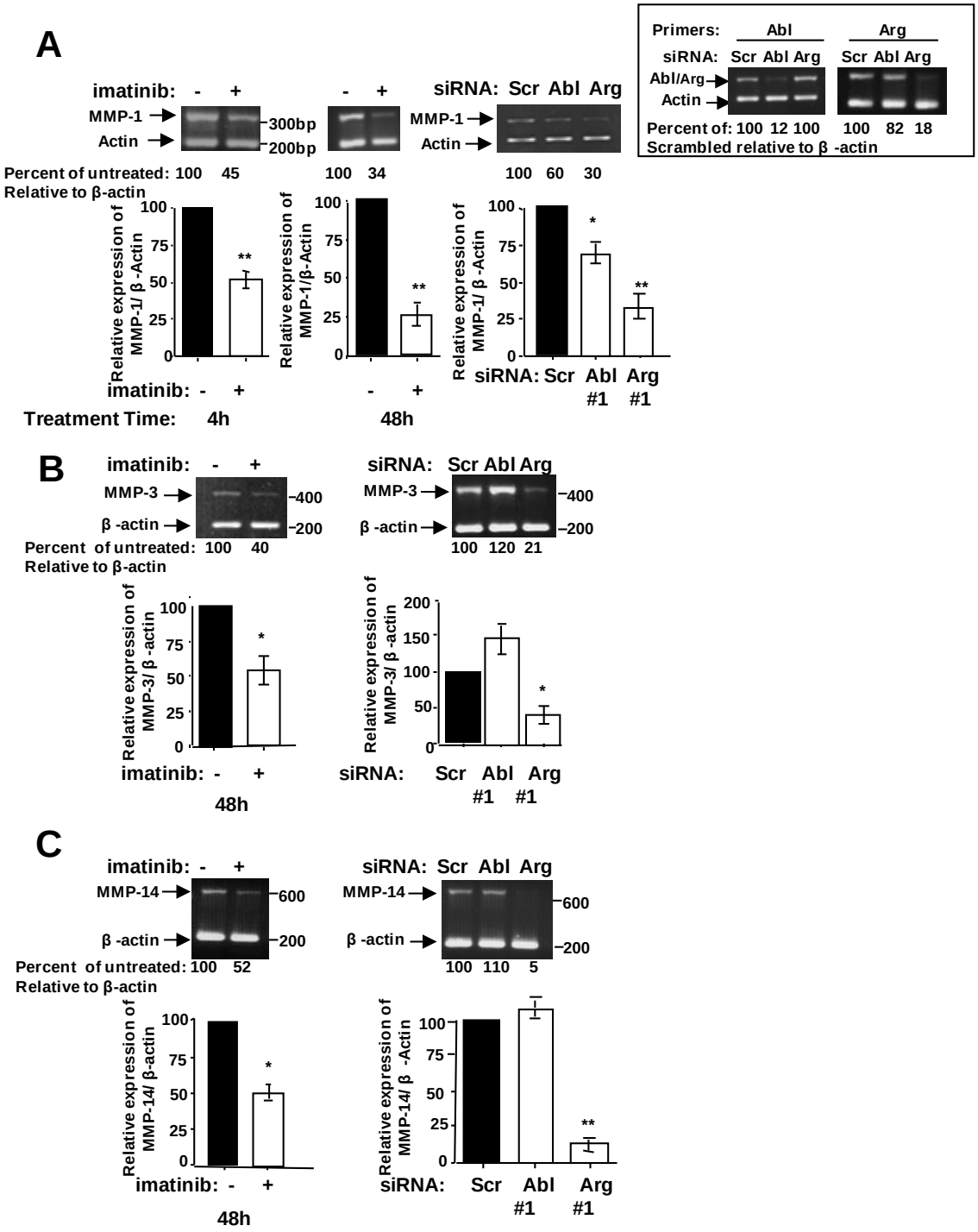


Figure 2.9 Abl kinases increase MMP-1, 3 and 14 transcription. 435s/M14 cells were plated, serum starved, and treated with the Abl inhibitor imatinib for 4h (**A**) or 48h (**A-C**) or were transfected with c-Abl/Arg siRNAs and serum-starved (**A-C**, Right,). Extracted RNA was subjected to semi-quantitative RT-PCR with MMP and internal control actin primers. Graphs are mean ± s.e.m.for three experiments. *p<0.05, **p≤0.01, ***p<0.001. Knockdown efficiencies were determined using c-Abl or Arg primers; a representative experiment is shown in inset. Representative blots are shown.

active forms of MMP-1, 3 and 14. Silencing c-Abl or Arg decreased the inactive and active forms MMP-1, consistent with the mRNA results, indicating a role of c-Abl and Arg in MMP-1 transcription (Fig 2.10A). Consistent with the mRNA levels, silencing Arg decreased the inactive and active forms of MMP-3 (Fig 2.10B). Since MMP-14 is trans-membrane MMP and isn't secreted we blotted the whole cell lysates with antibodies that recognize both the inactive and active forms. We found that silencing Arg significantly reduced the inactive and active forms of MMP-14 (Fig 2.11). The degree of inhibition in active band of MMP-1, and 3 is much more than the inactive band, indicating that probably Abl kinases induce activation and secretion of MMPs. Taken these results together we can conclude that Abl kinases upregulate the transcription, induce activation and secretion of MMP's in melanoma cells and thus promote melanoma cell invasion. MMP transcription is not always a measure of its activation as TIMPs inhibit the activation of MMPs while several other already active MMPs or several serine proteases activate MMPs. Inhibiting MMP activation by upregulating endogenous MMP inhibitors (TIMPs) could decrease the activation of MMPs. Our results indicate that Abl kinases promote the transcription and activation of MMP but the mode of activation needs to be determined.

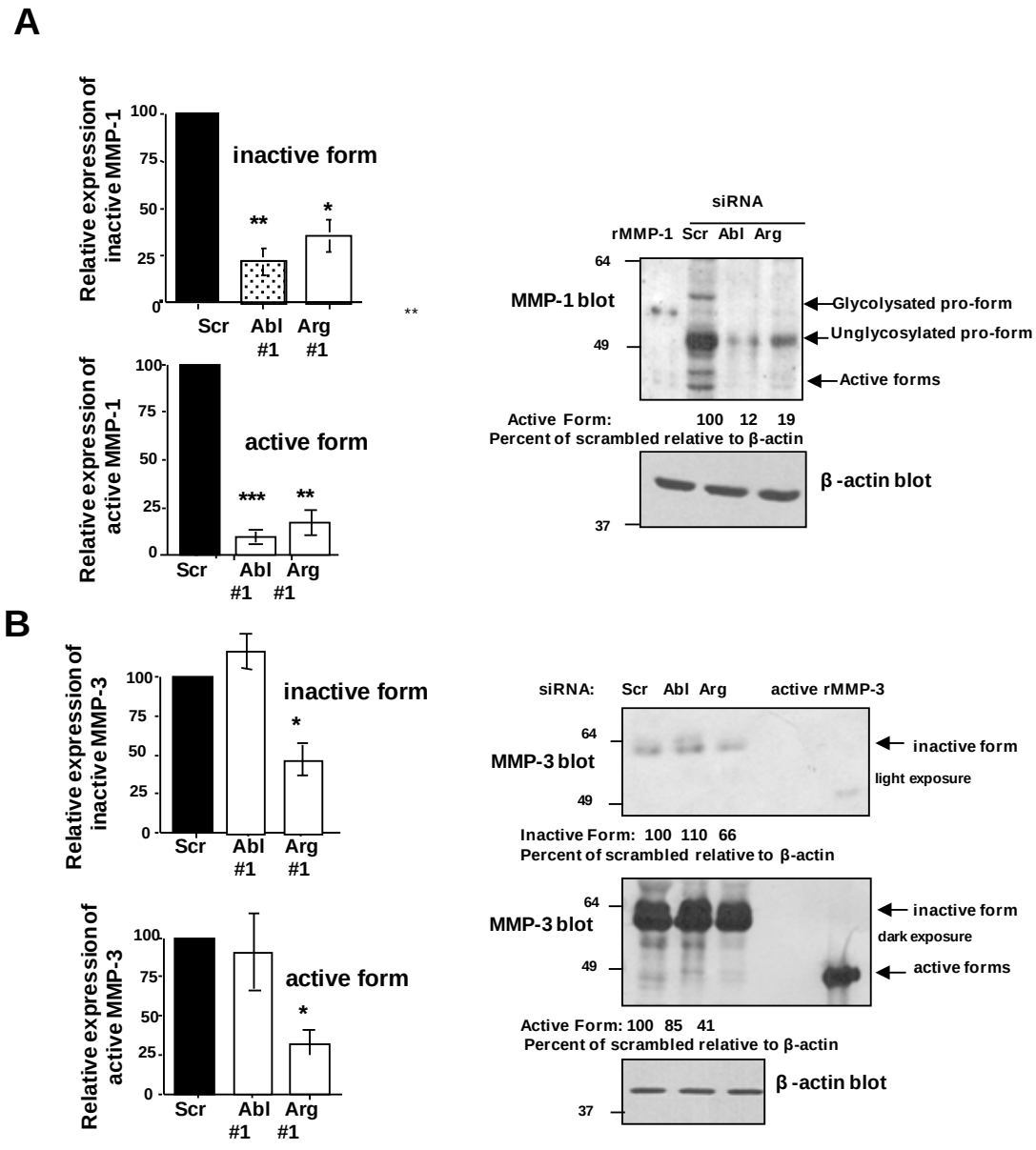


Figure 2.10 Abl kinases promote activation of MMP-1 and MMP-3. (A-B) 435s/M14 cells, transfected with c-Abl and Arg siRNAs were serum-starved for 48h and attached and detached cells were lysed and blotted for β-Actin. Media was collected and concentrated as described in Section 2.2, and loaded according to the β-Actin blot. Recombinant MMP-1 and MMP-3 proteins were used as positive controls for identifying the location of the inactive and active bands. Graphs are mean ± s.e.m.for three experiments and representative experiments are shown. *p<0.05, **p≤0.01, ***p<0.001

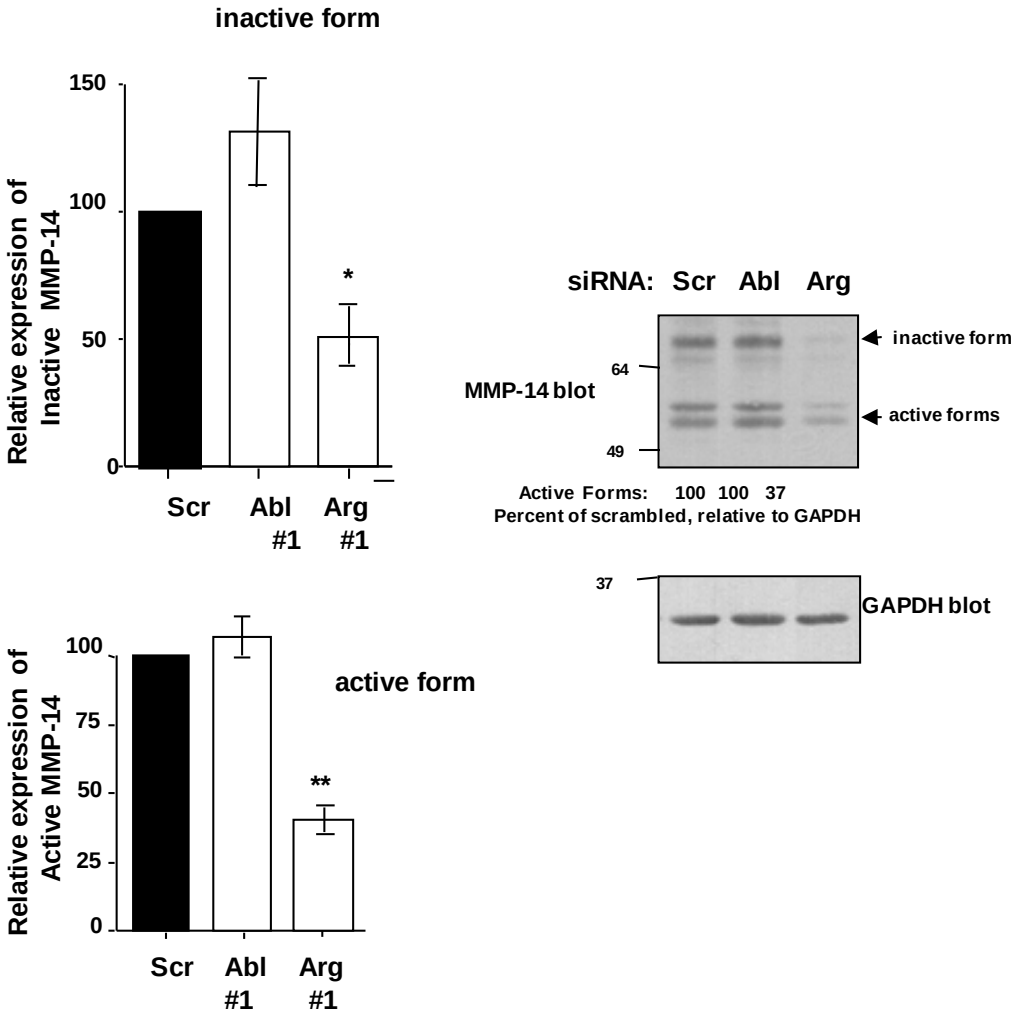


Figure 2.11 Arg increases MMP-14 expression. 435s/M14 cells transfected with siRNA against c-Abl, Arg or a scrambled control (Scr), were serum-starved for 48h, lysed, and blotted for indicated antibodies. Graphs are mean ± s.e.m.for three independent experiments. *p<0.05, **p≤0.01, ***p<0.001. Representative blot shown

2.3.4 Abl kinases induce MMP-1 transcription via activation of STAT3

STAT3 have been shown to induce the expression of MMP-1 in bladder and colon cancer cells [209, 222], and also is known to promote invasion in many cancers. Abl kinases activate STAT3 in a JAK-independent manner [64] and furthermore, the Abl kinase activator, Src phosphorylates STAT3 directly [223, 224]. Here, we set-out to investigate whether Abl kinases induce activation of STAT3 in 435s/M14 cells and in WM3248 cells under invasion assay conditions (serum-starved conditions). Inhibition of Abl kinase activity with imatinib inhibited STAT3 phosphorylation in 435s/M14 cells (Figure 2.12A) and in WM3248 cells (Figure 2.12B), and silencing c-Abl inhibited phosphorylation of STAT3 in WM3248 cells (Figure 2.12C). To examine whether Abl kinases induce the direct activation of STAT3 in the case for Src kinases, we overexpressed Flag-tagged wild type-STAT3 in a heterologous system, (HEK-293Tcells) and co-transfected with one of the Abl constructs: wild-type (Abl-WT or Arg-WT), constitutive forms (Abl-PP or Arg-PP), or kinase-inactive mutants of (Abl-KD and Arg-KD). Massive overexpression of Abl wild type is known to induce its activation in 293T cells [225]. Both Abl-PP and Arg-PP induced phosphorylation at STAT3-Y705, whereas the kinase-dead mutants of both c-Abl and Arg failed to induce phosphorylation of the Y705 residue of STAT3, indicating that active form of Abl and Arg induce STAT3 phosphorylation (Figure 2.13A). Abl kinases form a complex with STAT3 as wild-type and constitutively active forms of c-Abl and Arg co-immunoprecipitate with STAT3 in 293T cells (Divyamani Srinivasan, data not shown). To investigate whether c-Abl or Arg directly induced the phosphorylation of STAT3 we performed an *invitro* kinase assay. Following c-Abl-PP and Arg-PP

transfection in 293T cells, c-Abl or Arg was immunoprecipitated and incubated with GST-STAT3 in the presence of radiolabeled ^{32}P-ATP. Both c-Abl and Arg were unable to phosphorylate GST-STAT3 *in vitro* (Figure 2.13B), indicating that STAT3 is not a direct substrate of Abl kinases. Thus, Abl kinases indirectly induce the phosphorylation of STAT3 via a yet to be identified tyrosine kinase.

Since Abl kinases promote activation of MMP-1 and STAT3 in melanoma cells and STAT3 is known to have binding sites in the MMP-1 promoter, we investigated whether Abl kinases upregulate MMP-1 transcription via a STAT3-dependent mechanism. Silencing STAT3 in melanoma cells blocked MMP-1 transcription as assessed by semi-quantitative RT-PCR (Figure 2.14A). Using a constitutively active form of STAT3 (STAT3CA) overexpressed in 435s/M14 cells, we were able to rescue the inhibition of MMP-1 transcription observed following imatinib treatment of vector control cells (Figure 2.14B). These results indicate that STAT3 lies downstream of Abl kinases in regulating MMP-1 transcription.

Since Abl kinases and STAT3 both promote survival of cancer cells, we sought to investigate whether Abl kinases induce survival in a STAT3- dependent manner. Expression of a constitutively active form of STAT3 into 435s/M14 cells partially (30-40%) rescued STI571-mediated PARP cleavage during nutrient deprivation (Figure 2.15) These results indicate that c-Abl and Arg prevent apoptosis in response to serum-deprivation via STAT3-dependent and independent pathways.

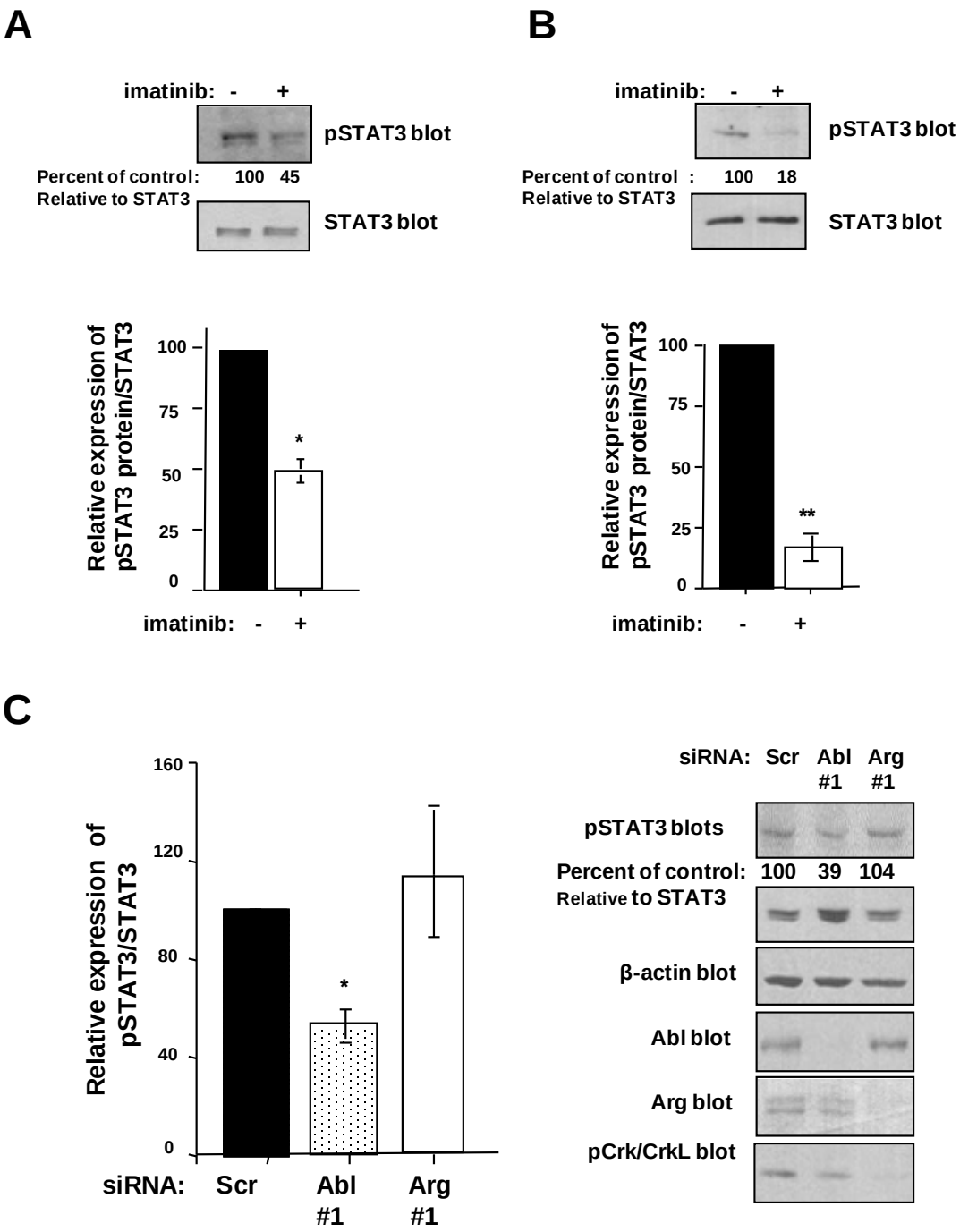


Figure 2.12: Abl kinases promote STAT3 phosphorylation.(**A**) 435s/M14 cells (**B**) WM3248 cells were serum starved, treated with imatinib (10μM) for 48h and lysates probed with the indicated antibodies. (**C**) WM3248 cells were transfected with c-Abl/Arg siRNAs and lysates probed with indicated antibodies. The graphs are Mean± s.e.m, n=3 *p<0.05, **p≤0.01, ***p<0.001. Representative blots are shown

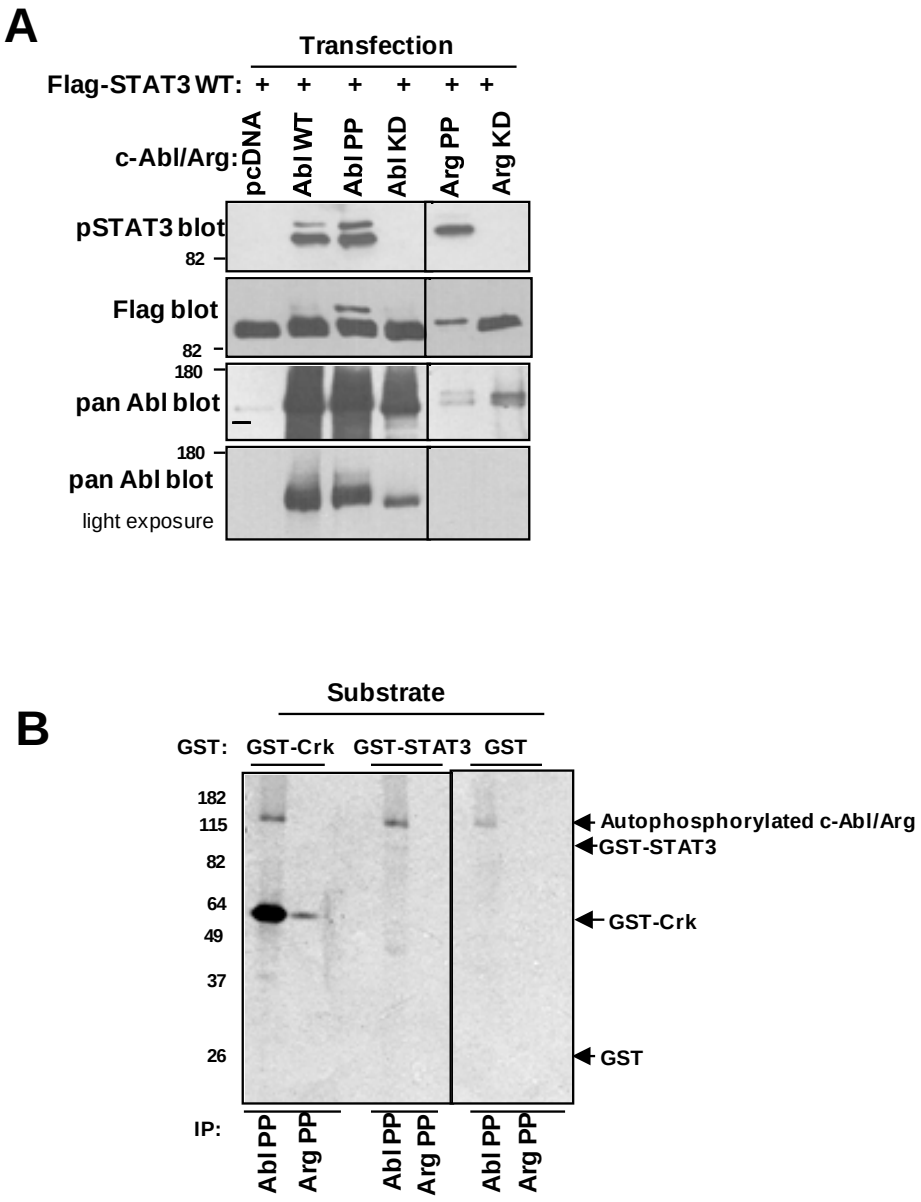
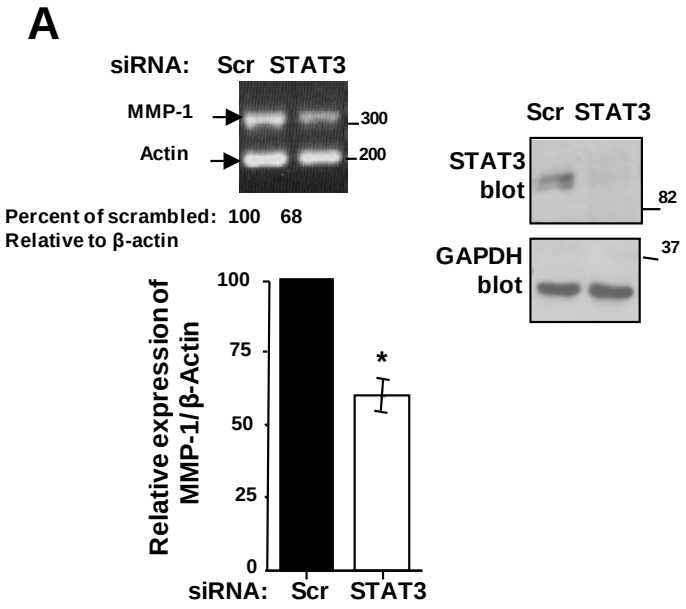


Figure 2.13: Abl kinases form a complex with STAT3, and indirectly induce STAT3 phosphorylation. (**A**) HEK-293T cells were co-transfected with plasmid encoding wild type or flag-tagged STAT-3 together with wild-type (WT), constitutively active (PP) or kinase dead (KD) forms of c-Abl or Arg, lysed and blotted with the indicated antibodies. **(B)** c-Abl and Arg were immunoprecipitated from lysates, and kinase activities were assessed by *in vitro* kinase assay using GST-Crk and GST-STAT3 as substrates. KD=Kinase Dead (K290R). PP=constitutively active Abl/Arg contain a mutation of proline 242 and 249 to glutamine (P242E/P294E). (A) Performed by Dr Divyamani Srinivasan.



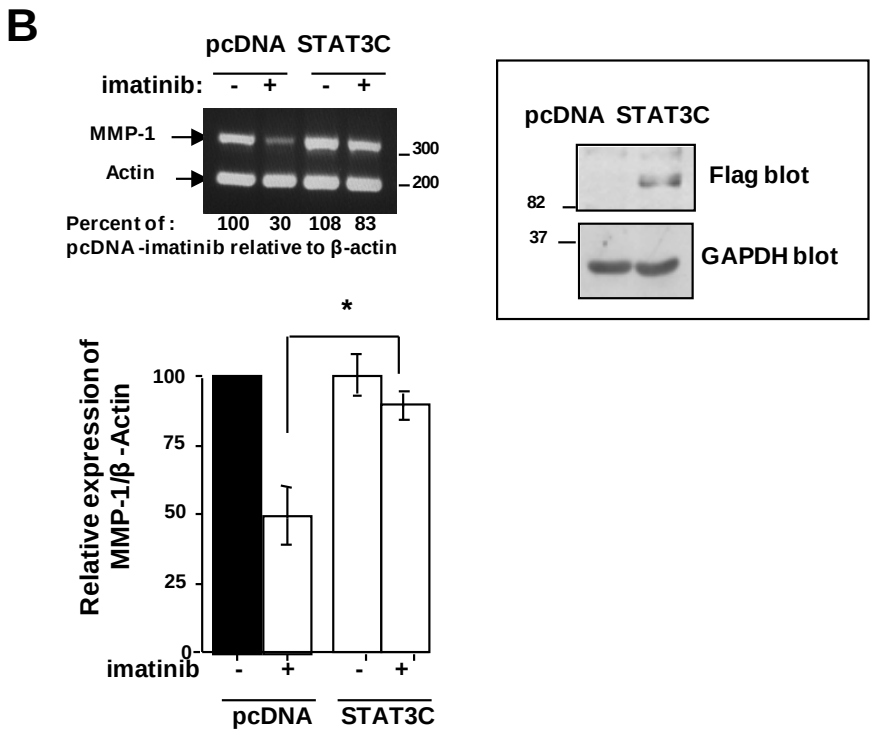


Figure 2.14: Abl kinases promote MMP-1 transcription via STAT3. (**A**) 435s/M14 cells transfected with STAT3 siRNA, were serum starved for 48 hr, and RNA was subjected to semi-quantitative RT-PCR. STAT3 knockdown was analyzed by western blotting. Mean ± s.e.m, n=3; *p=0.05 **(B)** 435s/M14 cells stably transfected with either vector (pcDNA) or a constitutively active form of STAT3 (STAT3C) were plated, serum starved, treated with imatinib for 48h, and MMP-1 transcript was analyzed by semi-quantitative RT-PCR. Mean ± s.e.m, n=3; *p <0.05;

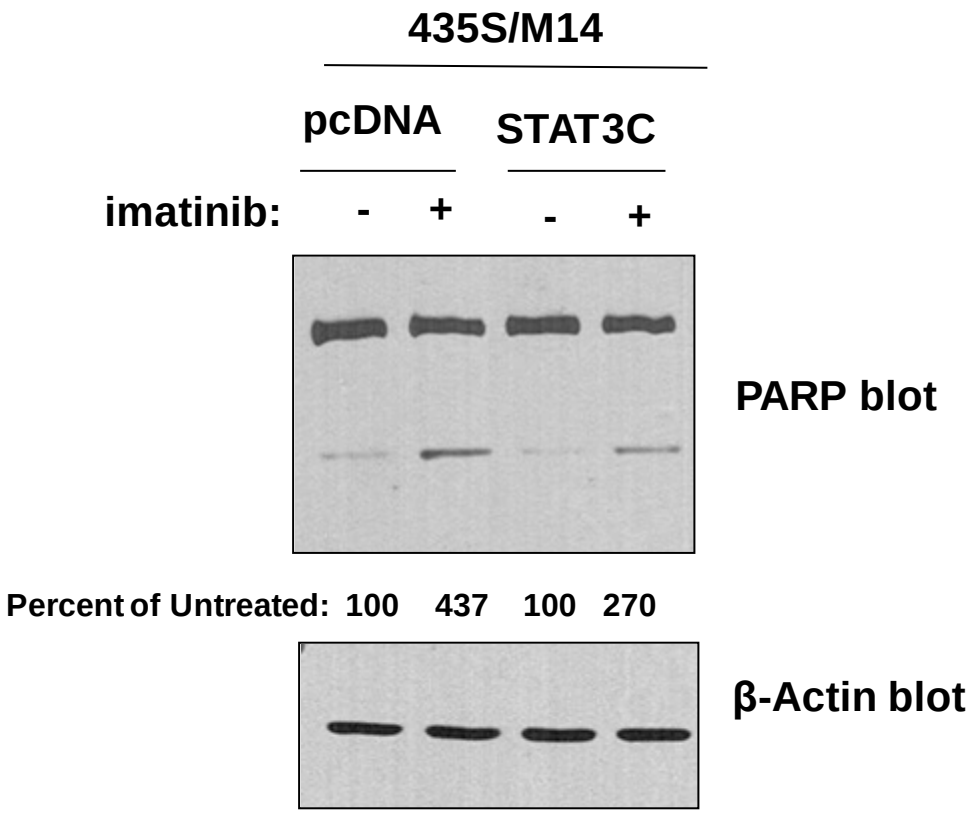


Figure 2.15: Abl kinases promote survival via STAT3-dependent and STAT3-independent pathways. 435s/M14 cells stably transfected with either vector (pcDNA) or a constitutively active form of STAT3 (STAT3C) were plated serum-starved, and treated with imatinib for 60h. Attached and detached cells were lysed and blotted with indicated antibodies

2.3.5 Abl Kinases promote TIMP-1 expression via a STAT3-dependent mechanism

TIMP-1 is an endogenous inhibitor on MMP-1, and has been reported to inhibit cancer cell invasion. To evaluate whether upregulation of MMP-1 by Abl kinases is dependent on suppression of the endogenous MMP-1 inhibitor, we wanted to determine the effect of inhibition of Abl kinases on TIMP-1 mRNA and protein levels in 435s/M14 cells. Inhibition of Abl kinases with imatinib significantly reduced TIMP-1 transcript and protein levels (Figure 2.16 A and B), indicating that Abl kinases promote TIMP-1 expression, contrary to our hypothesis that Abl kinases downregulated the expression of endogenous MMP inhibitor.In addition to negatively regulating MMPs, TIMPs also have been shown to have anti-apoptotic functions, and high TIMP-1 expression is correlated with decreased survival and a poor clinical outcome in breast, ovarian, lung and colon cancer patients [135,139]. Thus, it is possible that TIMP-1 may have anti-apoptotic functions in melanoma cells, and activated Abl kinases may induce this function by increasing TIMP-1 expression and secretion.

To investigate the molecular mechanism by which Abl kinases-mediate upregulation of TIMP-1, we investigated whether STAT3 can upregulate TIMP-1 transcription in 435s/M14 cells. Silencing STAT3 significantly downregulated TIMP-1 transcript levels (Figure 2.16C). Moreover, expression of a constitutively activate form of STAT3C rescued the effect of Abl kinases inhibition on TIMP-1 transcription and expression (Figure 2.17A and B), indicating that Abl kinases promote TIMP-1 expression via a STAT3-dependent pathway.

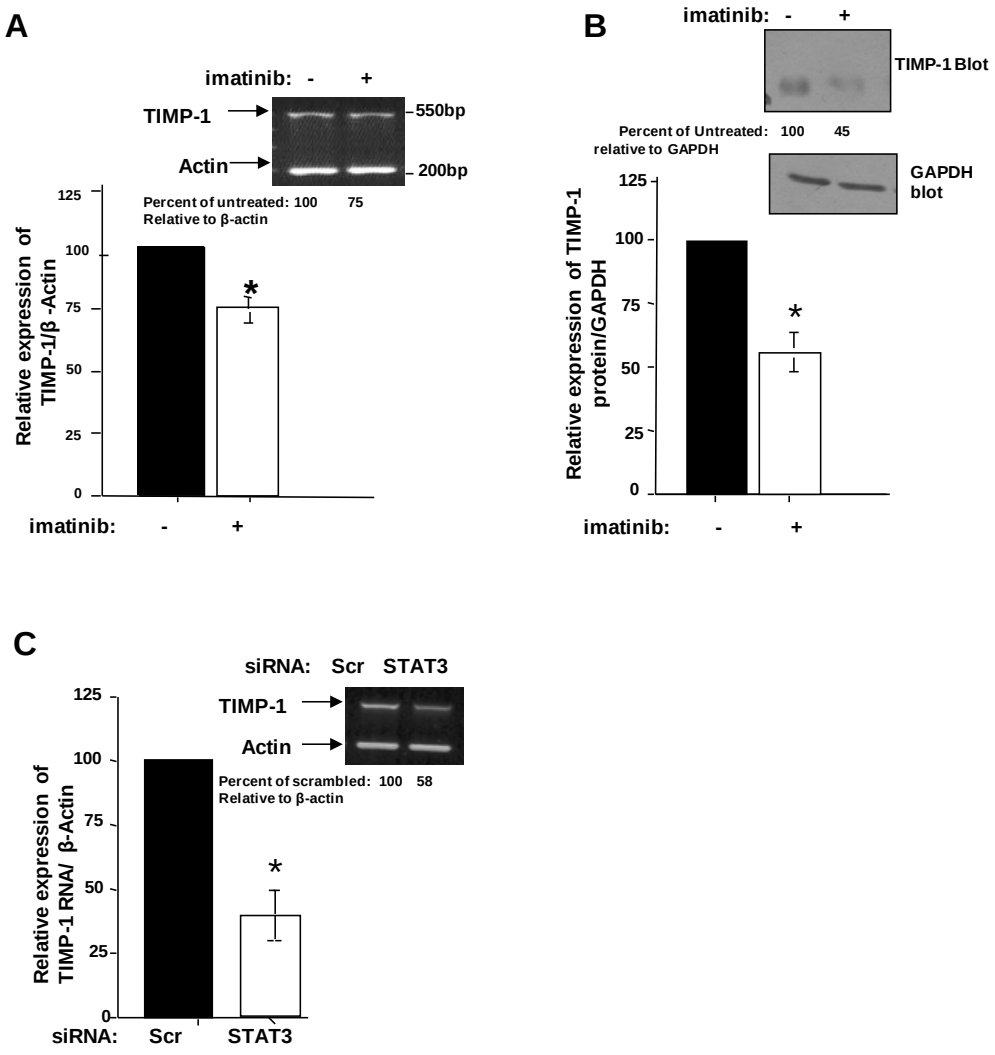


Figure 2.16: Abl kinases upregulate TIMP-1expression. 435s/M14 cells were plated, serum starved, and treated with the Abl inhibitor, imatinib (**A-B**) or cells were plated, transfected with siRNA against STAT3 or a scrambled control (Scr), serum starved for 48h, and extracted RNA was subjected to semi-quantitative RT-PCR. (**B**) Attached and detached cells were lysed and blotted for β-Actin, the media was collected concentrated, and loaded according to the β-Actin blot from cellular lysate. The graphs are Mean± s.e.m, n=3 *p<0.05, **p≤0.01, ***p<0.001. Representative blots are shown.

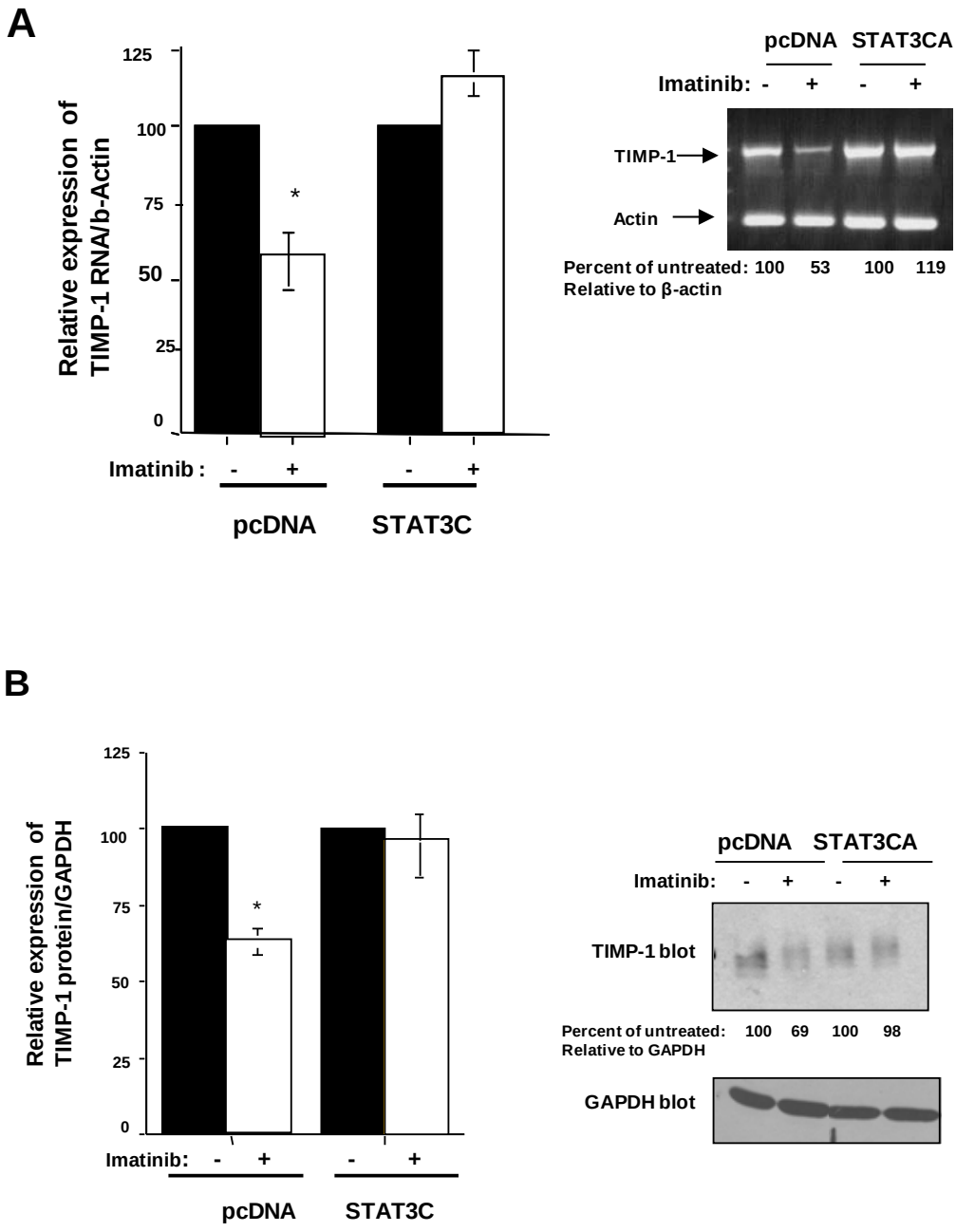


Figure 2.17 Abl kinases promote TIMP-1 expression via STAT3-dependent pathway. (A,B) 435s/M14 cells stably transfected with either vector (pcDNA) or a constitutively active form of STAT3 (STAT3C) were plated, serum starved, treated with imatinib for 48h, and TIMP-1 transcript (**A**) and protein (**B**) was analyzed by semi-quantitative RT-PCR and by western blot of conditioned media respectively, Mean ± s.e.m, n=3; *$0.01 \leq p < 0.05$; **$0.001 \leq p < 0.01$. Representative blots shown.

2.3.6 c-Abl promotes invasion via a STAT3-dependent MMP-1 pathway whereas Arg increases invasion in a STAT3-independent manner through MMP-1 and MMP-3

Silencing either c-Abl or Arg significantly inhibited invasion in melanoma cells, indicating that c-Abl and Arg are required for melanoma cell invasion. Inhibition of Abl kinases significantly downregulated STAT3 activation in 435s/M14 and WM324 cells, indicating that Abl kinases activate STAT3 in melanoma cells. Since silencing STAT3 in 435s/M14 cells also decreased invasion (Figure 2.18A), we investigated whether c-Abl and Arg mediate invasion in a STAT3-dependent manner. Matrigel invasion assay were conducted after silencing c-Abl or Arg in 435s/M14 cells expressing a constitutive expression of the active form of STAT3 (STAT3CA). Silencing c-Abl or Arg inhibited invasion in vector control cells (Figure 2.18B) whereas in STAT3CA cells, silencing of c-Abl failed to inhibit invasion to the basal level (28% inhibition). In contrast silencing Arg reduced invasion to basal levels as seen in the vector control cells (Fig 2.18B). Thus, STAT3CA significantly rescued the block in the invasion induced following silencing c-Abl, indicating that c-Abl drives invasion in a STAT3- dependent manner and Arg promotes invasion in a STAT3-independent manner. c-Abl and Arg were efficiently silenced in vector-control and STAT3CA cell lines (Figure 2.18B right) indicating that the results observed were not due to inefficient inhibition of c-Abl/Arg in 435s/M14-STAT3CA cells.

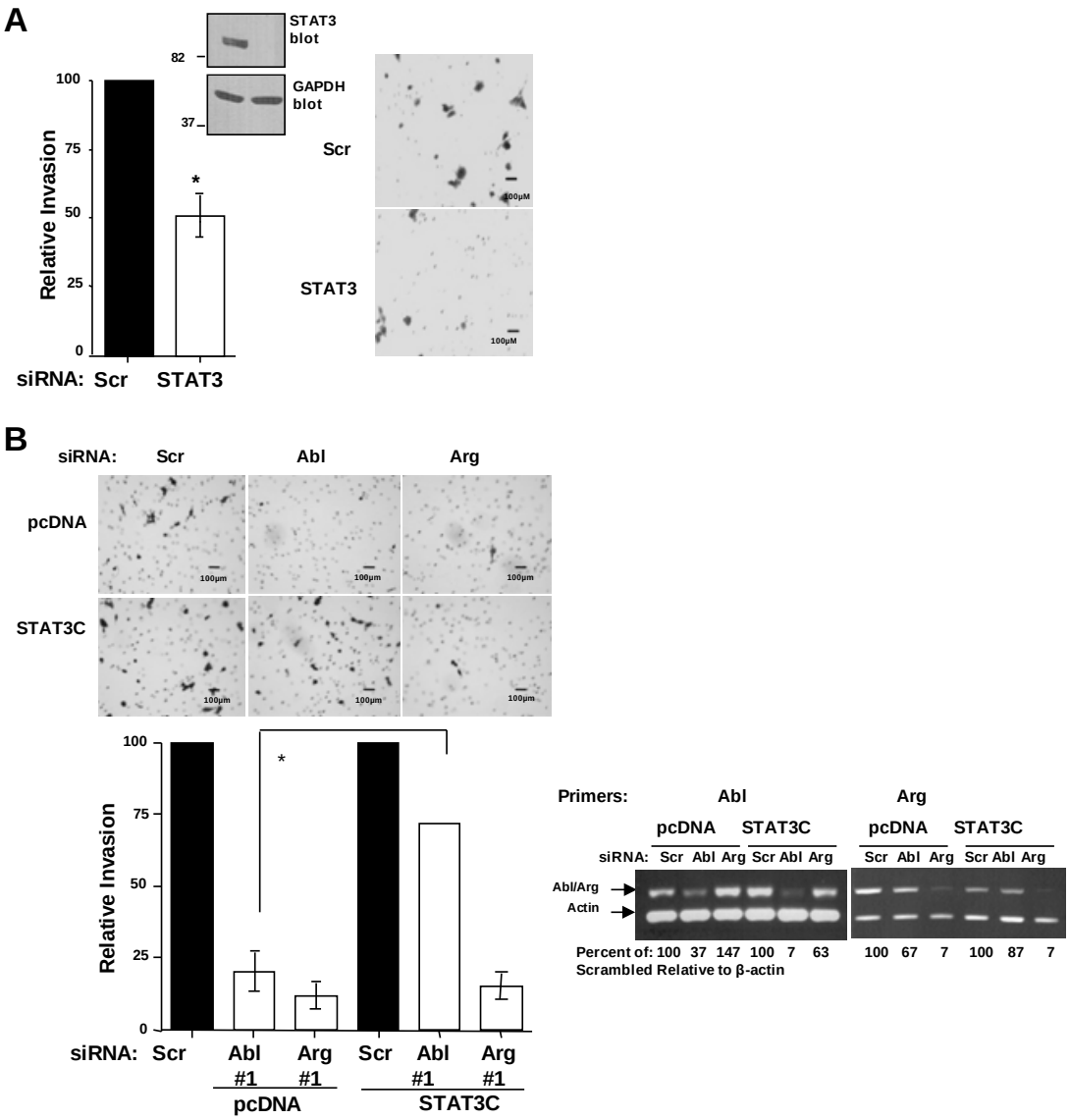


Figure 2.18: c-Abl promotes invasion in a STAT3-dependent manner. (A) 435s/M14 cells were transfected with siRNA against STAT3 or a scrambled control (Scr) and invasion assays were performed. Knockdown efficiency was determined by taking an aliquot from cells used in the assay and probed for indicated antibodies (B). Invasion assays were performed on 435s/M14 cells stably expressing vector or STAT3C, transfected with c-Abl/Arg siRNAs. Knockdown efficiency was determined by semi-quantitative RT-PCR using c-Abl/Arg primer. Mean ± s.e.m, n=3, normalized to scrambled. *$0.01 \leq p < 0.05$; **$0.001 \leq p < 0.01$. Pictures were taken in 100x magnification.

Abl kinases promote the transcription and activation of MMPs, and MMP-1 and 3 drive invasion in 435s/M14 cells (Figure 2.8-2.10). Thus, to evaluate whether c-Abl and Arg promote invasion in a MMP-1 and MMP-3 dependent manner, we performed rescue invasion assays. Constitutive expression of MMP-1 in 435s/M14 cells significantly rescued the block in invasion following inhibition of c-Abl or Arg, however the rescue observed was partial (40%). Using a complementary approach, we also treated the upper well of invasion chambers with recombinant active MMP-1 in cells silenced with c-Abl or Arg and observed the same level of rescue as seen with cells over-expressing the MMPs (Figure 2.19B). Since the rescue was partial using recombinant MMP-1, we hypothesized that MMP-3 might also play a part in Arg-mediated invasion. Using recombinant MMP-3, we found that inhibition of invasion was partially rescued in Arg silenced, 435s/M14 cells (Figure 2.19C). These results indicated that c-Abl mediates invasion via a STAT3 and MMP-1-dependent pathway, whereas Arg drives invasion in a MMP-1 and MMP-3-dependent pathway, which is STAT3-independent. These results also show for the first time that that c-Abl and Arg promote invasion through distinct mechanisms.

2.3.7 Activated Abl kinases promote late stages of melanoma metastasis

Cancer cells invade the extracellular matrix (ECM), intravasate into the blood vessels, extravasate out of the blood vessels to ectopic sites, seed, and

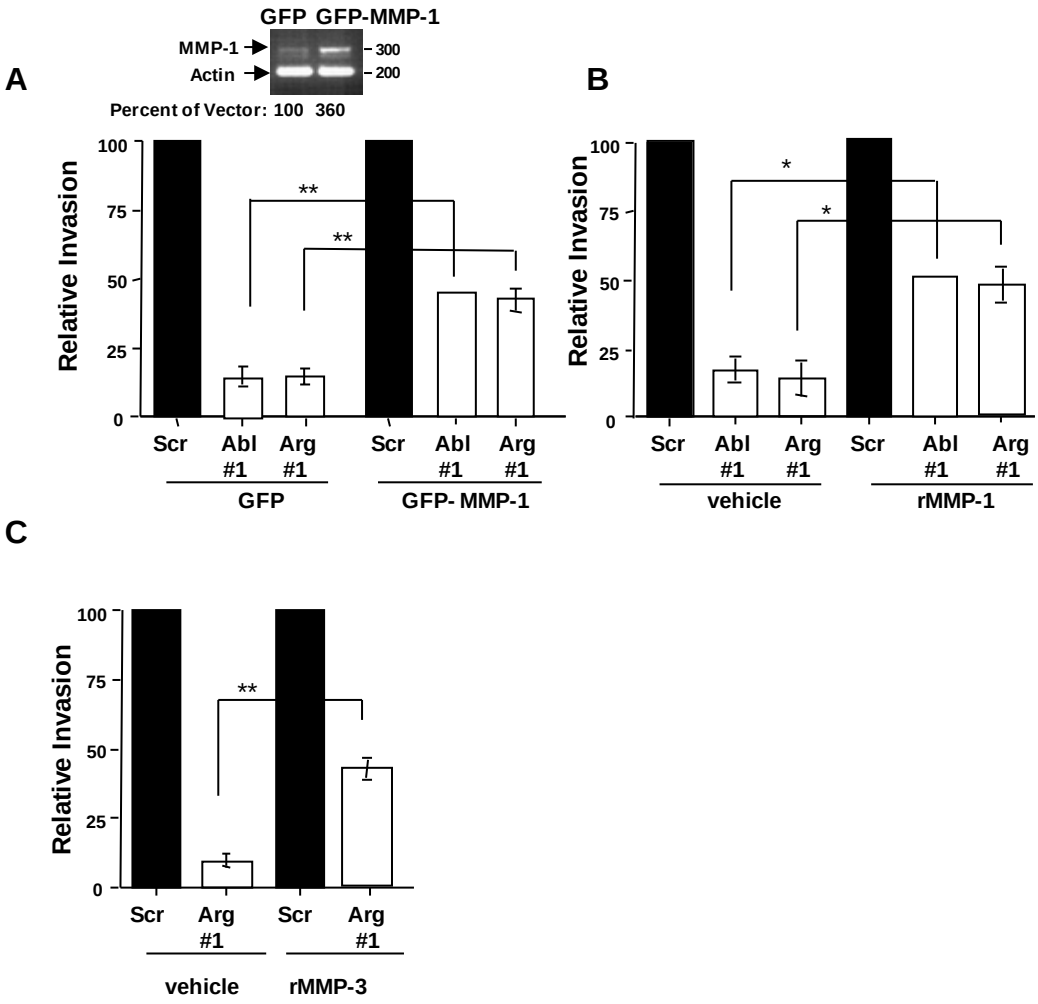
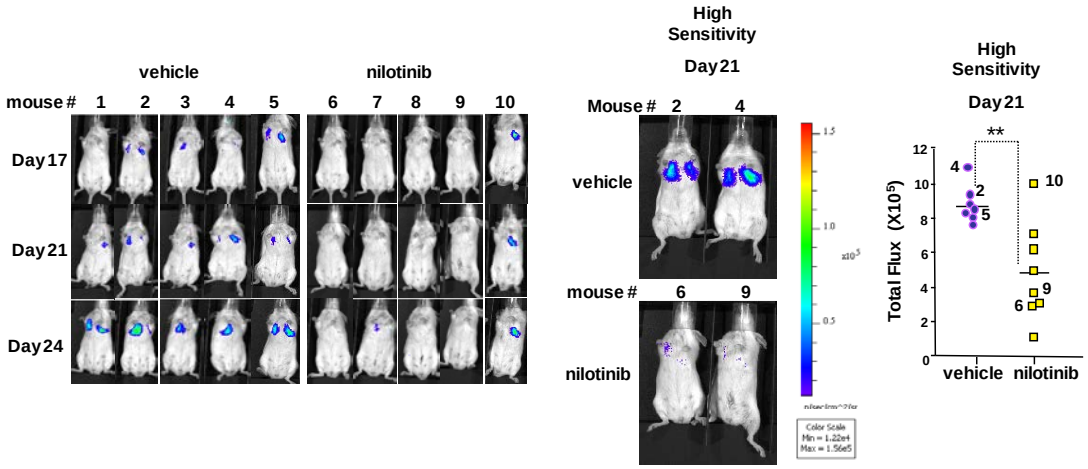


Figure 2.19: c-Abl and Arg promote invasion in a MMP-dependent manner. (**A**).435s/M14 cells stably expressing GFP or GFP-MMP-1 were transfected with c-Abl or Arg siRNAs and invasion assays were performed. The efficiency of MMP-1 overexpression was determined by semi-quantitative RT-PCR (**B** and **C**). 435s/M14 cells transfected with c-Abl or Arg siRNAs were incubated with recombinant MMP-1 or MMP-3 (25 ng/ml) during the invasion assays. Mean ± s.e.m, n=3, normalized to scrambled. *0.01 ≤ p <0.05; **0.001 ≤p<0.01.

proliferate to form tumors in the ectopic sites. This process is termed as metastasis. In experimental metastasis assay, cells are injected directly in the blood stream, via a tail vein injection. In this process, the cancer cells bypass the few initial steps of cancer metastasis cascade, like primary tumor growth, invasion through the extracellular matrix, and intravasation into the blood stream. However, the cells still have to extrasavate out of the blood stream and invade the new organ site to form micrometastasis in the ectopic site, survive in nutrient free conditions, and proliferate to form macrometastasis. Since c-Abl and Arg promoted proliferation, invasion and survival *in-vitro* conditions we assessed whether active Abl kinases drive late stages of melanoma metastasis by performing experimental metastasis assays. 435s/M14 cells tagged with GFP and luciferase was injected into the tail vein of SCID-beige mice. As shown in Figure 2.20A, in experiments using 435s/M14 cells vehicle-treated mice had metastatic lung colonization from Day 17 to Day 24, indicating that the cells are proliferating in the lung microenvironment. Treatment of mice with the c-Abl and Arg inhibitor, nilotinib, significantly decreased lung metastasis, as measured by IVIS imaging. Quantifying metastasis-using IVIS imaging at Day 21, we found a significant decrease in lung metastasis in nilotinib treated mice, as compared to vehicle treated mice (Figure 2.20A right). Immunohistochemical analysis of nilotinib responsive mice lungs indicated a decrease in the phospho Crk/CrkL staining as compared to lungs from vehicle-treated. A mouse that did not respond to nilotinib (#10) had minimal suppression of c-Abl and Arg activity

A



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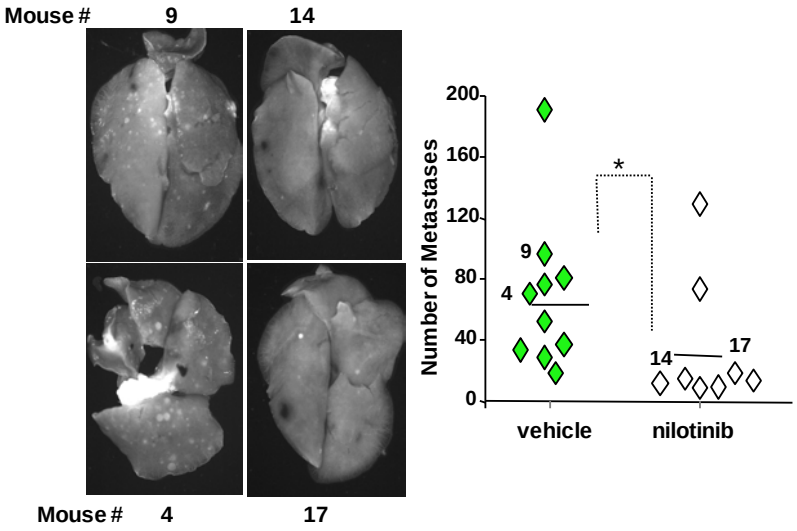


Figure 2.20: Activation of Abl kinases promote melanoma metastasis (**A**) 435s/M14 cells, labeled with GFP and luciferase ($2X10^6$), were injected into the tail vein of SCID-beige mice. Mice were treated with vehicle or the pharmacological Abl kinase inhibitor, nilotinib (30mg/kg; b.i.d) by oral gavage, and imaged with IVIS following luciferin D injection (i.p.). (**A**; left) IVIS imaging of representative mice on day 17, 21 and 24 post-injection. (**A**; right) Representative mice on day 21 imaged shown with high integration such that low-level fluorescence could be detected with quantification below. Numbers refer to mouse numbers shown in (A). **$0.001 \leq p < 0.01$. **(B)** WM3248 cells expressing GFP, were injected intravenously (tail vein) into nude mice, and lungs were fixed, photographed and metastases quantified on day 34.*$p<0.05$ using a student's t-test.

(Figure 2.21). Furthermore, inhibition of c-Abl/ Arg activity was inversely correlated with IVIS fluorescence in all the nilotinib-treated mice, indicating that the anti-metastatic capacity of nilotinib is linked to inhibition of c-Abl and Arg kinase activity.

To validate our findings we extended our study to another melanoma cell line, WM3248. Consistent with our data using 435s/M14 cells, inhibition of Abl kinase activities with nilotinib for a period of 34 days significantly reduced lung metastasis of WM3248/GFP cells in a nude mouse model, as assessed by scoring GFP-fluorescing metastatic nodules under a fluorescent dissecting scope (Figure 2.20B). Summarizing these results, we can conclude that nilotinib decreased melanoma metastasis by specifically inhibiting c-Abl and Arg, and for the first time we report that active Abl kinases promote melanoma metastasis.

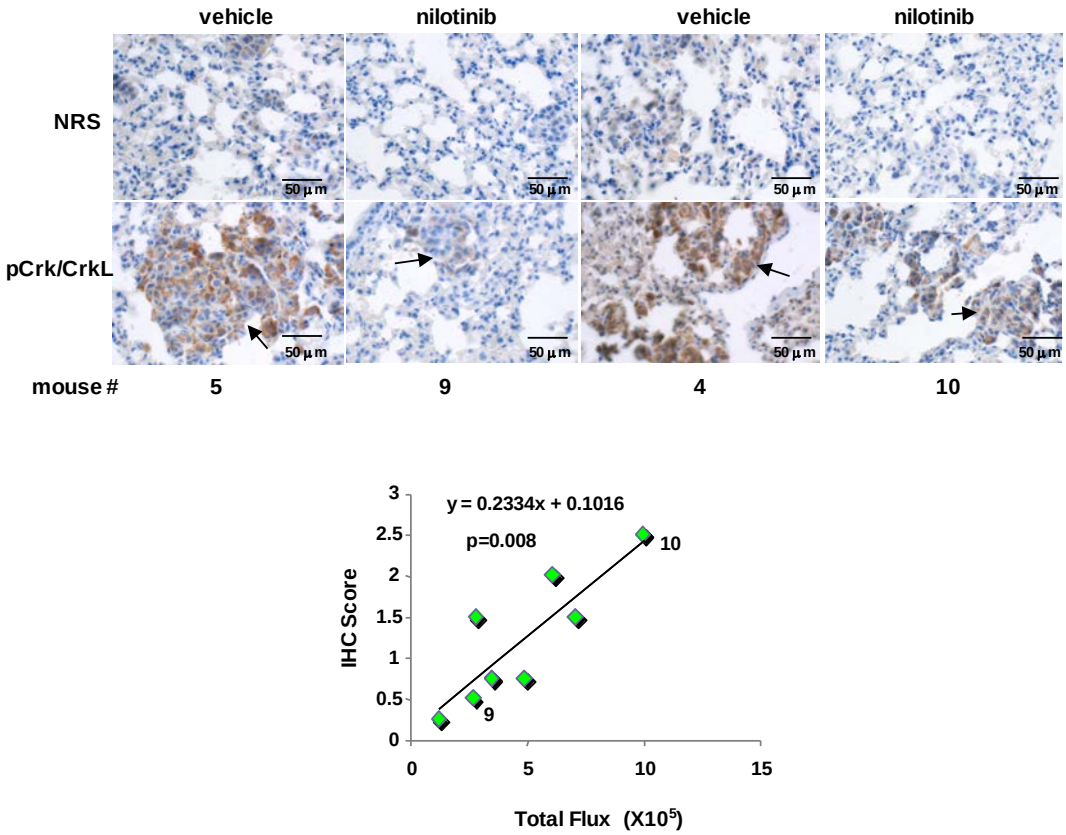


Figure 2.21: Positive correlation between Abl kinase activity and metastasis burden in nilotinib-treated mice. Lungs from the indicated mice (graphs is in Figure 2.20 A) were fixed in formalin on day 24, paraffin-embedded, sectioned, stained with phosphorylated Crk/CrkL (bottom panel), or normal rabbit serum (top panel). Photographs are 400X magnification. The IVIS fluorescence values from Figure 2.20 A were plotted against pCrkL/Crk score from the lung metastases in nilotinib treated mice. A positive correlation was noticed. Lungs were sectioned, and stained by Leann Fiore and Matthew Thacker

2.4 Discussion

Malignant melanoma accounts for about three fourths of all skin cancer deaths, and is refractory to all known treatments. Although there is evidence for the presence of activated Abl kinases in several solid tumors, activation of Abl kinases in melanoma had, as of yet, remained unexplored. In this study, we report that c-Abl and Arg kinases activities are elevated in some primary melanoma tumor samples, as compared to benign nevi, and that many human metastatic melanoma cell lines have higher c-Abl kinase activity as compared to non-metastatic cell lines and primary melanocytes. These results suggest that activation of Abl kinases in melanoma is one of the prerequisite steps in melanoma metastasis and progression. Activating B-RAF mutations are seen in 80% of benign nevi [226] cases whereas Abl kinases are elevated in only 33% of cases, indicating that unlike B-RAF, activated Abl kinases probably do not play a role in the melanoma initiation, but may play a role in later stages, such as transforming cells from the RGP to VGP stage. Our work also establishes a link between Abl kinases and metalloproteinases. MMPs are critical in converting non-invasive RGP to invasive VGP, so c-Abl and Arg are likely to have a role in this process. This study also reports for the first time that active Abl kinases promote invasion, proliferation, and survival, and late stages of cancer metastasis (experimental metastasis) of two human melanoma cell lines. Thus, Abl kinases may be considered a novel drug-able target in metastatic melanoma patients harboring high Abl kinases activities.

c-Abl and Arg kinase activities are highly elevated in aggressive melanoma cells (WM3248 and 435s/M14), but, in low invasive cells, like WM239, c- Abl and Arg activities are low. We show that in 435s/M14 and WM3248 cells harboring high c-Abl and Arg kinase activities, inhibition of Abl kinases by nilotinib suppressed invasion to comparably, but in WM278 cells, which have minimal to no Abl kinase activities, there was no inhibition of invasion observed after nilotinib treatment. These results indicate that active Abl kinases are necessary to drive the process of invasion in melanoma cells. In order to test whether activation of Abl kinases is sufficient to drive the process of invasion in melanoma, future experiments we will need to test whether stable constitutively activated Abl kinases increases invasion of non/low invasive cells (WM239).

Our results indicate that Abl kinases promote melanoma proliferation in contrast to their role in other cell types such as fibroblasts. Overexpression of c-Abl causes G_1 cell cycle arrest in fibroblasts, while we found that activation of Abl kinases enhances G_1/ S phase transition in 435s/M14 cells [55] and inhibition of Abl kinases resulted in a block in proliferation in breast cancer cells. We found that in 435s/M14 cells, inhibition of Arg but not c-Abl decreased proliferation [55]; however interestingly, in WM3248 cells both c-Abl and Arg promoted proliferation. Furthermore, active c-Abl and Arg also increased proliferation in a panel of melanoma cell lines. It is intriguing that the contribution of c-Abl and Arg to proliferation differs in 435s/M14 and WM3248 cell lines since both these lines have the same level of c-Abl and Arg kinase activation and both regulate invasion in a similar fashion. In addition the finding that active Abl kinases drive

proliferation in a panel of melanoma cell lines indicates that Abl kinases are indispensable for driving proliferation in melanoma. Since, proliferative index is usually considered a standard for identifying patients who have a high risk of developing metastasis; proteins that can drive proliferation are good markers for targeted chemotherapy [227, 228]. Cancer cells after they extravasate out of the blood vessels survive and have to proliferate to form micrometastasis and proliferate further to form macrometastasis. Hence, Abl kinase inhibitors might be able to be used to block melanoma progression in a select group of patients having activated Abl kinases.

Abl kinases promote breast cancer cell survival during nutrient deprivation [55], and here, for the first time, we report that Abl kinases also promote the survival of melanoma cells during the stress of nutrient deprivation. Apoptosis is a major hindrance in tumor growth and metastasis, as tumor cells have to survive in nutrient free conditions after they extravasate out of the blood vessels. Drugs that promote apoptosis are attractive agents in the clinic to prevent metastasis [229,230]. Thus our data indicate that the Abl kinase inhibitor, imatinib, may hold promise in treating patients with metastatic melanoma.

Recently, Smith-Pearson et al reported that Abl kinases promote gelatinase activity of MDA-MB-231 breast cancer cells and Src-transformed NIH3T3 cells. The authors indicated that the mechanism involved Abl-dependent phosphorylation of MMP-14 and subsequent localization to the plasma membrane. They also showed that, in 239T cells, overexpression of c-Abl and Arg induced the phosphorylation of MMP-14 and silencing Arg inhibited the

localization of MMP-14 in the plasma membrane [62].However, the authors failed to show endogenous c-Abl/MMP-14 complexes and Abl-dependent tyrosine phosphorylation of endogenous MMP-14 in human cancer cells. Here, we show that Abl kinases promote the transcription and activation of MMPs, including MMP-14. c-Abl induces the transcription and activation of MMP-1 while Arg induces the transcription and activation of MMP-1, 3 and MMP-14. We also show that silencing c-Abl and Arg inhibited single-cell 3D single cell invasion, due to the inability of these cells to degrade the collagen bed. These results indicate that Abl kinases promote collagenase activity of melanoma cells, an important prerequisite for promoting invasion. Since collagen I is an important constituent of blood vessels and extracellular matrix, thus indicates that activation of Abl kinases is likely to be important for melanoma cells to invade in (intrasavation), and out of blood vessels (extrasavation).

Our results demonstrate that c-Abl and Arg form a complex with STAT3 and induce its phosphorylation. In our previous studies, we showed that Abl kinases activate STAT3 in a JAK-independent manner [55]. Here we report that the phosphorylation is not direct but through a yet to be identified kinase. Phosphorylation of STAT3 at Y705 residue correlates with the transcriptional activity of STAT3 [232], and thus, Abl kinases are likely to induce the transcriptional activation of STAT3. Abl kinase signaling via STAT3 is required for melanoma cell survival as expression of a constitutively active form of STAT3 partially rescues the block in survival of melanoma cells treated with imatinib. Since this rescue is partial, it indicates that Abl kinases also promote survival via

a STAT3-independent mechanism as well. The PI3K/AKT pathway is one of the major survival pathways studied in melanoma and selective activation of Akt3 has been shown to promote melanoma cell survival [231,233]. These data indicate that future studies should be directed to investigate this pathway as a possible additional mechanism by which Abl kinases may promote survival in response to nutrient deprivation. STAT3 plays a critical role in tumor progression by controlling important processes such as proliferation, survival, and invasion. STAT3 has been reported to be involved in tumorigenesis in many types of cancer, including melanoma, and silencing of STAT3 signaling inhibited melanoma growth in mice [74, 75, 79, 82, 86, 91]. Thus, it is possible that many of these effects might be regulated by Abl kinases-dependent STAT3 activation. Activation of STAT3 and MMPs is critical for transforming non-invasive RGP melanomas to invasive VGPs. Since Abl kinases modulate the activity of STAT3 and MMPs, they may also play a critical role in RGP to VGP transition.

Arg-dependent proliferation in 435s/M14 cells does not occur via STAT3 signaling (demonstrated by Divyamani Srinivasan), indicating that not all properties of Abl kinase-mediated oncogenesis occurs via Abl kinase-mediated activation of STAT3. Abl kinases are known to promote PDGF-mediated cell cycle progression by activating the Rac/JNK pathway and upregulating c-myc expression [234] ; thus Arg might promote proliferation via such a pathway, which is independent of STAT3. Phosphorylation of STAT3 by Abl kinases might also be important in other biological processes known to be mediated by c-Abl/Arg and STAT3, such as anchorage independent growth.

Our findings show that activated Abl kinases upregulate invasion in melanoma cells (Figure2.3 and [55]). Here, we report for the first time, that c-Abl and Arg signal through divergent pathways to mediate the same biological function. c-Abl signals through a STAT3/MMP-1 pathway to promote invasion of melanoma cells (Figure 2.18 and 2.19), whereas Arg promotes invasion in a STAT3-independent manner via MMP-1 and MMP-3. We also demonstrate that c-Abl activates STAT3, which promotes invasion in a MMP-1-dependent manner. STAT3, activated by EGF stimulation, has been shown to bind to the MMP-1 promoter by interacting with the AP-1 component c-Jun, and promotes bladder cancer cell invasion and metastasis [222]. Thus, it would be interesting to investigate whether STAT3 interacts with an AP-1 element to promote MMP-1 transcription in an IGF-1R-dependent manner since 435s/M14 cells express IGF-1R receptors, rather than EGFR [55], and IGF-1 activation of STAT3/MMP-1 has not yet been demonstrated.

Although, we have identified the mechanism by which c-Abl promotes invasion, it is unclear how Arg promotes MMP expression and invasion. It is possible that Arg might increase invasion through modulation of Akt-2/Protein Kinase B, or via the transcription factor NF-κB. Activation of Akt is linked to the activation of NF-κB in melanoma [235]. Overexpression of Akt2, in MDA-MB-435 cells increases invasion through matrigel [236], and thus, it is possible that Arg may signal through Akt-2 to regulate invasion in melanoma cells. Indeed, we found that Abl kinases upregulate phospho-pAkt levels and Akt isoforms 1, 2, and 3 in 435s/M14 cells (S. Ganguly, unpublished data). In addition, our lab

recently showed that Abl kinases promote transcriptional activation of the p65 subunit of NF-κB [237]. MMP-3 is activated by NF-κB, although there no NF-κB binding site has been identified in the MMP-3 promoter region [238], indicating that this interaction is likely to be indirect and mediated by yet another yet to be identified transcription factor. Thus future experiments will test whether Arg mediates invasion through a NF-κB and/or PI3K/Akt-dependent pathway.

There is currently controversy in the literature regarding the role of Abl kinases in solid tumors. Studies from our lab and others have shown that Abl kinases are activated in some solid tumors, and once activated they promote invasion, anchorage-independent growth, proliferation, and survival in cancer [54]. Although there are numerous papers substantiating our finding that Abl kinase activation promotes solid tumor progression, some data from other laboratories demonstrate that Abl kinases inhibit invasion and metastasis progression [239,240,241]. However, these studies did not examine the activation status of Abl kinases, or have inhibited Abl kinases in cells having low Abl kinase activity. One study showed that treatment of MDA-MB-435 cells with ephrinB2, transiently activated EphB4 and c-Abl/Arg, which inhibited proliferation, invasion, MMP expression and xenograft tumor growth, and these effects were blocked by inhibition of Abl kinases by imatinib [241]. In this case Abl kinase-mediated downregulation of invasion could be also explained by the fact that, unlike growth factors which promote invasion, this stimulus ephrin-B2/EphB4, inhibits invasion. Moreover, the stimulation with ephrin B2 may not be physiological relevant in this context as ephrinB2 expression is lost in metastatic cancer cells [241].

Our studies show that inhibition of Abl kinases, using nilotinib, abrogated the process of experimental metastasis. However, there are other reports that have demonstrated some contrasting evidence in Abl kinases-mediated breast cancer tumor growth, although they did not investigate the effects on metastasis[239,240] However, their contrasting results can be attributed to the use of mouse cell rather than human cancer cells and also overexpression of a mutated, constitutively active form of c-Abl in mouse cancer cell lines. This constitutive, mutated form of c-Abl is naturally not present in solid tumors. These authors also showed that low doses of imatinib did not have any effect on tumor growth in mice. These results may have been obtained due to inadequate imatinib dosing. The half-life of imatinib mesylate is 4 to 5h, and plasma levels decrease to less than 1% in 12 h [242] and, the authors only used low doses of imatinib once daily. It is also possible that imatinib might not inhibit Abl kinases at the threshold level needed to inhibit metastasis, and a more specific Abl kinase inhibitor like nilotinib, as used in our study is required to inhibit metastasis in an animal model. In support of this hypothesis, we found that imatinib mesylate could not inhibit experimental metastasis of 435s/M14 cells (data not shown). Since Abl kinases promote experimental metastasis and invasion, we can hypothesize that Abl kinases will also upregulate spontaneous metastasis, which involves invasion of cells from the primary tumor, intravasation, and all the other steps involved in experimental metastasis. Future experiments are aimed at testing this hypothesis.

Our study reports for the first time, that Abl kinases are activated in metastatic melanoma, promote many important steps of cancer progression *in vitro*, and promote late stages of metastasis. Thus, our data indicate that targeting Abl kinases with nilotinib may be effective for treating metastatic melanoma. Unlike the successful targeting of c-Kit, with imatinib in gastrointestinal tumors, a number of clinical trials using imatinib have not been successful [54,243,244]. However, there were many limitations in these clinical trials. First of all, these trails were conducted on untargeted populations, where the activation status of Abl kinases in tumors was not evaluated. Secondly, maximal doses of imatinib were not used to inhibit Abl kinases in these patients, and thus, it isn't clear whether the kinases were inhibited. Here, we demonstrate that nilotinib inhibit metastasis, whereas imatinib failed, which could be due to increased potency and selectivity of nilotinib towards Abl kinases [46]. Hence, clinical trials using nilotinib should be conducted on patients whose melanomas harbor active c-Abl and Arg. In summary, even though there is some conflicting evidence for the use of imatinib or nilotinib in solid tumors, our work indicates that in metastatic melanoma patients whose tumors are positive for active Abl kinases, c-Kit, and/or PDGFR might benefit from nilotinib treatment.

CHAPTER 3: Abl kinases Promote Invasion Via Cathepsin-Mediated Lysosomal Degradation of A Metastasis Suppressor, NM23-H1

3.1 Introduction

NM23-H1, the first metastasis suppressor identified, has decreased expression in metastatic cancer cell lines. Its expression also is inversely correlated with metastatic progression in a large number of cancers. Although NM23-H1 is extensively studied in suppressing cancer progression, no mechanism has been elucidated for its loss of expression metastatic cancers like melanoma, breast and colon. It also is not known how NM23-H1 is degraded, and how its re-expression can be induced in metastatic tumors to inhibit cancer progression [145-150].

Cathepsins, known to promote invasion and metastasis of cancer cells, can also degrade proteins in acidic lysosomes. In cancer cells, cathepsin expression is dramatically increased [177,178,190]. In melanoma, overexpression of cathepsin leads to disease propagation and cathepsin B and L are critical for melanoma progression. Extracellular cathepsin B and L are secreted by tumor cells, and as a result promote invasion and metastasis by degradation of extracellular matrix proteins, activating proteases (MMP) or by inactivating TIMPs [178,182,190,192,194,195]. Although, the role of intracellular cathepsins in cancer cell invasion and metastasis is not well studied, few studies indicate that intracellular cathepsins promote invasion perhaps by degrading collagen or by degrading proteins involved in invasion/progression such as E-

cadherin [179,204]. Recently it has been reported that Abl kinases upregulate the expression of cathepsin B and L in lung cancer cells, resulting in autophagy [245], indicating that Abl kinases promote cathepsin activation and thus, most likely also affect the endocytic/lysosomal pathway.

In Chapter 2, we elucidated the mechanism by which c-Abl promotes invasion by demonstrating that it activates a STAT3-dependent MMP-1 pathway, whereas Arg promotes invasion in a STAT3-independent, MMP-1 and 3-dependent manner. However, the rescue we observed with MMP-1 overexpression or recombinant protein is partial (40%), indicating that there is likely another parallel pathway that Abl kinases affect to regulate invasion in melanoma cells.

In a uveal model of melanoma, imatinib treated tumors cells, when extracted and cultured *in vitro,* showed an increase in the expression of KISS1, a metastasis suppressor gene, indicating that inhibition of Abl kinases might promote the expression of metastasis suppressor genes [73]. Given that Abl kinases promote melanoma invasion and progression whereas NM23-H1 inhibits progression, we hypothesize that Abl kinases promote melanoma progression by downregulating the metastasis suppressor NM23-H1.

This chapter will address if Abl kinases can downregulate the expression of NM23-H1 in melanoma cells, and will evaluate the mechanism by which Abl kinase degrades NM23-H1. We will also investigate the biological significance associated with the Abl kinases -mediated NM23-H1 degradation.

3.2 Materials and Methods

Cell Lines:

435s/M14 and BT-549 cell lines expressing NM23-H1shRNA were created by lentiviral-mediated infection of sigma Mission shRNA constructs 182s1c1 or non-target shRNA (PLKO1). Cells were plated and a day later 8µg of polybrene was added per milliliter of media prior to infection with 20µl of the virus (MOI 10).8h after infection, cells were refreshed with fresh media. The cells were selected with Puromycin, clones were pooled together and stable cell line was made. L. Fiore established these cell lines.

Other cell lines are as described before in Chapter 2.

Reagents:

The following antibodies were purchased commercially: NM23-H1 (sc-465; immunofluorescence), LAMP1, NM23-H1 (C-20) was purchased from Santa Cruz Biotechnology; Santa Cruz, CA; EEA1, Rab7, NM23-H1/H2 (D141), NM23-H1/H2 (D98; immunohistochemistry, western blotting) (Cell Signaling; Danvers, MA), Cathepsin L was from Sigma (St. Louis MO), Cahepsin B was from Abcam (Cambridge, MA) and HRP-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), and fluorescently labeled secondary antibodies (Alexafluor 488 and 555) were from Cell Signaling (Danvers, MA). pcDNA-c-Abl-PP and pcDNA-Arg-PP constructs were described previously (Plattner et al 1999). Recombinant NM23-H1 was a kind gift from Drs Rob

McCorkle and David Kaetzel [Ma et al 2004]. All other antibodies were described Chapter 2.

siRNA: Pre-validated siRNA against NM23-H1 (s9588; 1nM); cathepsin L1 (s3753; 5nM); cathepsin B (s3740; 5nM), scrambled control #1 (control for non-silencer select siRNAs), silencer select scrambled #1 (for silencer select siRNAs) were purchased from Applied Biosystems/ Ambion; Carlsbad, CA. Scrambled siRNA concentrations were used at concentrations equivalent to the test siRNA. Other RNAi are described in Chapter 2.

Procedures

Western Blots: Described in Chapter 2.

Soft agar Colony Formation Assay [64]: Cells stably expressing PLK01 vector or NM23-H1 shRNA (4 X10^3 cells) were plated in 0.3% soft agar on top of 0.6% agar in 60mm dishes, in the absence or presence of 10µm imatinib. Cells were fed with media containing 10µm imatinib or vehicle every week once and after 3 weeks of incubation; colonies (≥ 100 microns) were counted. Experiments were done in triplicates and repeated twice.

Immunohistochemisty: Details are in Chapter 2.

Invasion assay: Details are in Chapter 2.

Single-cell 3D Invasion assay: Details are in Chapter 2.

***In-vitro*-Proteolysis Assay** [246]**:** Recombinant NM23-H1 (1ul; 875ng) was mixed with 3ul Buffer L (25 mM HEPES pH 7.4, 5 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, 5 mM DTT), 5ul of recombinant cathepsin B or L, 5ul 3X reaction buffer (150mM sodium acetate pH 6.0, 12mM EDTA, 24mM DTT) and 1ul water. Reactions were incubated for the indicated times at 37^0C, terminated by addition of SDS-PAGE buffer, ran in 20% SDS-PAGE gel, and blotted for C-termini (C-20; NM23H-1; Santa Cruz) and N-termini (D-141, NM23-H1; Cell signaling) recognizing NM23-H1 antibodies.

Mass Spectrometry and Edman Degradation N-terminal sequencing. Cathepsin B (2.5ug) or L (160ng) were incubated for 30' with 875ng of NM23-H1 as described above, and reactions terminated by addition of 15% glacial acetic acid. Due to the presence of glycerol and detergent in the samples, mass analysis was performed with an AB Sciex (Framingham, MA) 4800 MALDI TOF/TOF mass spectrometer in linear, mid-mass mode (2-20 kDa) using the Chait thin layer method [247] by Carol Beach in the University of Kentucky Proteomics Facility. From the stock reaction mixture a 1:10 dilution of sample was analyzed which consisted of 10,000 laser shots at a detector voltage of 1.971 kV, and the signal was averaged. Substrate (NM23-H1; 1:10) and enzymes (cathepsins, 1:10) were also measured using the same criteria. Cathepsin L spectra could not be visualized due to the low amount of enzyme utilized. For N-terminal sequencing, proteolysis reactions were run on 20% SDS-PAGE gels, transferred to Immobilon P^{SQ} membrane, stained with Coomassie Blue, and de-stained until bands were visible. C-terminal fragments were

sequenced by Edman Degradation in the Protein Structure Core Facility at the University of Nebraska Medical Center (Omaha, Nebraska).

Immunofluoresence and Confocal Microscopy: 435s/M14 cells plated on glass coverslips in 6 well plates, were allowed to settle overnight, and treated with imatinib for 4h. Cells were fixed in 4% paraformaldehyde, blocked in 5% goat serum, incubated with primary antibodies (EEA1-1:100, Rab7-1:25, NM23-H1 sc465-1:400, LAMP1-1:10) in PBS/1%BSA/0.3% Triton-X-100, followed by 1h incubation with fluorescent-conjugated secondary antibodies (Alexafluor 488-green or 555-red (1:1000)) (Cell Signaling, Danvers, MA) and mounting in ProlongGold Antifade solution (Invitrogen, Grand Island, NY). Photographs were taken on an Olympus FV1000 laser scanning confocal microscope, with 60 X objectives (oil emersion) with 488nm and 550nm laser lines. Magnified images are 3X.

3.3 Results

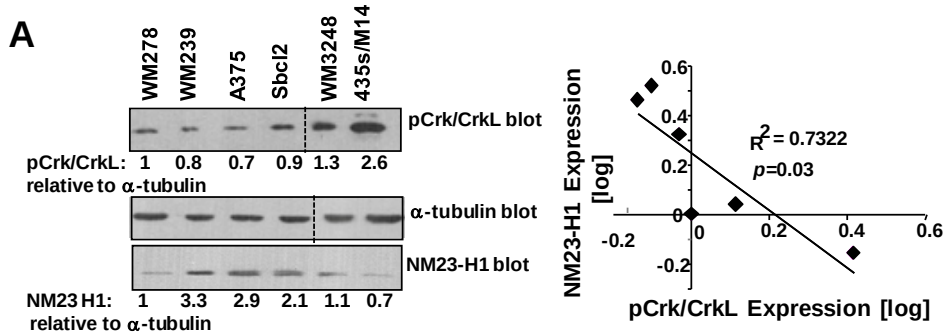
3.3.1 c-Abl and Arg induce the downregulation of metastasis suppressor, NM23-H1

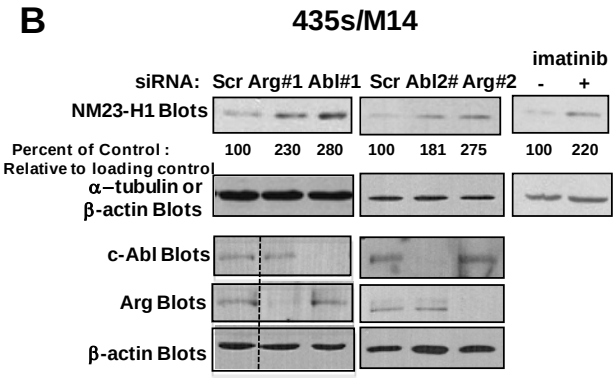
Abl kinases mediate melanoma invasion partially through MMP-1 and MMP-3 (Chapter 2), indicating that Abl kinases will also promote invasion via another mechanism. In the search for some another mechanism, we screened a panel of melanoma cells for the presence of a metastasis suppressor NM23-H1. As shown in Figure 3.1A, phosphorylation of Abl kinase substrates, Crk/CrkL, was inversely correlated with NM23-H1 expression in melanoma cells (Figure 3.1A right). pCrk/CrkL can be used as a read out of c-Abl and Arg activity as inhibition of c-Abl and Arg with imatinib mesylate in breast cancer and melanoma cell lines suppress pCrk/CrkL levels in a dose-dependent manner similar to silencing c-Abl or Arg [55,64]. 435s/M14 and WM3248, which express high levels of phosphorylated Crk/CrkL ,express low levels of NM23-H1 as compared to the low invasive cell line, WM239, which has high levels of NM23-H1. To assess whether Abl kinases mediate downregulation of NM23-H1 in invasive melanoma cells, both pharmacological and siRNA-mediated inhibition approaches were used. Silencing of Abl kinases with 2 independent siRNAs targeted against c-Abl or Arg respectively, stabilized NM23-H1 expression in 435s/M14 cells. Leann Fiore, a graduate student in our lab, found that inhibition of Abl kinases by the pharmacological inhibitors, imatinib or nilotinib, also stabilized NM23-H1 expression, in 435s/M14 cells (Figure 3.1B and 3.2, data not shown). To substantiate that this is not a cell-specific phenomenon, we examined whether

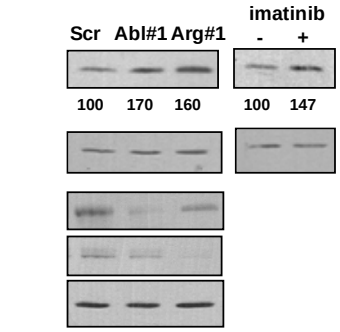
inhibiting/ silencing Abl kinases induced NM23-H1 upregulation in other cell lines (WM3248-melanoma, BT-549-breast cancer). Indeed, NM23-H1 upregulation was observed in both cell lines and tumor types (melanoma, breast) (Figure 3.1C,D and 3.2). To validate our loss of function studies, we also used a gain of function approach. Constitutive forms of c-Abl and Arg (Abl-PP/Arg-PP) were transiently expressed in a low invasive cell line expressing low c-Abl and Arg kinase activities (WM164-melanoma). Mutation of the two-proline residues in the interlinking region between the SH1 and SH2 domains to glutamate generates Abl-PP and Arg-PP, resulting in the loss of auto-inhibition, and subsequent constitutive activation. Overexpression of constitutively active forms of c-Abl and Arg induced the downregulation of NM23-H1 in WM164 cells (Figure 3.1E and 3.2 C). These results indicate that c-Abl and Arg are necessary and sufficient to downregulate NM23-H1 in invasive melanoma cells.

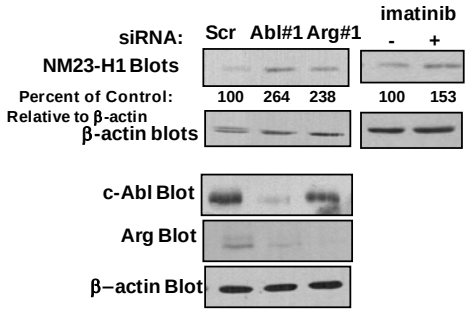
3.3.2 NM23-H1 is degraded via the lysosomal, cysteine cathepsins

To assess the mechanism by which Abl kinases degrade NM23-H1 in melanoma cells, Leann Fiore, first investigated whether the downregulation of NM23-H1 by Abl kinases is transcriptionally regulated. She found out that siRNA -mediated knock down of c-Abl or Arg did not have any significant effect on the transcript levels of NM23-H1 as assessed by semi-quantitative RT-PCR (data not shown).









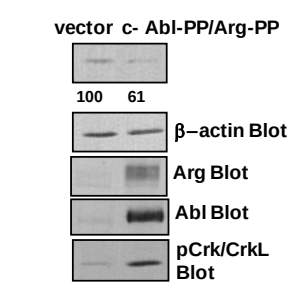
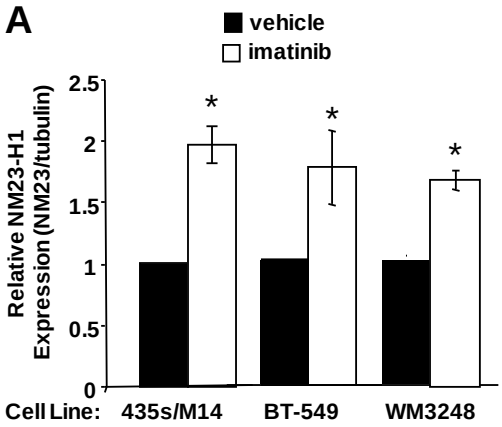
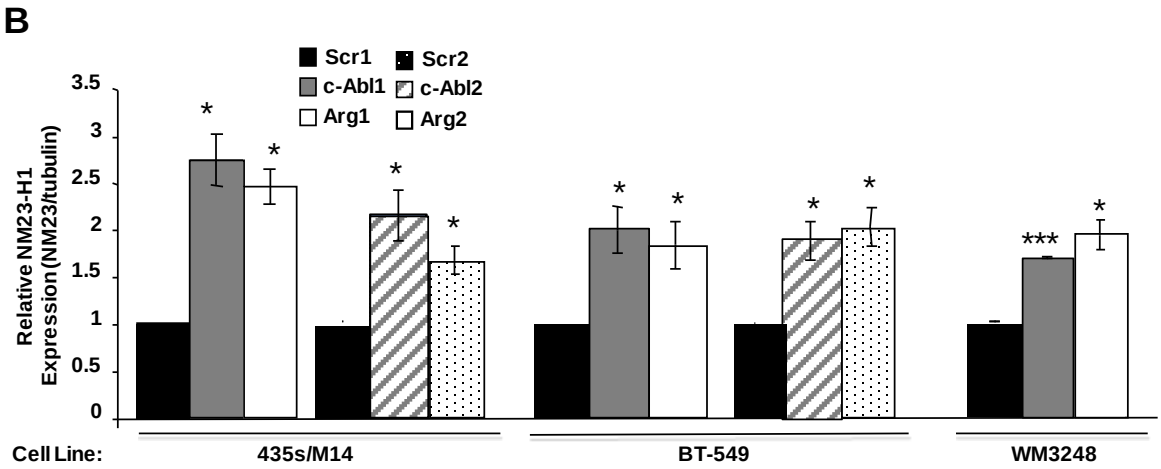


Figure 3.1 c-Abl and Arg is necessary and sufficient for NM23-H1 loss in cancer cells.(**A**) c-Abl/Arg activities were assessed indirectly via phosphorylation of Crk/CrkL in melanoma cell lines, lysates were blotted with indicated antibodies, and log-transformed values for c-Abl/Arg activity plotted against log-transformed NM23-H1 expression values (**A** right). An inverse correlation was found between c-Abl/Arg activity and NM23-H1 expression, which was statistically significant: Pearson's correlation coefficient =-0.85, 95% confidence interval -0.98 to -0.139, p=0.03. Done in collaboration with W. Friend **(B-D)** Cancer cell lines containing highly active c-Abl/Arg were transfected with c-Abl or Arg siRNAs or treated with vehicle or c-Abl/Arg inhibitor imatinib (10µM) for 8h, and blotted for indicated antibodies. NM23-H1 expression was quantified and expressed relative to loading controls and relative to scrambled or vehicle-treated cells. Knock-down efficiency was determined by blotting with c-Abl and Arg antibodies. Figure 2B left panel (Set #1 siRNA) was done by Dr R. Plattner. Figure 3.1**B** and Figure 3.1 **D** right panel (imatinib treatments) was performed by L. Fiore **(E)** WM164 melanoma cells were transiently transfected with constitutively active forms of c-Abl and Arg (PP), lysed three days after transfection, blotted for indicated antibodies and efficiency of overexpresison was determined by blotting for c-Abl, Arg and pCrk/CrkL.





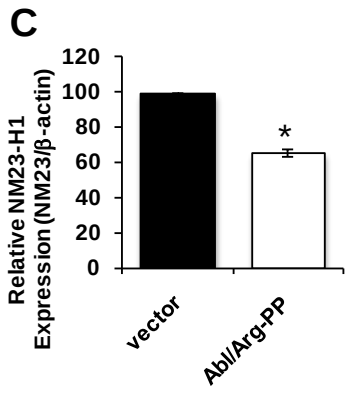


Figure3.2: Activation of c-Abl and Arg reduces expression of NM23-H1.

Highly invasive cancer cell lines known to express activated c-Abl/Arg, were treated with imatinib (10µM) for 8h (**A**)**,** or were transfected with 2 independent sets of siRNA directed against c-Abl and Arg or scrambled control (**B**) as described and shown in Figure 3.1 (**C**). Low-invasive WM164 cells were transiently transfected with c-Abl-PP and Arg-PP (constitutively active forms), and lysates blotted with the indicated antibodies. In all graphs relative NM23-H1 expression was quantified and expressed relative to loading controls and relative to scrambled or vehicle-treated cells. (Mean± s.e.m for 3-4 independent experiments). *p<0.05, **p<0.01, ***p<0.001 using single-sample t-tests.

In addition, treatment with the proteosomal inhibitor, MG132, also did not result in stabilization of NM23-H1 in 435s/M14 cells, but did stabilize p27, a protein known to be degraded by the proteosomal pathway. These data indicate that NM23-H1 is not degraded via the proteasome (data not shown). Interestingly, treatment of 435s/M14 cells with the lysosomal protease inhibitors, ammonium chloride and chloroquine or with a cell permeable cysteine protease inhibitor (E64D), induced NM23-H1 protein expression (Figure 3.3A). These results indicate that NM23-H1 could be degraded by the lysosomal cysteine proteases E64D is known to inhibit a wide range of cysteine proteases including cathepsin B and L, which have known roles in cancer invasion and metastasis. Overexpression of cathepsin B and L are reported in many carcinomas and is frequently associated with a poor prognosis. Silencing cathepsin B or L in 435s/M14 cells induced the expression of NM23-H1 (Figure 3.3B). Taken together these results indicate that NM23-H1 is degraded via cathepsin B and L. Since degradation of NM23-H1 is dependent on cathepsin B and L, we investigated whether cathepsin B and L directly cleave/degrade NM23-H1 *in vitro*. To test this hypothesis, recombinant, NM23-H1 was incubated with recombinant, active forms of cathepsin B or L in acidic conditions which favor cathepsin activation. Cathepsin L cleaved NM23-H1 as evidenced by the appearance of a 10kD band detected by blotting with an antibody, which detects the C-terminus of NM23-H1 (Figure 3.4 A). Using an antibody that recognizes N-terminus of NM23-H1, we also detected a 6kD band.

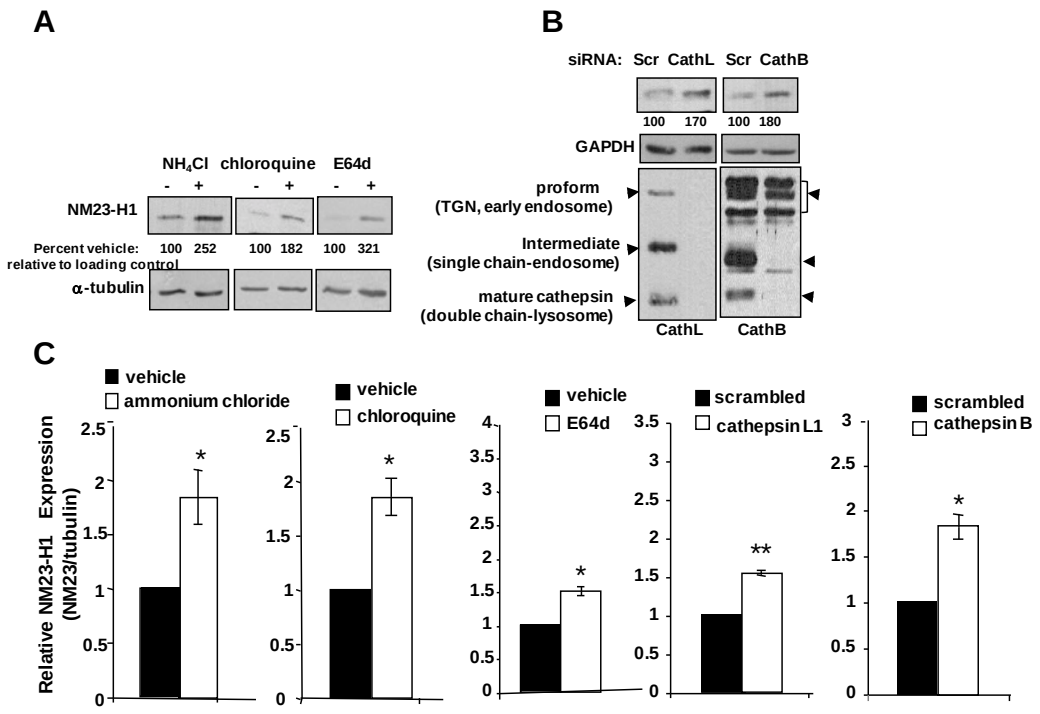


Figure 3.3: NM23-H1 is degraded via the lysosomal cysteine proteases, cathepsin B and L in 435s/M14 cells. 435s/M14 **c**ells were treated with lysosome inhibitors (ammonium chloride, 60mM; chloroquine, 100mM, E64D-cysteine protease inhibitor, 20mM) for 8h (**A**), or transfected with siRNAs against cathepsin B and L (**B**), lysed, and blotted with the indicated antibodies. Representative experiments are shown and graphed (**C**) relative to vehicle-treated or scrambled control as shown in (**A-B**). Graphs are Mean±s.e.m for 3 independent experiments; *p<0.05, **p<0.01 using one-sample t-tests. These experiments were performed by L.Fiore

Cathepsin L directly cleaved NM23-H1 in a dose- and time-dependent manner (Figure 3.4A). Similar results were observed with cathepsin B (Figure 3.4B). These results indicate that cathepsin B and L directly cleave NM23-H1.

To more accurately assess the mass of the cleavage products, the reactions were analyzed by mass spectrometry (performed by University of Kentucky Proteomics facility; Carol Beach). An aliquot of the in vitro proteolysis samples were run in a SDS-PAGE gel and blotted with antibody to the NM23-H1 C-terminus to confirm the efficiency of the reaction (Figure 3.5, 3.6; inset). Mass Spectrometry analysis of the mock reaction resulted in the visualization of a 17 kD peak, which corresponds to the full length NM23-H1. Incubation of NM23-H1 with the cathepsins led to the appearance of 9.7 kD peak and 6.6 kD peaks, in addition of some smaller species (Figure 3.5). The C-terminal NM23-H1 fragments were sequenced (Edman Degradation), and their N-termini identified as “YMHSGP” (Figure3.4C); (University of Nebraska Protein Structure Core Facility).

We detected 5-6kd, N-terminal NM23-H1 fragments following incubation with cathepsins as determined by SDS-PAGE analysis. However, the predicted weight of N-termini fragment should be 7.6 kD if the cathepsins only one time at the "YMHSGP" cleavage site. Since the predicted mass is more than the actual mass detected, this indicates that the N-terminal fragment probably was cleaved more than once resulting in smaller fragments that cannot be detected on a 20% SDS-PAGE gel. These results indicate that cathepsin B and L directly cleave

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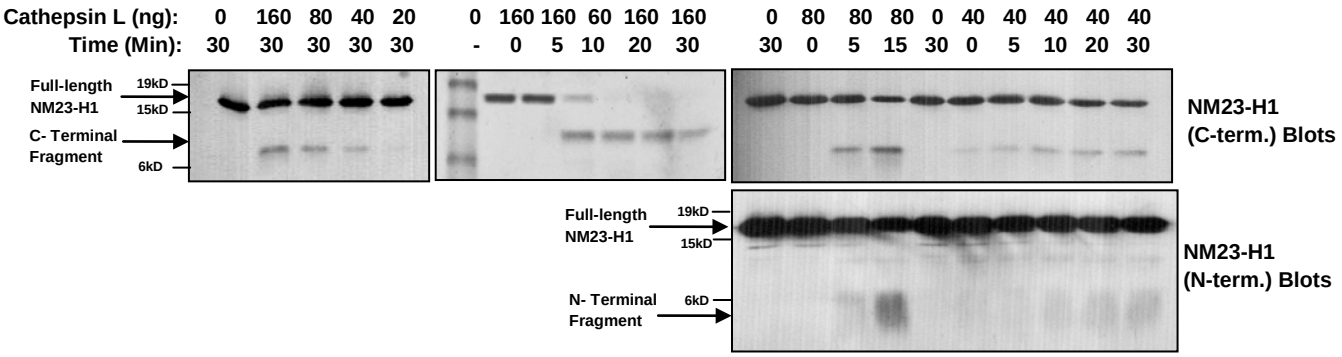

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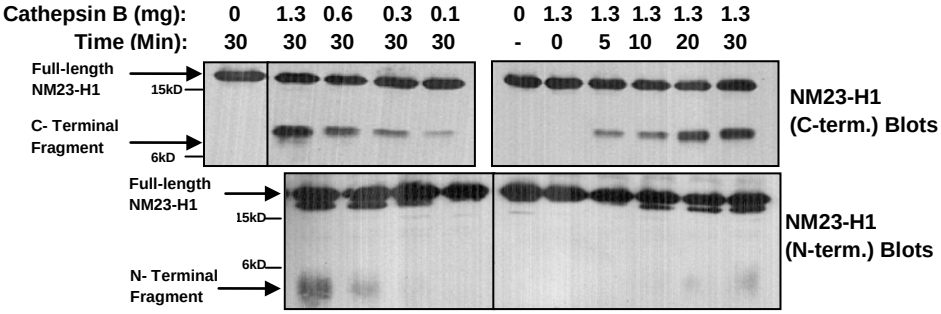

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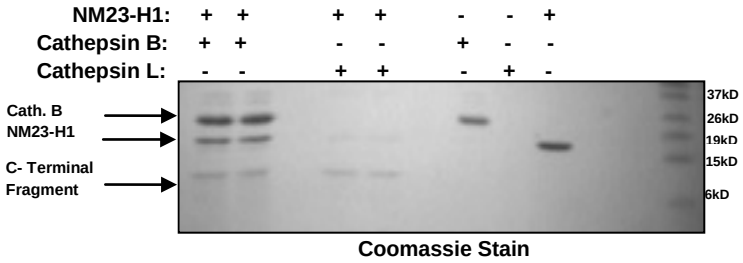


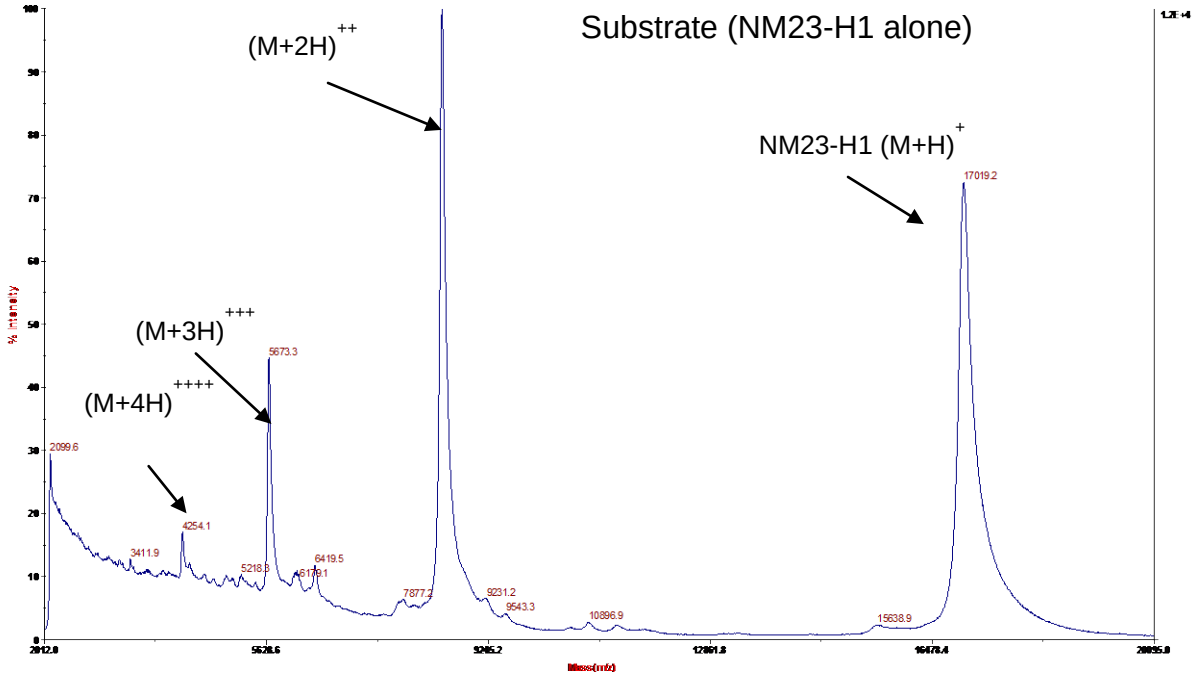
Coomassie Stain

NM23-H1 Variant 2 Sequence

MANCERTFIAIKPDGVQRGLVGEIIKRFEQKGFRLVGLKFMQASEDLLKEHYVDLKDRPFFAGLVK↓
YMHSGPVVAMVWEGLNVVKTGRVMLGETNPADSKPGTIRGDFCIQVGRNIIHGSDSVESAEKEI
GLWFHPEELVDYTSCAQNWIYE

Figure 3.4: Recombinant cathepsin B and L directly cleave NM23-H1.

Recombinant active cathepsin L (**A**) and B (**B**) were incubated with NM23-H1 (875ng) at 37^{o}C in acidic conditions as described in Section 3.2, for the indicated time, run in 20% SDS-PAGE gel, Coomassie-stained or transferred to nitrocellulose and probed with the indicated antibodies.



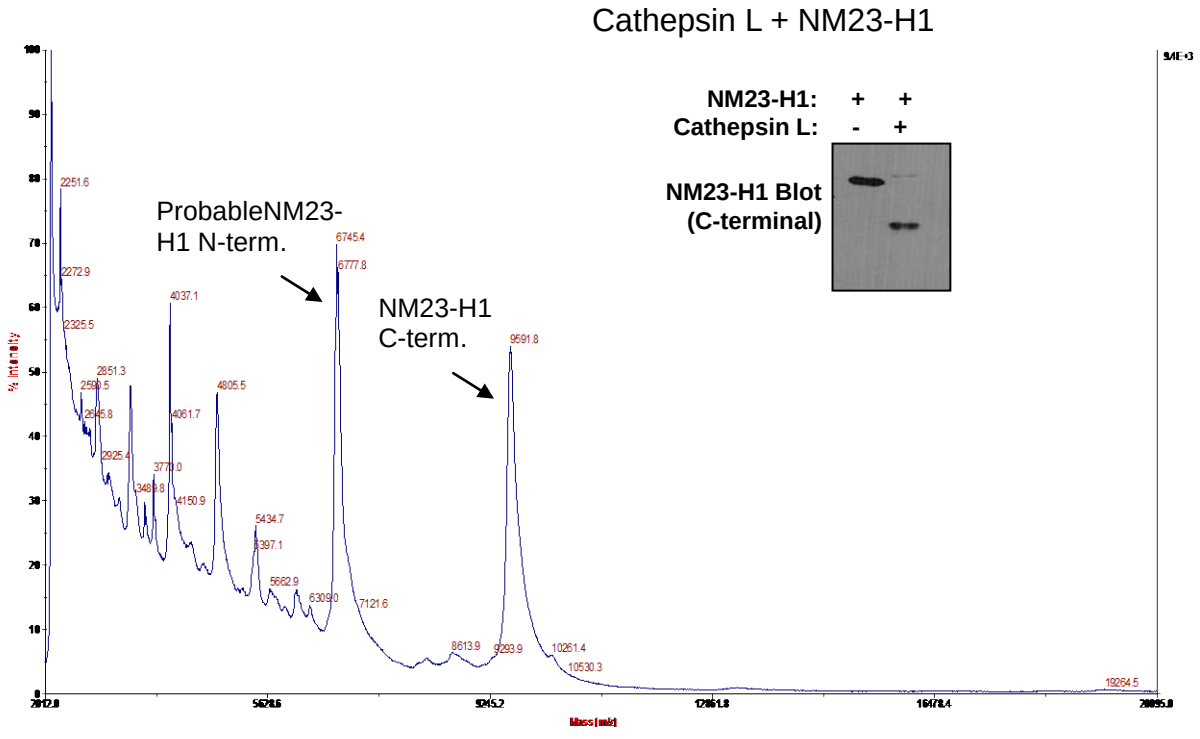
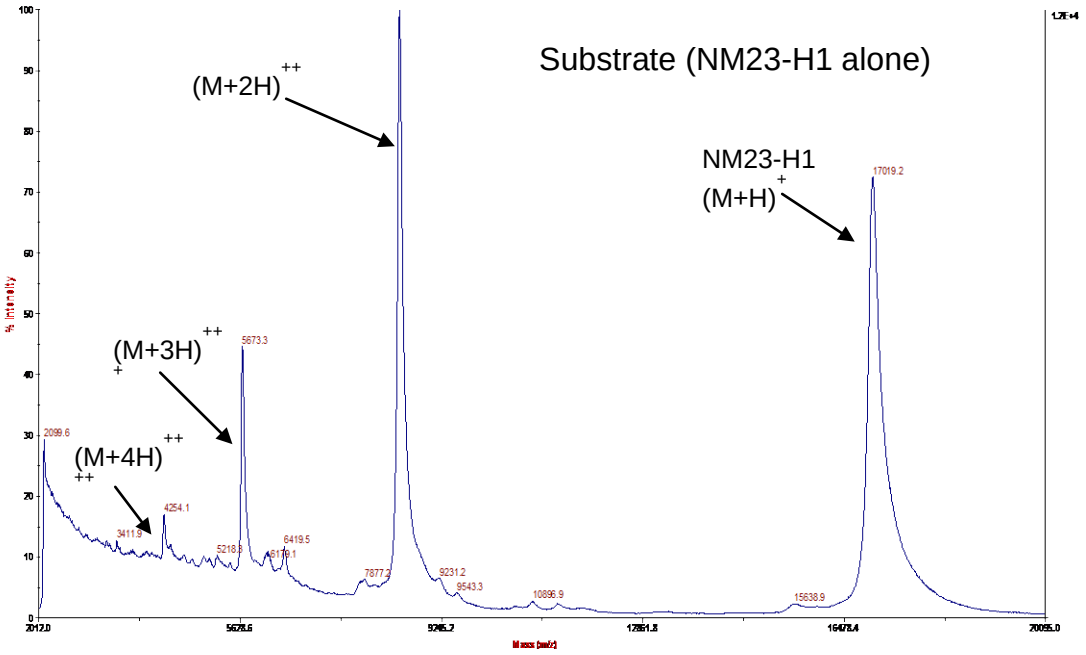


Figure 3.5: Active cathepsin L directly cleaves NM23-H1. Recombinant active cathepsin L was incubated with recombinant NM23-H1 for 30' at 37^{o}C as described in Figure 3.4 the reaction was stopped with 15% glacial acetic acid and analyzed by MALDI MS using the Chait thin-layer technique. $(M+2H)^{++}$or $(M+3H)^{+++}$ indicates multiple charging due to oxidation. The NM23-H1 protein was completely cleaved in the reaction (bottom panel). An aliquot of the reaction was, run on a 20% SDS-PAGE gel, and probed for indicated antibody (as shown in the bottom panel)



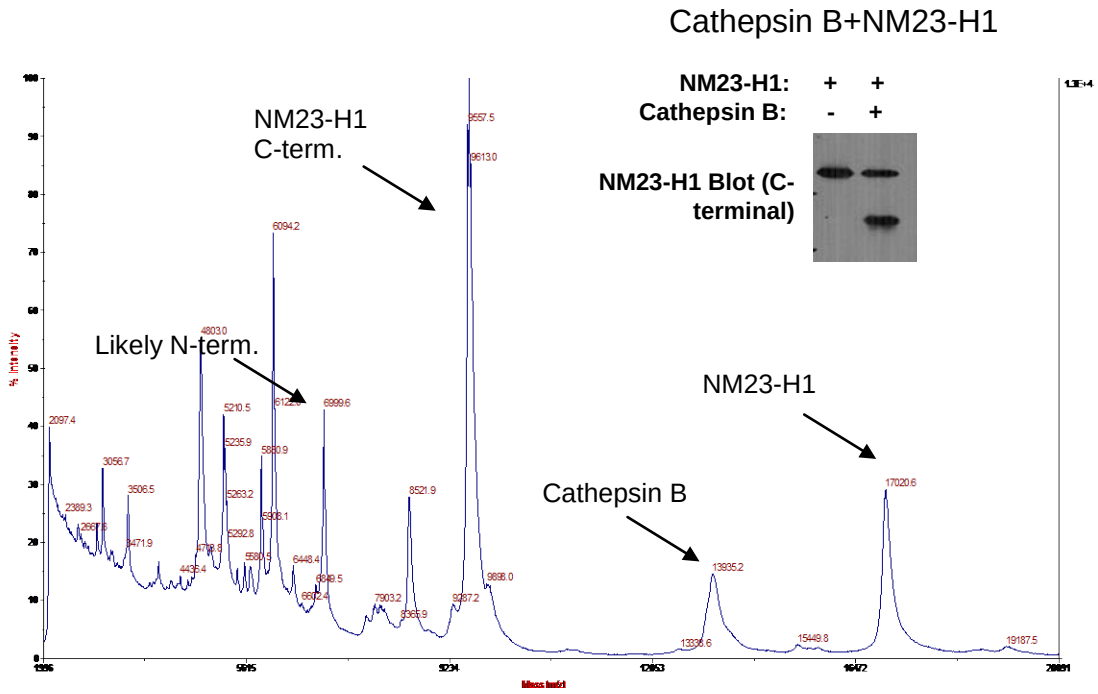


Figure 3.5: Active cathepsin B directly cleaves NM23-H1. Recombinant active cathepsin B was incubated with recombinant NM23-H1 for 30' at 37^{o}C as described Figure 3.4. The reaction was terminated by the addition of 15% glacial acetic acid and analyzed by MALDI MS using the Chait thin-layer technique. $(M+2H)^{++}$or $(M+3H)^{+++}$ indicates multiple charging due to oxidation. The NM23-H1 protein was partially cleaved in the reaction (bottom panel). An aliquot of reaction was taken, run on 20% SDS-PAGE gel, and probed with the indicated antibody as shown in bottom panel.

NM23-H1 at the same site, and given the role of cathepsin B and L in degrading proteins in lysosomes, these results give additional evidence that NM23-H1 may be degraded in the lysosomes. This hypothesis will be addressed in section 3.3.4

3.3.3 c-Abl and Arg promote cathepsin expression and activation in melanoma cells

Abl kinases downregulated NM23-H1 in melanoma cells, and degradation of NM23-H1 is dependent on cathepsin B and L. Thus we tested whether Abl kinases upregulate the expression and activation of cathepsin B and L. Silencing c-Abl or Arg in 435s/M14 cells, significantly reduced the expression of the proform (located in transgolgi and early endosome) of cathepsin B and L, indicating that c-Abl and Arg promote overall cathepsin expression. Interestingly, the lysosomal mature double chain active form of cathepsin B and L was significantly decreased with silencing of c-Abl (Figure 3.7), indicating that c-Abl activates the cathepsins .To investigate the mechanism by which c-Abl and Arg increase the procathepsin expression, we assess whether Abl kinases transcriptionally regulate cathepsin expression. Leann Fiore found out that silencing of c-Abl or Arg, significantly decreased cathepsin B and L mRNA levels in 435s/M14 cells, indicating that cathepsin upregulation occur at the mRNA level (data not shown). These results indicate that although both c-Abl and Arg upregulate cathepsin expression, activation is promoted only by c-Abl.

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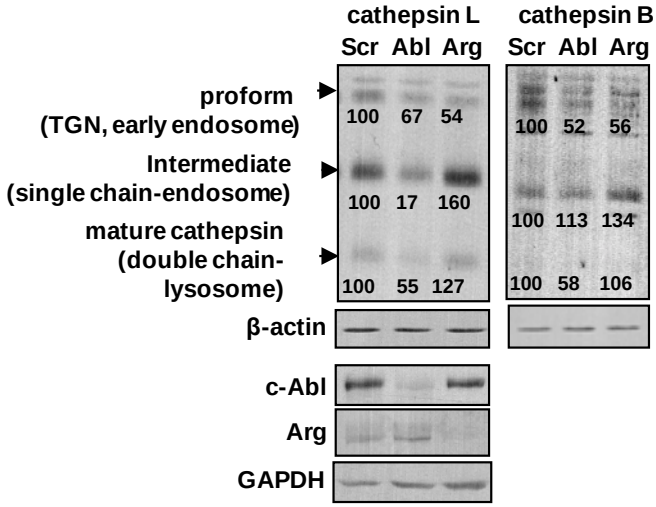

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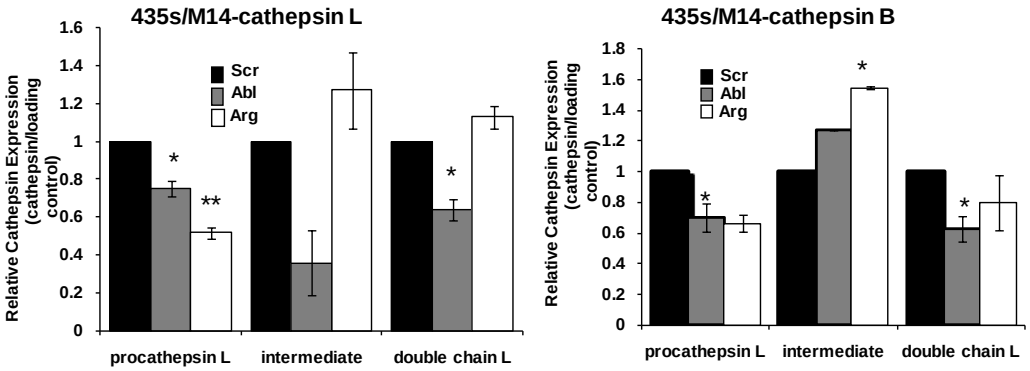


Figure 3.7: c-Abl promotes cathepsin B and L activation. 435s/M14 cells transfected with scrambled, c-Abl or Arg siRNAs were split 2 days after the first transfection, and lysed one day later when the cells were of equal confluency. The lysates were probed for indicated antibodies (**A**) and graphed (**B**) normalizing to loading and scrambled controls. Graph are Mean ±s.e.m *p<0.05, **p<0.01, ***p<0.001 using one-sample t-tests.

3.3.4 c-Abl promotes endosome-lysosome trafficking

We found that cathepsin B and L directly cleave NM23-H1, and that cathepsins B and L are activated by c-Abl. Since cathepsins cleave various proteins in the lysosome, we tested whether NM23-H1 is degraded in the lysosomes in a c-Abl - dependent manner. To confirm that NM23-H1 is localized in the endosomes/lysosome, and to test whether inhibition of c-Abl and Arg alters the distribution/localization of NM23-H1 in these vesicles, we performed immunofluorescence using antibodies that recognize specific surface markers on vesicles within the pathway as well as NM23-H1 antibody. We found that NM23-H1 is located in the cytoplasm in the majority of cells, and silencing of NM23-H1, abrogated all NM23-H1 (sc 465) staining indicating the specificity of this antibody for NM23-H1 (Figure 3.8).

To examine whether NM23-H1 is localized within endosomes, we co-stained cells with antibodies to EEA1 (early endosome marker and effector of Rab5). In vehicle-treated cells, small NM23-H1 puncta partially colocalized with EEA1; however inhibition of Abl kinases with imatinib for 4h induced enlarged EEA1-positive vesicles. Imatinib treatment also increased the clustering of EEA1 positive vesicles in one perinuclear region (Figure 3.9 and 3.10) and increased the staining intensity. Silencing c-Abl also considerably increased the clustering of EEA1-positive vesicles in a perinuclear region (Figure 3.9 and 3.10). Moreover, we observed a greater colocalization between NM23-H1 and EEA1 in cells in which c-Abl was silenced as compared to scrambled control cells. Silencing Arg increased the EEA1 perinuclear localization; however, the vesicles

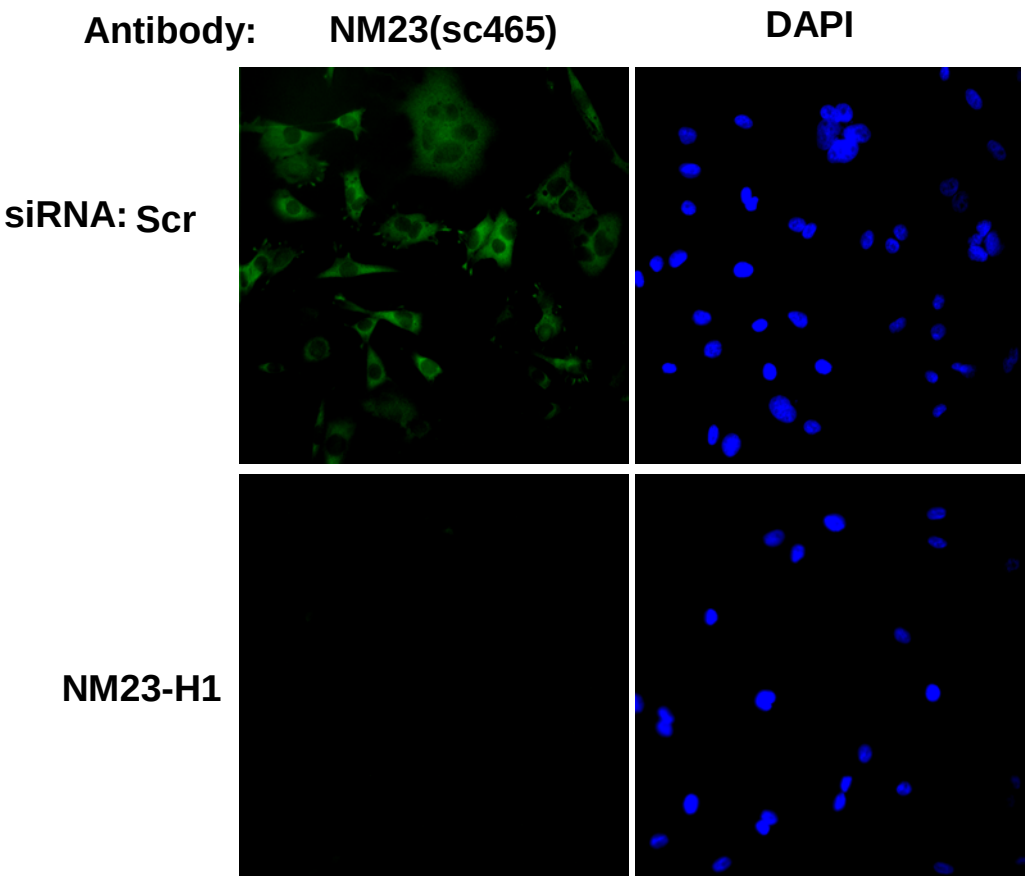
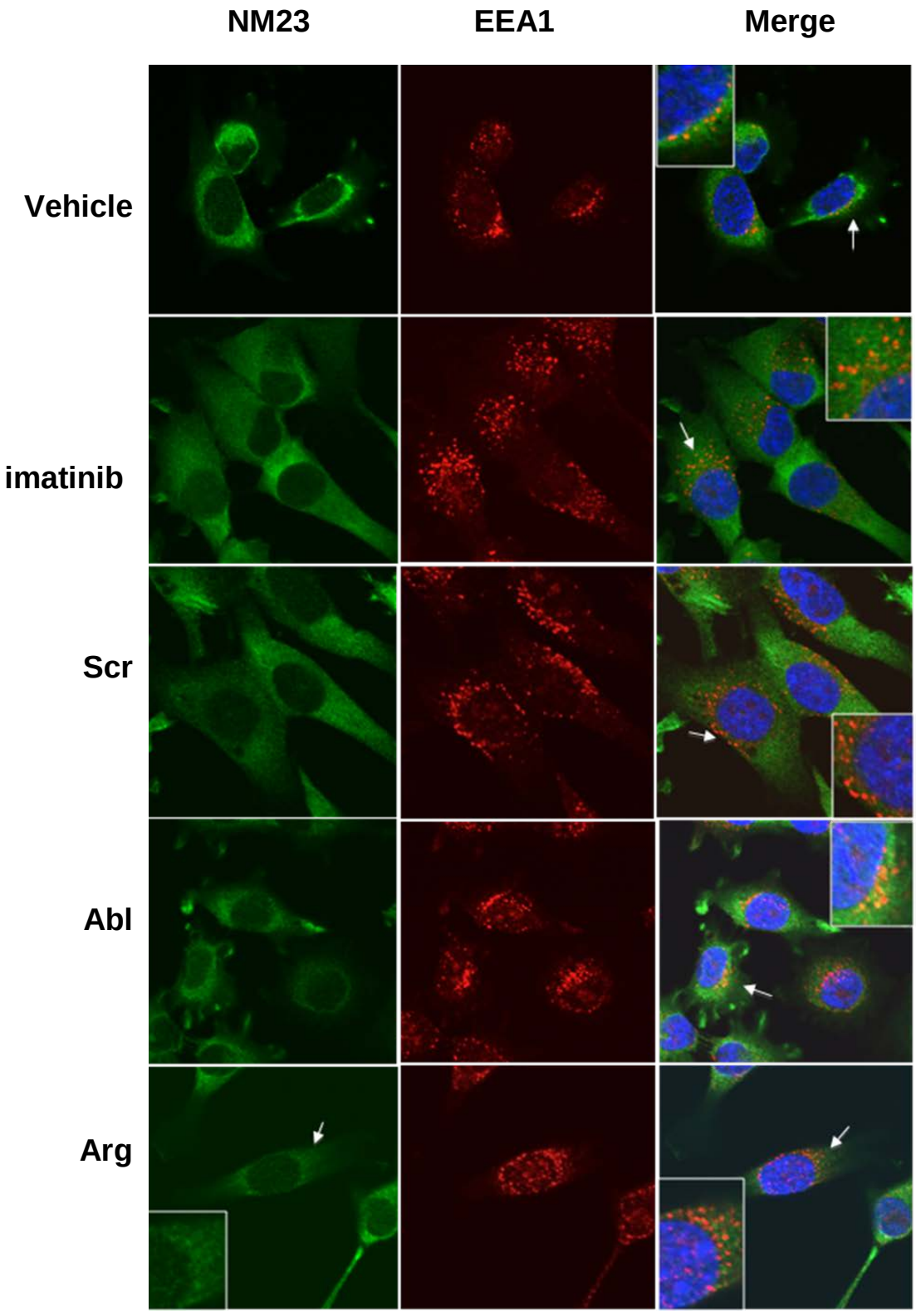


Figure 3.8: The NM23-H1 antibody specifically stains NM23-H1 in 435s/M14 cells. 435s/M14 cells, transfected with scrambled control or NM23-H1 siRNAs, were stained with NM23-H1 antibody (sc-465, used for immunofluorescence) followed by fluorescent-conjugated secondary antibody, and counterstained with DAPI.



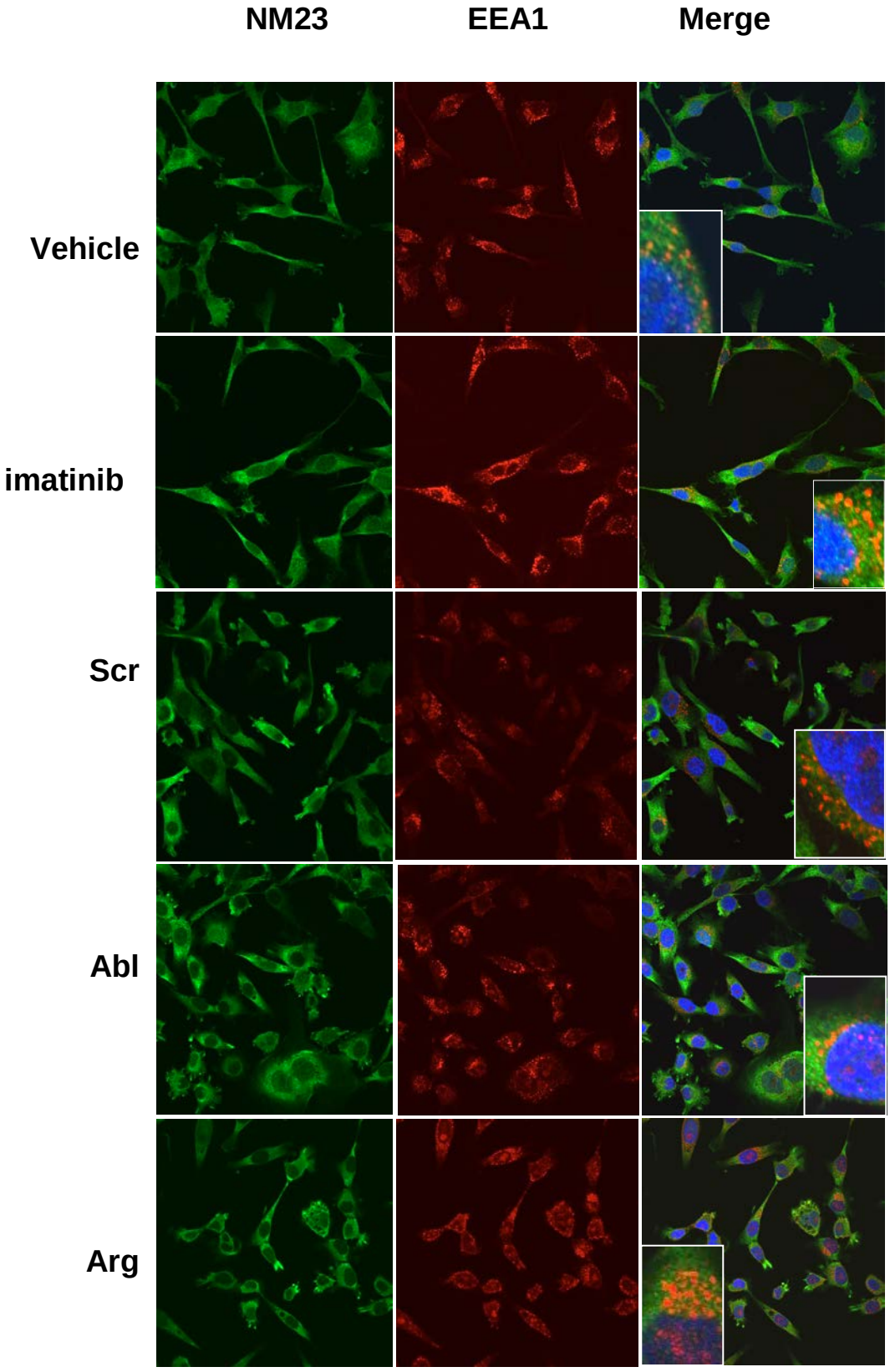


Figure 3.9 and Figure 3.10: Silencing/inhibiting c-Abl alters the localization and trafficking of early endosome. 435s/M14 cells were treated with imatinib for 4h or transfected with siRNAs against c-Abl and Arg (Set #1). The cells were stained with indicated antibodies, and counterstained with DAPI. Pictures were taken under confocal microscope using the brightest EEAI focal plane with a 60X oil-emersion objective. 3X magnified images from original images are in Figure 3.9. Insets show enlarged areas from the focal plane shown with an arrow.

were not confined to one perinuclear region observed following silencing c-Abl. During endosome maturation early endosomes are acidified and move along the microtubules to travel to theperinuclear region. The endosomes become enlarged during the acidification process [248,249]. To test whether c-Abl affects the acidification process, we treated cells with chloroquine. Chloroquine is lysosomotrophic reagent, which prevents the acidification of endosomes, and thus prevents the endosomes from moving to the perinuclear region. Chloroquine treated cells had bigger early endosomes, but they were not confined to one perinuclear region in contrast to the effects observed when cells were treated with imatinib or silenced with c-Abl RNAi (Figure 3.11). Since the effects of inhibiting/silencing c-Abl were distinct from those obtained with chloroquine, we hypothesize that c-Abl promotes endosomal maturation independent of the acidification process.

To analyze whether these large EEA1-positive perinuclear early vesicles, induced by c-Abl silencing, express late endosome marker (Rab7), an important step in endosome maturation, we stained cells with Rab7 (an effector for late endosomes). Rab 5 to Rab 7 conversion is essential in early-to-late endosome maturation. The transition between early to late endosome occurs by switching small GTPase Rab5 (early endosome) to Rab7 GTPase (late endosomes) [248]. Unlike EEA1, inhibition of Abl kinases or silencing c-Abl or Arg did not change the localization of Rab7 (Figure 3.12), indicating that the early endosomes

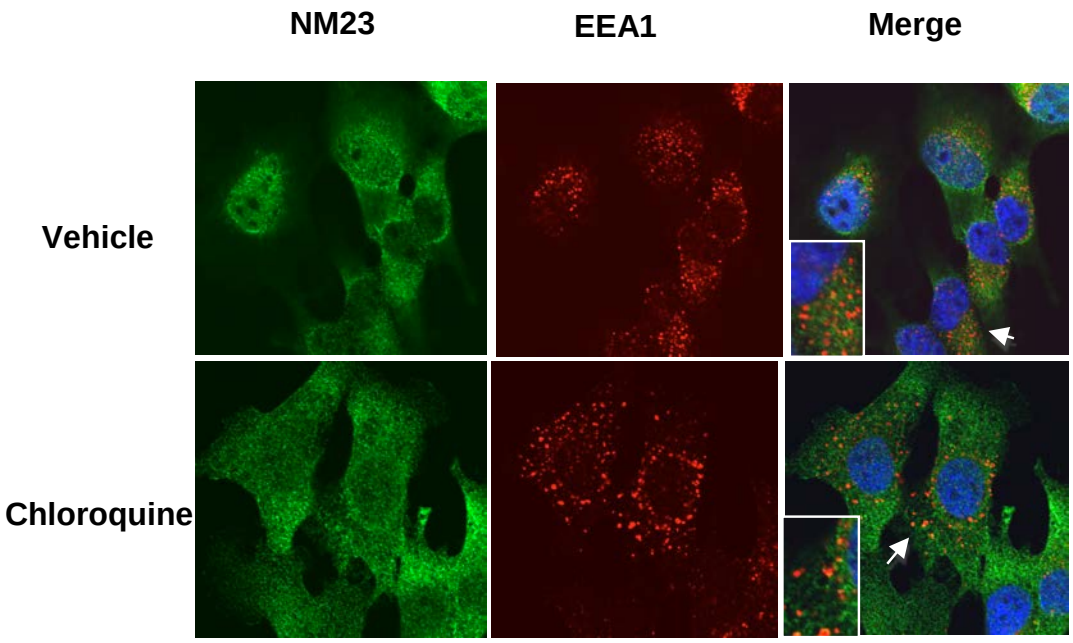


Figure 3.11: Inhibition of endosome/lysosome acidification by chloroquine induces larger/clumped non-perinuclear early endosomes. 435s/M14 cells were treated with chloroquine (100mM; 4h), stained with indicated antibodies and counterstained with DAPI. Pictures were taken under confocal microscope in the brightest EEAI focal plane using 60X oil emersion objective. 3X magnified images from 60x magnification pictures are shown here.

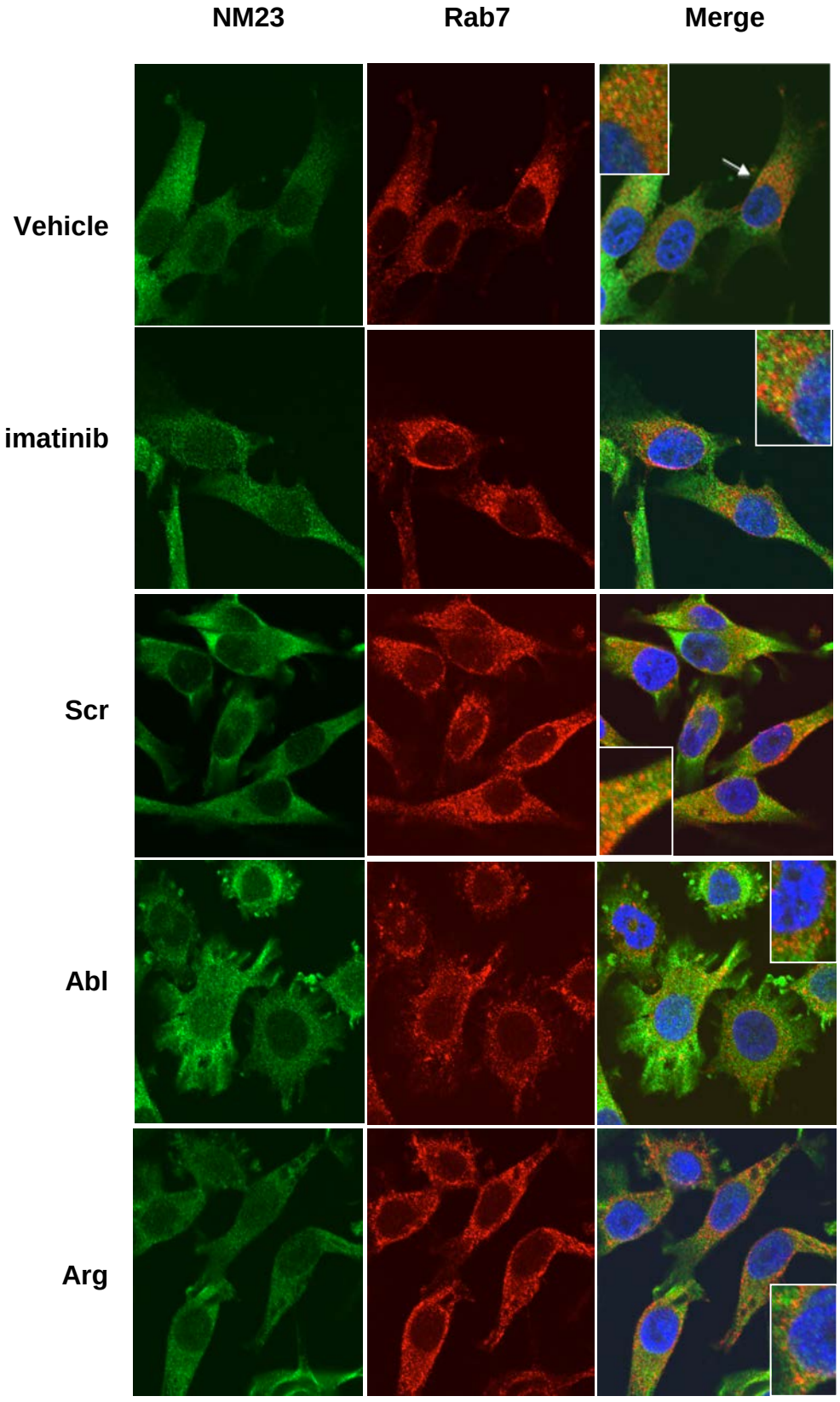


Figure 3.12: Silencing/inhibiting c-Abl has no effect on late endosome distribution, and NM23-H1 partially colocalizes with late endosomes. 435s/M14 cells were treated with imatinib for 4h or transfected with siRNAs against c-Abl and Arg (Set #1). The cells were stained with the indicated antibodies, and counterstained with DAPI. Pictures taken under confocal microscope in the brightest RAB7 focal plane using 60X oil-emersion objective and 3X zoom pictures from 60X are shown here.

detected previously were not hybrid early/late endosomes. Moreover, in vehicle-treated or scrambled control cells, unlike EEA1, there was considerable colocalization of NM23-H1 with Rab7 (Figure 3.12), indicating that, NM23-H1 is present in Rab7-positive vesicles. Silencing, c-Abl decreased Rab7 staining intensity. Furthermore, although silencing c-Abl did not change the distribution of LAMP1 positive vesicles (lysosomes) the intensity of LAMP-1 staining decreased, similar to Rab7 staining (Figure 3.13). These results indicate that inhibition of c-Abl likely decreased the total Rab7 (late endosomes) and LAMP1 positive vesicles (lysosomes) present. Thus, c-Abl inhibition results in the accumulation of early endosomes, and depletion of the late endosomes and lysosomes, which indicate that c-Abl likely promotes early to late endosome maturation.

3.3.5 c-Abl and Arg mediate invasion of melanoma cells in a NM23-H1-dependent manner

Forced expression of NM23-H1 in invasive cancer cells inhibits invasion, anchorage-independent growth, and metastasis [145,148.149, 159,160]. Our lab previously demonstrated that Abl kinases upregulate invasion, anchorage-independent growth [54] and single-cell 3D invasion in melanoma cells (described in Chapter 2). To investigate whether Abl kinases mediate invasion via degradation of NM23-H1 in 435s/M14 cells, we stably expressed NM23-H1 shRNA in order to prevent stabilization of NM23-H1 following c-Abl/Arg inhibition.

In vector-infected 435s/M14 cells, imatinib treatment significantly reduced matrigel invasion and single-cell 3D invasion; however, these effects were rescued in cells expressing a NM23-H1 shRNA (Figure 3.14 A and B). Thus the downregulation of NM23-H1 is required to promote matrigel invasion and single-cell 3D invasion in 435s/M14 cells. However, in BT549 cells the rescue in invasion using the NM23-H1 shRNA was only partial (Figure 3.15), indicating that Abl kinases mediate invasion via a NM23-H1-dependent and -independent pathways. Interestingly, inhibition of NM23-H1 in 435s/M14 cells increased soft agar growth as compared to the vector control cells, and silencing NM23-H1 partially enhanced anchorage-independent growth in the presence of imatinib (Figure 3.16), indicating that Abl kinases promoted anchorage- independent growth in a NM23-H1-dependent and -independent manner.

In Chapter 2 we have described that active Abl kinases promote late stages of melanoma metastasis (Figure 2.20A), and showed that c-Abl/Arg kinase activity (assessed by pCrk/CrkL immunohistochemistry (IHC) staining) correlated with metastatic burden (IVIS luminescence) in lung nodules from nilotinib-treated mice injected with 435s/M14-GFP/luciferase cells (Figure 2.21). Mouse that did not respond to nilotinib had large nodules, stained intensely with pCrk/CrkL antibody as compared to mouse which responded to nilotinib, indicating that the anti-metastatic capability of nilotinib is linked to inhibition of c-Abl/Arg kinase activity. To determine whether anti-metastatic property of nilotinib, is also associated with upregulation of NM23-H1 *in vivo*, we stained lungs from nilotinib-treated mice (Figure 2.21) with NM23-H1 antibody. Significantly, the

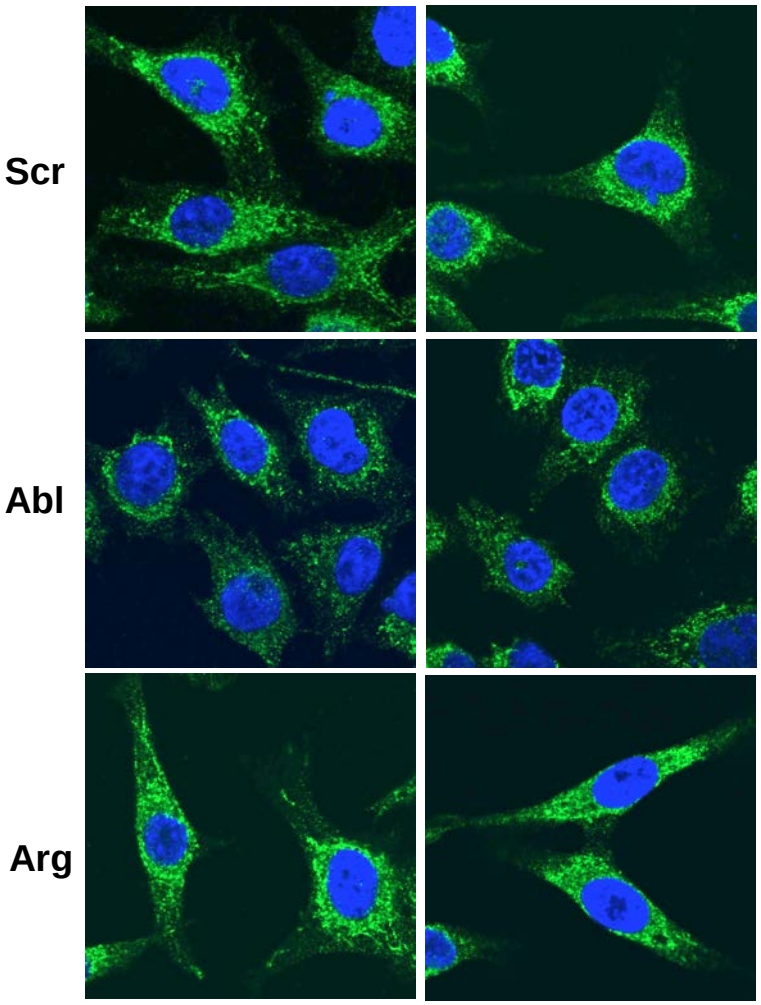


Figure 3.13: Silencing c-Abl decreases the intensity of lysosome staining (LAMP1). 435s/M14 cells were transfected with siRNAs directed against c-Abl and Arg (Set#1), stained with LAMP1 antibody, and counterstained with DAPI. Fields from two independent experiments are shown**.** Pictures were taken on a confocal microscope using the brightest LAMP1 focal plane using 60X oil-emersion objective and 3X zoom**.**

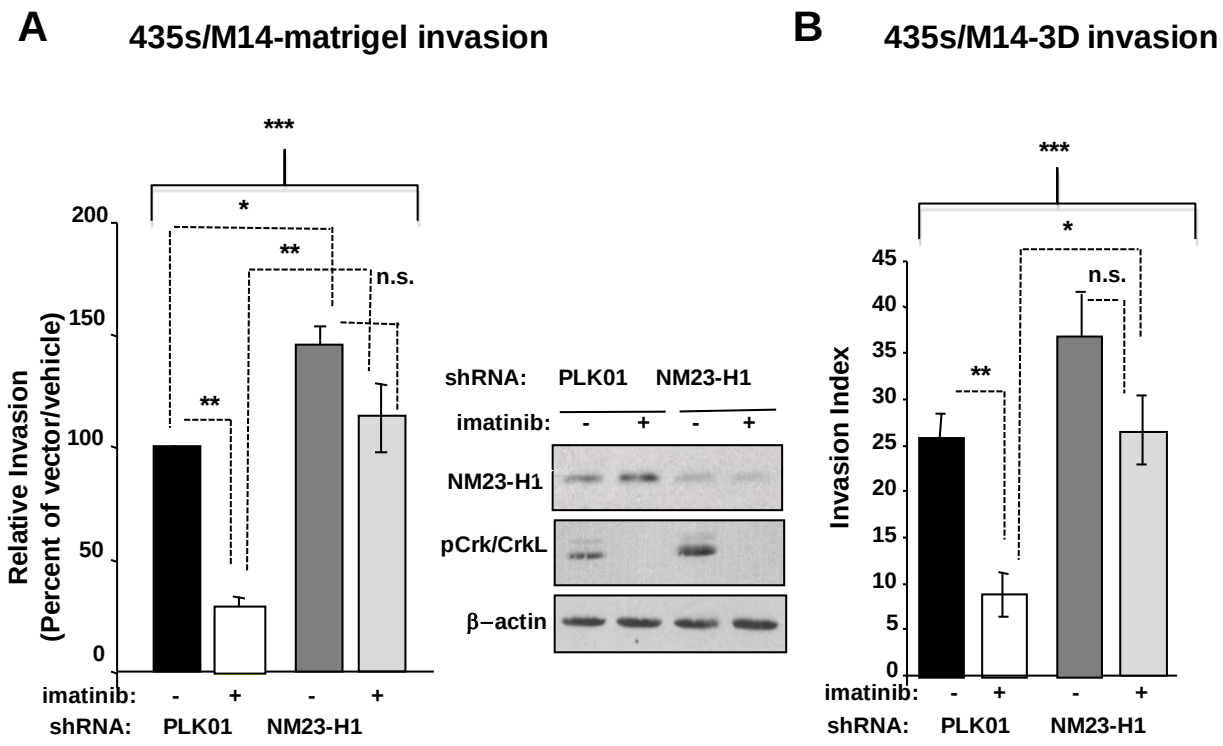


Figure 3.14: Abl kinases promote invasion of melanoma cells by the down regulation of NM23-H1. (**A-B**) 435s/M14 cells transected stably with NM23-H1 shRNA or vector control (PLKO1), were treated with imatinib for 20h and serum-starved (**A**), and used for a matrigel invasion assay for 48h(**A**; 48h timepoint) or single-cell 3D invasion assay (**B**; 2h timepoint). The graphs are mean± s.e.m (n=3 independent experiments), normalized to vehicle treated cells. Aliquots of cells were taken, lysed, and probed for indicated antibodies to assess knockdown efficiency. Representative blots are shown. One-way ANOVAs followed by Tukey posthoc tests were used to measure statistical significance. *p<0.05, **p<0.01

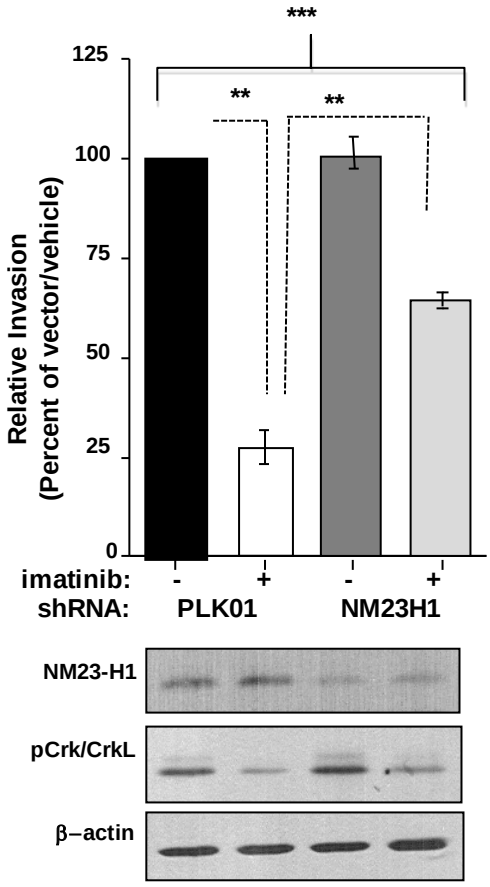


Figure 3.15: Abl kinases promote breast cancer cell invasion via down regulation of NM23-H1. BT549 cells, transfected stably with NM23-H1 shRNA or vector control (PLKO1), were treated with imatinib for 20h, serum-starved and used in a matrigel invasion assay for 48h. The graphs are mean± s.e.m (n=3 independent experiments), normalized to vehicle-treated cells. Aliquots of cells were taken, lysed, and probed with the indicated antibodies to determine knockdown efficiency. Representative blots are shown. One-way ANOVAs followed by Tukey posthoc tests were used to measure statistical significance. *p<0.05, **p<0.01

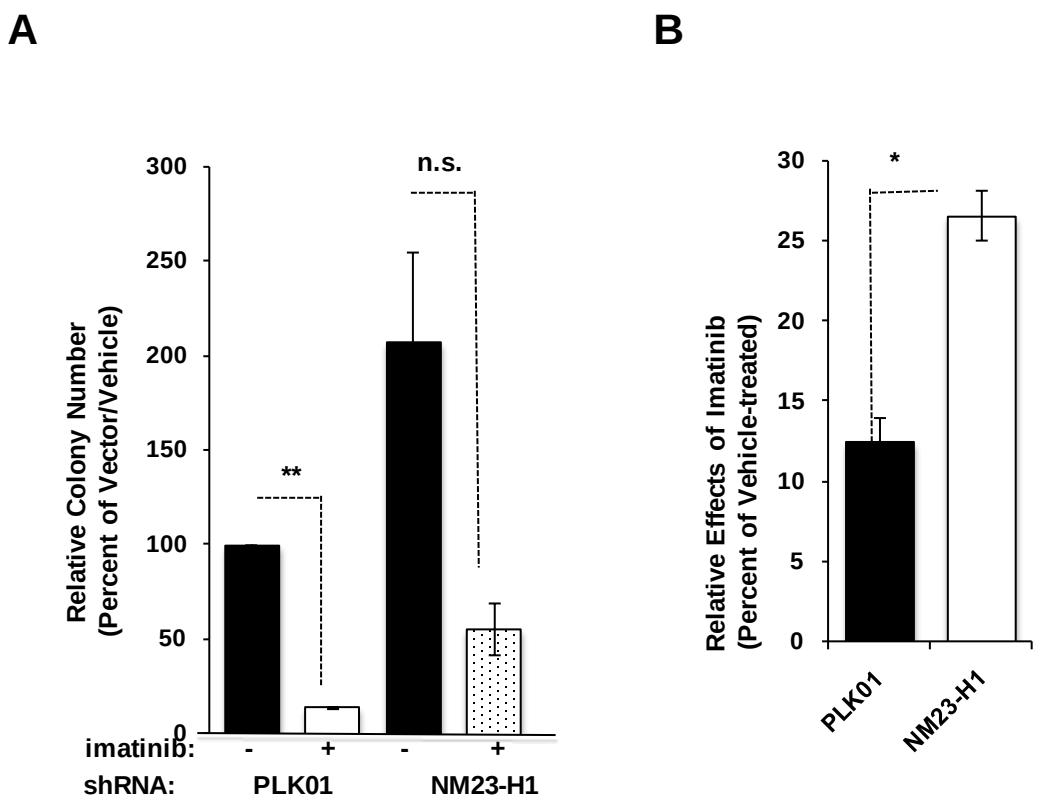


Figure 3.16: c-Abl/Arg promotes anchorage-independent growth via NM23-H1-dependent and- independent pathways. (A,B) Soft agar assays were conducted with 435s/M14 cells, transected stably with NM23-H1 shRNA or vector control as described in Chapter 3.2. After 3 weeks of incubation, colonies (>100mm) were counted and graphed by normalizing to (**A**) PLKO1(vehicle alone), vehicle-treated cells or (**B**) by comparing the effects of imatinib treatment in both cell lines used. Experiments were performed in triplicate. Graphs are Mean±s.e.m for two independent experiments.

mice that responded to nilotinib, having lesser metastatic burden (lesser Flux number), with small metastatic lesions had more NM23-H1 expression as compared to the mouse that did not respond to nilotinib. NM23-H1 expression was inversely correlated with c-Abl/Arg activity and metastatic burden in all nilotinib-treated mice (Figure 3.17), indicating that c-Abl/Arg inhibition induces NM23-H1 stabilization, *in vivo*. Summarizing these findings, we can conclude that Abl kinases promote matrigel invasion and single-cell 3D invasion assay and experimental metastasis via the degradation of the metastasis suppressor, NM23-H1, whereas soft-agar growth is only partially dependent on NM23-H1 degradation.

3.3.6 c-Abl and Arg activity and NM23-H1 expression are inversely correlated in primary melanomas

As described in Chapter 2, using a commercially available melanoma TMA, we have shown that c-Abl/Arg is activated in some primary melanomas. In contrast to B-RAF, which is activated in benign nevi, c-Abl/Arg activity was more highly elevated in primary melanomas and metastasis as compared to benign nevi. This is in contrast to NM23-H1 expression, which is induced in primary melanomas, and suppressed at later stages during progression. To investigate whether activation of Abl kinases is inversely associated with NM23-H1 expression in melanoma, Leann Fiore examined c-Abl/Arg activity (assessed via pCrk/CrkL staining) and NM23-H1 expression in commercially available primary melanoma

TMAs. As shown in Figure 3.18, cores with high pCrk/CrkL-staining intensity had very low NM23-H1 staining and vice-versa, indicating that c-Abl/Arg activity is inversely correlated with NM23-H1 expression in primary melanomas. Thus, the signaling axis that we have identified in melanoma cell line also exists in the human disease.

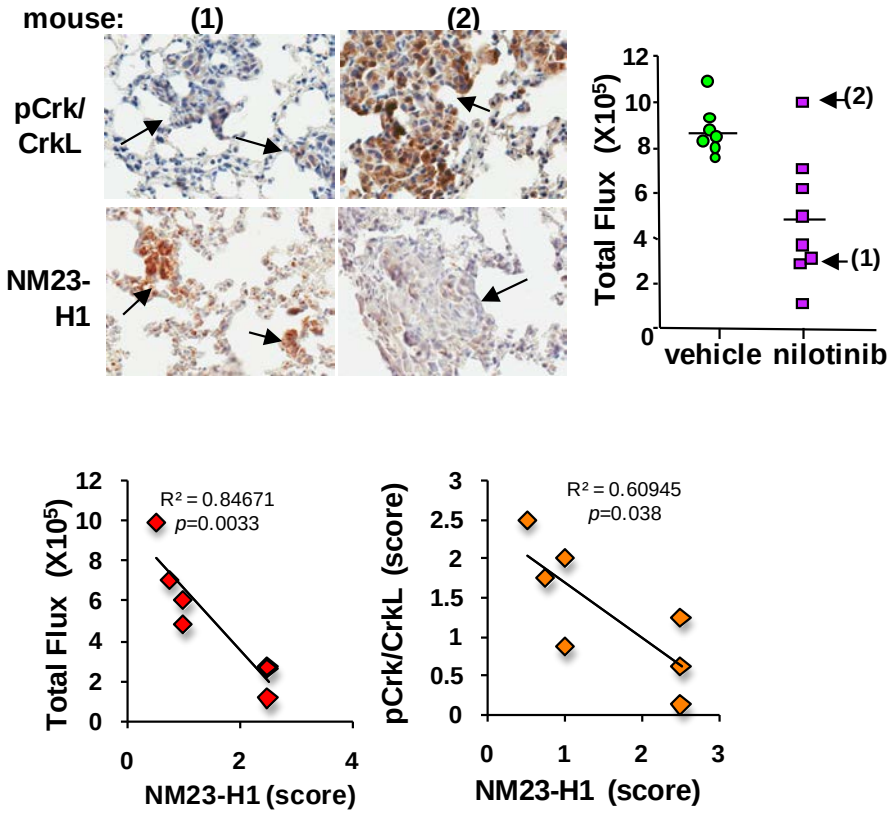


Figure 3.17: c-Abl/Arg induces NM23-H1 degradation *in vivo*. Lungs sections from mice injected with 435s/M14/GFP/luciferase, treated with nilotinib (Chapter 2), were stained with pCrk/CrkL and NM23-H1 antibodies. Representative lungs section shown from mice which responded to nilotinib treatment (mouse 1) and mouse which did not respond to the treatment (mouse 2) are shown. IVIS flux values for all mice are shown (top right). Arrows indicate the mice, lungs shown in top left. NM23-H1 IHC scores were plotted with IVIS flux values (bottom left) or pCrk/CrkL values (bottom right). 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. Lungs were stained by L.Fiore

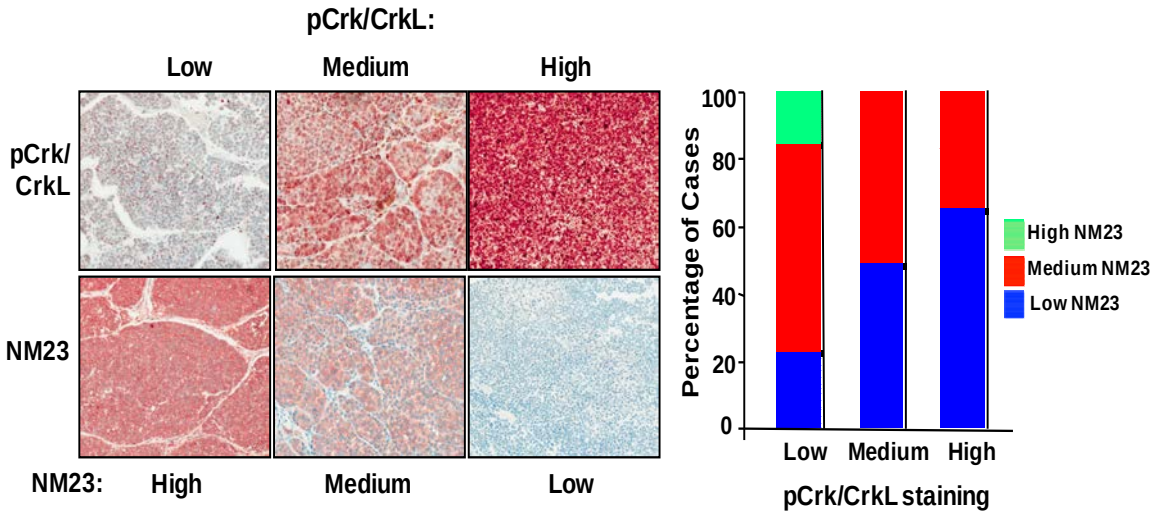


Figure 3.18: c-Abl/Arg activities and NM23-H1 expression are inversely correlated in primary melanomas. An NCI Progression TMA containing nevi (n=81), primary melanomas (n=60), and lymph node/organ metastases (lymph, n=37; organ, n=67) were stained with pCrk/CrkL antibody as described in Chapter 2.2. The cores were blindly scored by pathologists as described earlier in Figure 2.2. Representative regions of the slides shown with screen shots obtained with Aperio software (left); Mean pCrk/CrkL scores (Mean±SEM, right). Bracket indicates *p*-value for one-way ANOVA, **p≤0.01. This experiment was performed by L. Fiore and scored by Dr. Michael Cibull MD.

3.4 Discussion

Although there is extensive literature describing the metastasis suppressor function of NM23-H1, there is little evidence of how it is degraded in invasive cancer cells. Here, we report for the first time that Abl kinases downregulate NM23-H1 in a cathepsin-dependent manner. We also identify that cathepsin B and L directly cleave NM23-H1at the same site, which results in protein degradation. To date, no studies report the mechanism by which NM23–H1 is degraded in invasive cancer cells. Our work provides the first evidence that NM23-H1 is degraded in the acidic lysosomes, in a cathepsin B and L-dependent manner, and we also provide the first evidence demonstrating that a proto-oncogene degrades a metastasis suppressor to promote invasion (Figure 3.19). We also show that the pathway we identified has clinical relevance as c-Abl/Arg activation is inversely correlated with NM23-H1 expression in primary melanomas.

In the previous chapter, we have shown that Abl kinases activity is highly upregulated in melanoma cells, and once activated, Abl kinases promote invasion, survival, proliferation and metastasis of melanoma cells. Here, we show that c-Abl/Arg activity is inversely correlated with NM23-H1 expression in melanoma cells. Very low invasive WM239 and Sbcl2 cells have very high levels of NM23-H1 expression as compared to the invasive WM3248 and 435s/M14 cells, indicating that low Abl kinase activity and high NM23-H1 expression render a cell with low invasive capacity, in contrast to 435s/M14 and WM3248 cells, which are highly invasive and have low NM23-H1 expression and high c-Abl/Arg

activity. Silencing c-Abl and Arg induces stabilization of NM23-H1 in 435s/M14 and WM3248 cells as well as in BT549 breast cancer cells. Inhibition of Abl kinases by imatinib also stabilizes NM23-H1 to a similar extent as silencing of c-Abl/Arg, indicating that the effects that we observe are dependent on the kinase activities of c-Abl/Arg. Moreover, transient transfection of constitutive active Abl and Arg-PP into low invasive WM164 melanoma cells induces downregulation of NM23-H1 in low invasive melanoma cells. Thus we have used gain-of-function and loss-of- function approaches to show that c-Abl/Arg are required and sufficient for NM23-H1 downregulation in cancer cells.

Extracellular cathepsins play a pivotal role in the invasion of cancer cells; however, not much is known regarding how intracellular cathepsins promote invasion. Intracellular cathepsins may promote invasion by cleaving proteins such as laminin, type IV collagen, the cell adhesion protein, E-cadherin, as well as dynamin, which functions during endocytosis of cells [179, 200-204, 207]. We demonstrate a previously unrecognized role for intracellular cathepsins: degradation of the metastasis suppressor, NM23-H1 via cathepsins B and L. Using an *in vitro* cleavage assay, we also show that cathepsin B and L cleave NM23-H1 directly in the same site in a low acidic environment, indicating that both these cathepsins might have redundant functions in regulating NM23-H1 activity. Silencing c-Abl suppresses the activation of cathepsin B and L while silencing Arg had no effect on cathepsin double chain processing. However, Arg promotes the expression of cathepsin B and L and also induces NM23-H1 degradation, indicating that Arg probably affects some other cathepsins important

in NM23-H1 degradation. Non-metastatic melanoma cells can be transformed to metastatic cells by overexpressing cathepsin L [195] indicating that Abl/Arg-PP overexpression in non-metastatic melanoma cells can make them metastatic via the upregulation of cathepsins L and subsequent downregulation of NM23-H1.

Abnormal vesicular trafficking plays a crucial role in cancer progression. Endocytosis permits the internalization of signaling receptors, and targets them to be destroyed in the acidic lysosomes [250]. For example, EGFR and TGFβ-R are endocytosed resulting in recycling back to the plasma membrane or degradation [251]. In the case of a metastasis suppressor, endocytosis might be considered an attenuator of signaling as degradation of the metastasis suppressor can lead to cancer progression. In a recent study, it was demonstrated that, in human astrocytoma cells, ARF6 recruits NM23-H1 and regulates clathrin- and dynamin- dependent $P2Y_1$ and $P2Y_{12}$ internalization. $P2Y_1$ and $P2Y_{12}$ are purinoreceptors whose activation by ADP is required for normal platelet aggregation, and thus, facilitating thrombus stability [252]. Hus T et al showed that von Hippel-Lindau tumor suppressor protein requires the activity of NM23-H1 to endocytose FGFR1, and thus, prevents abnormal activation of FGFR1 [253]. In Dictyostelium, NM23-H1 reduces both macropinocytosis and exocytosis, thus, increasing the time for complete digestion of the nutrients present in the vesicles [254]. Since c-Abl and Arg activation induces NM23-H1 degradation, there is a possibility that this will result in decreased receptor endocytosis, resulting in sustained signaling and efficient metastatic progression.

In this chapter, we demonstrate that Abl kinases play an important role in the endocytotic pathway. Abl kinases affect endosome maturation as early endosomes increase in size following inhibition of Abl kinases. Acidification is an essential step for the perinuclear localization of endosomes, as demonstrated by treatment of 435s/M14 cells with chloroquine (Figure 3.11). This is in contrast to the effects observed following inhibition of Abl kinases, indicating that Abl kinases do not effect endosome maturation at the acidification step. Inhibition of Abl kinases might affect endosome maturation by promoting the movement of the early endosomes along microtubules. Alternatively, inhibition of Abl kinases and silencing c-Abl shows similar effects as overexpressing Rab5 or expressing a GTPase -eficient Rab7, which results in the presence of giant enlarged endosomes [255]. Thus, Abl kinases might prevent the switch between Rab5 and Rab7. Inhibition of this switch does not allow early endosomes to fuse with late endosomes, and thus, the endocytic cargo fail to traffic to the acidic lysosomes for degradation. Silencing c-Abl also decreases LAMP staining intensity, indicating that fewer lysosomes are present. In the absence of Rab7, the endocytic cargo is entrapped in the late endosomes /multivesicular bodies, and lysosomal activity is decreased, as indicated by the presence of fewer lysosomes [Vanlandingham et al]. Inhibition of Abl kinases and silencing c-Abl exerts similar effects on lysosomes, indicating that Rab5 activity is overexpressed, or there is a decrease in Rab7 expression following inhibition of Abl kinases. Inhibition of Abl kinases may thus prevent the switch between Rab5 and Rab7, leading to an

accumulation of NM23-H1 in early endosomes and preventing its degradation in lysosomes. Future experiments are ongoing to test this hypothesis.

Abl kinases also have been shown to have a role in endocytosis. c-Abl has been shown to inhibit EGFR internalization, which results in sustained EGFR signaling and cellular transformation [256]. Likewise, here we show that Abl kinases promote invasion/metastasis by inducing endocytosis of NM23-H1, a metastasis suppressor protein (Figure 3.19). In another report, RIN1 activation by c-Abl was shown to prevent endocytosis, and promote cell surface expression of EGFR following EGF stimulation of Hela cells [257], indicating that Abl kinases promote oncogenesis by preventing the endocytosis of EGFR. In non-transformed cells, endogenous c-Abl induces the endocytosis of B-cell receptor (BCR) by cytoskeletal remodeling, and regulates the extent and time of BCR activation, which is essential for the antigen presenting function of BCR [258]. Furthermore, in a mouse model of cutaneous leishmaniasis, inhibition of Abl kinase decreases lesion development and parasite burden by phagocytosis of Leishmania [259]. In summary, all these results indicate that Abl kinases prevent endocytosis of proteins critical for cancer or disease progression and here, for the first time, we report that Abl kinases promote endocytosis of NM23-H1, and alter its stability in invasive cancer cells, thus promoting cancer progression.

In this chapter, we demonstrate that Abl kinases mediate invasion via the downregulation of NM23-H1, as silencing NM23-H1 rescued imatinib effects (almost 80%), indicating that most of invasive properties of c-Abl/Arg are mediated through the downregulation of NM23-H1 in 435s/M14 cells. In Chapter

2, we demonstrated that Abl kinases mediate invasion by upregulating MMP-1 and -3, but the rescue of silencing c-Abl or Arg effects by MMP-1 overexpression was only 40%. It is possible that these two mechanisms are parallel pathways, or alternatively, there might be crosstalk between these two pathways. Rescue invasion assay needs to be conducted to test whether Abl kinases promote MMP-1 and MMP-3–dependent invasion via the downregulation of NM23-H1.

Here we show that silencing NM23-H1 with shRNA increases single-cell 3D invasion through collagen I, which support the results by Bossian et al, who reported that NM23-H1 loss increased both gelatin degradation and invasion of native type I collagen and that NM23-H1 silencing promotes MMP-14 activity. We showed that Arg kinase promotes MMP-14 expression/activity, and is possible that the pathway by which Arg-mediates MMP-14 activity might be dependent on NM23-H1 degradation. NM23-H1 depletion is casually involved in increasing the expression of MMP-1 and- 3 [159] and silencing NM23-H1 promotes collagen I degradation. Taken these results and our findings, we can hypothesize that Arg promotes MMP-14 activation and collagen degradation via the downregulation of NM23-H1. Future experiments will examine if Arg/NM23-H1/ and MMP-14 lie in the same pathway.

This work shows for the first time that Abl kinases and NM23-H1 are inversely correlated in melanoma cell lines and primary melanomas. Moreover, we show that activated Abl kinases-mediate the degradation of NM23-H1 in a cathepsin B and L-dependent manner. Research since the discovery of NM23-H1 has demonstrated the degradation of this metastasis suppressor in many

cancers, including melanoma and agents that can induce its re-expression are being pursued in the clinic [171]. In this chapter, we show that Abl kinases inhibitors promote the upregulation of NM23-H1 in invasive cancer and targeting Abl kinases in metastatic melanoma can be of therapeutic benefit.

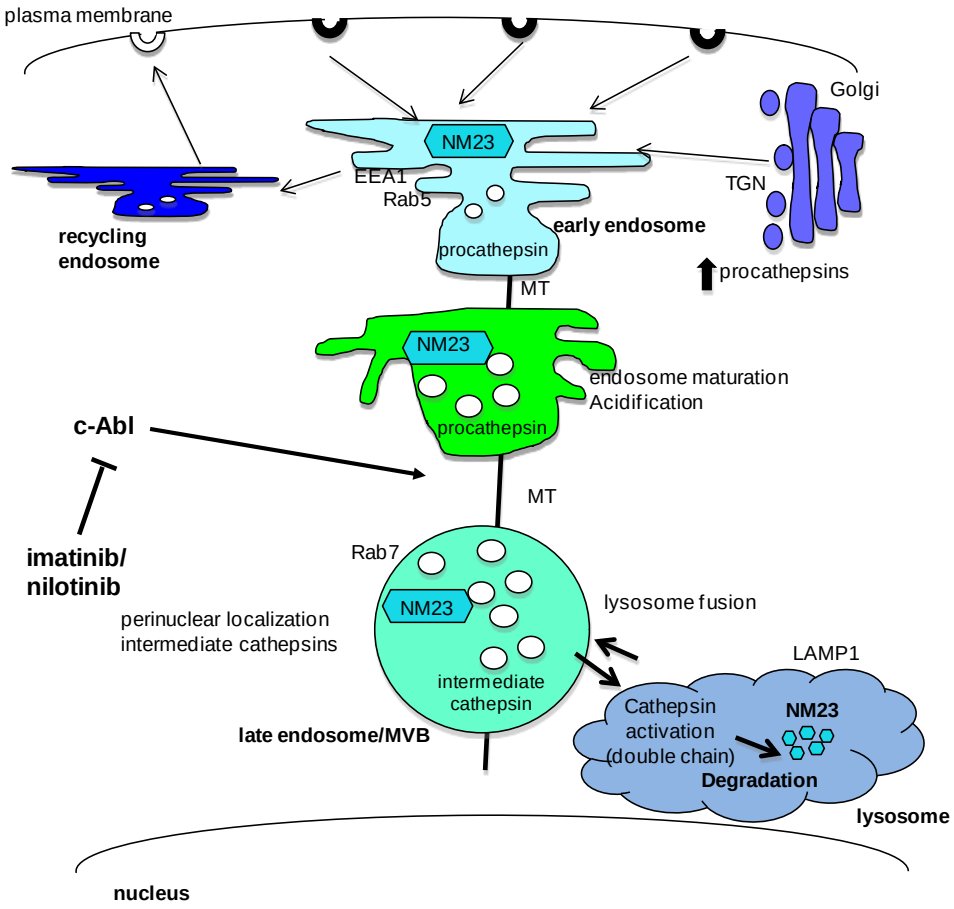


Figure 3.19. Model for the mechanism by which c-Abl/Arg promote vesicular trafficking and NM23-H1 degradation. c-Abl induces cathepsin activation by promoting endosome to lysosomal trafficking. Endosomal NM23-H1 is transported to lysosomes and degraded by lysosomal protease cathepsin B and L. Inhibition or silencing c-Abl/Arg prevents cathepsin expression and activation, which leads to stabilization of NM23-H1. MT=microtubules, MVB=multi-vesicular body. Model provided by Dr R. Plattner

Chapter 4: Conclusions and Future Directions

4.1 Conclusions

Melanoma, the most lethal form of skin cancer, represents only 4% of the total number of skin cancer diagnoses, but accounts for approximately 80% of skin cancer-related deaths [1-3], with a 5-year survival rate of under 5%. This survival rate has not improved in the past decade. Recent advances in the development of new drugs for treating melanoma have raised enthusiasm for the identification of new therapeutic targets. However, the paucity of understanding of the mechanisms that lead to melanoma progression has unfortunately led to the failure of many of these drugs. These clinical failures underscore the need for identification and characterization of the molecular mechanism(s) that drive melanoma progression, thus leading to identification of new targets for its treatment.

While c-Abl and Arg are known to play an important role in breast, lung, prostate and liver cancer progression, little is known about their role in melanoma. Our study demonstrates a previously unrecognized role for c-Abl and Arg in melanoma progression and shows that c-Abl and Arg are activated in primary melanomas (60%), benign nevi (33%) and in some human melanoma cells lines in comparison to primary melanocytes. Our data also indicate that active c-Abl and Arg drive invasion, proliferation, and metastasis as well as survival following nutrient deprivation. Furthermore, we demonstrate that c-Abl upregulates melanoma invasion through a STAT3/MMP-1-dependent pathway,

whereas Arg promotes invasion through STAT3- independent MMP-1 and MMP-3 pathways. To our knowledge we are the first to report that c-Abl and Arg promote the same biological process via distinct mechanisms.

As a likely parallel pathway in melanoma progression, we show that Abl kinase activities are inversely correlated with expression of the metastasis suppressor, NM23-H1, in human melanoma cells. We further show that c-Abl and Arg are required for NM23-H1 degradation. NM23-H1 is degraded in a lysosomal cysteine cathepsin-dependent manner and that cathepsins B and L directly cleave NM23-H1. Moreover, we show that c-Abl promote the activation of cathepsins B and L. Our data indicate that c-Abl promotes the degradation of NM23-H1 by altering the endocytic pathway and that c-Abl likely promotes early to late endosomal maturation. The effects we observe have biological consequences as c-Abl/Arg promote melanoma cell invasion and metastasis by inducing NM23-H1 degradation, and promote anchorage-independent growth partially via NM23-H1 loss. The Abl/NM23-H1 pathway is clinically relevant as there is an inverse correlation between c-Abl/Arg activity and NM23-H1 expression in primary melanomas. Thus, this study identifies for the first time that a proto-oncogene has the ability to promote invasion and metastasis via degradation of a metastasis suppressor.

4.2 Future Directions

Our lab has previously shown that Abl kinases are activated by growth factor receptors and SFKs. Here we demonstrate that activated c-Abl and Arg promote melanoma proliferation, survival, invasion and late stages of metastasis. Our study in Chapter 2 shows that MMPs play an important role in c-Abl and Arg-mediated invasion of melanoma cells, indicating that targeted therapy directed against c-Abl and Arg may also inhibit MMPs. Inhibition of MMP-1 in cancer cells has been shown to inhibit collagen degradation and decrease tumor size in mice [123]. Conversely, over-expression of MMP-1 in the skin of transgenic mice increased their susceptibility to skin carcinogenesis [260,261]. Thus, future directions could include testing whether c-Abl and Arg promote melanoma metastasis in an MMP-dependent manner. To test this, we would need to perform immunohistochemisty on lung samples from nilotinib-treated mice to determine whether expression of MMPs is decreased as compared to vehicle-treated animals. We could also perform rescue experiments by injecting 435s/M14 cells overexpressing MMPs into mice to investigate whether c-Abl and Arg promote melanoma metastasis in a MMP-dependent manner.

In the past decade, multiple MMP inhibitors such as the peptide MMP inhibitors, Batimastat and Marimastat, have been used in the clinical setting [262,263]. However, none showed promising results in extending patient survival, indicating that MMPs may not be good targets for inhibiting cancer progression. Careful appraisal of these failures will help to identify the cause(s) of the problems encountered with the use of these drugs in these clinical trials.

Batimastat had poor solubility and could not be administered orally and hence was replaced by Marimastat. Marimastat is a broad-range MMP inhibitor, similar to Batimastat and thus, it also lacked specificity. Other non-peptidic MMP inhibitors, BAY-12-9566 and AG3340, were also used in clinical trials but showed poor clinical efficacy [262-265]. In addition, all these MMP inhibitors showed significant side effects such as musculoskeletal syndrome, which manifests as pain and immobility in the shoulder joints and contractures in the hands. These side effects were specifically seen when MMP-1 was targeted, which led to the development of MMP inhibitors that do not target MMP-1. In this study we show that MMP-1 is critically important in promoting invasion of melanoma cells. Thus, drugs targeting upstream regulators of MMP-1, such as STAT3 or c-Abl and Arg, might be better alternatives than MMP inhibitors and may have fewer side-effects.

Due to intolerable side effects associated with MMP inhibitors, such as musculoskeletal pain and tendinitis, many patients withdrew from the clinical study, thus, decreasing the group size in the treatment arm, rendering evaluation of the drug efficacy difficult. Thus, in follow-up trials, either MMP-specific small molecules or antibody-based inhibitors should be used. In mouse models, MMP inhibition has been effective in preventing cancer progression perhaps because they were used at an early stage of disease, prior to the presence of overt metastases. However, in human clinical trials, these inhibitors were used in patients who were in advanced stages of diseases, after metastatic progression, and were not utilized in combination with other chemotherapeutics [262-265].

These drawbacks may explain the poor performance of these drugs in the clinic. Our studies indicate that MMPs are important targets in melanoma metastasis and that they can be targeted using Abl kinase inhibitors, which do not exhibit any of the side-effects associated with MMP-1 inhibitors.

Recent data indicate that c-Abl and Arg promote gelatinase activity of breast cancer cells [62]. Our data suggest that c-Abl and Arg also promote collagen I degradation (single-cell 3D invasion assay). c-Abl/Arg promote the activation of MMP-1, -3 and -14, and all of these are known to degrade collagen I. Future experiments designed to demonstrate that c-Abl and Arg induce degradation of fluorescently-labeled collagen I could answer the question whether c-Abl and Arg promote collagenase activity in melanoma cells via the upregulation of MMPs. siRNA-mediated inhibition of MMP-1, -3 and -14 will identify which of the MMPs play a major role in collagen degradation in 435s/M14 cells. The collagenase activity could also be quantified using another type of assay, an *in vitro* collagen degradation assay. In this assay, 435s/M14 cells transfected with c Abl or Arg siRNAs would be incubated with collagen I, the collagen/gel solution allowed to gel followed by the addition of basal media on top of the gel and incubation at 37^0C [123]. The weight of the media at the start of the assay, subtracted from the total media weight at the end of the assay, would allow one to quantify the extent to which Abl kinase inhibition prevents collagen degradation. Future experiments will also be designed to investigate whether c-Abl and Arg-mediated collagen degradation is MMP-dependent by performing rescue experiments using MMP overexpression cells transfected with

c-Abl or Arg siRINA. These experiments will establish the role of c-Abl and Arg-mediated MMP upregulation in collagen degradation, a prime constituent in ECM and will also identify if c-Abl/Arg promote melanoma metastasis in a MMP-dependent pathway.

Our studies indicate that c-Abl promotes melanoma invasion in a STAT3-dependnet manner (Chapter 2) and activated c-Abl and Arg promote STAT3 phosphorylation, in a JAK-independent manner [64]. Phosphorylation of STAT3 at Y705 induces its transcriptional activity [232], but we have not yet demonstrated that c-Abl and Arg promote STAT3 transcriptional activity. Thus, future experiments will involve the use of luciferase reporter assays to confirm that c-Abl and Arg promote STAT3 transcriptional activity. Likewise, one could also test whether inhibition of c-Abl and Arg decrease the nuclear pool of phospho-STAT3 in melanoma cells. It also would be interesting to examine the STAT3 activation status in WM164 cells, which have low invasive potential and concomitantly have low c-Abl and Arg kinase activities as compared to the more highly invasive WM3248 and 435s/M14 cells. If transfection of constitutively-active c-Abl and Arg -(PP) into WM164 cells increases invasion by activating STAT3, we could conclude that activation of STAT3 is essential for c-Abl-mediated invasion as we showed in invasive 435s/M14 cells.

Our results indicate that c-Abl and Arg form a complex with STAT3 and induce its phosphorylation and activation. We also report that the phosphorylation of STAT3 is indirect and probably mediated by another tyrosine kinases. Src kinases can phosphorylate STAT3 directly [223, 224].Our lab has

previously shown that PDGFR-β phosphorylates c-Abl *in vitro*, and overexpression of constitutively active c-Abl induces the phosphorylation of kinase-dead PDGFR, indicating a bi-directional signaling pathway [266]. c-Abl can also phosphorylate EGFR and prolong EGFR signaling in the membrane [256]. Based on these data it would be interesting to investigate if c-Abl can phosphorylate Src kinase in a bi-directional manner and therefore activate STAT3 in a Src kinase-dependent manner and thus promote invasion. To test this hypothesis we would perform co-immunoprecipitation experiments to investigate if the c-Abl/STAT3 complex is disrupted following silencing of Src, and additionally, we could test whether expression of constitutively active c-Abl can induce the phosphorylation of a kinase-dead mutant of Src kinase.

In colorectal cancer, the transcription factor, STAT3, is known to bind to the promoter region of MMP-1, but the adjacent AP-1 binding site also has been shown to be essential for STAT3-mediated transcription of MMP-1 [209]. Moreover, in Chapter 2 we report that c-Abl promotes MMP-1 transcription via a STAT3-dependent mechanism. Thus, future experiments will be designed to test whether c-Abl promotes the direct binding of STAT3 to the MMP-1 promoter region. A reporter gene construct, including a promoter sequence from the human MMP-1 gene driving luciferase expression, will be transfected into 435s/M14 cells to test whether inhibition of c-Abl/Arg can repress MMP-1 promoter-driven luciferase activity. Rescue experiments utilizing this reporter construct transfected into 435s/M14-STAT3CA (cells expressing constitutively active STAT3) would be expected to result in rescue of the inhibitory effects of

imatinib. Furthermore, experiments involving mutation of the STAT3 binding sites in the MMP-1 promoter region could also address the question whether this binding occurs directly through STAT3. To investigate the necessity of the AP-1 element in this c-Abl/STAT3/MMP-1 interaction, AP-1 binding sites will be mutated in the presence of intact STAT3 binding sites. If the interaction is dependent on AP-1, the luciferase activity will be inhibited in the absence of the AP-1 promoter element.

The hepatocyte growth factor (HGF) has been shown to signal through c-Met to promote melanoma progression. In human squamous cell carcinomas, the HGF/c-Met/STAT3 signaling pathway promotes cancer cell invasion [267]. Blocking constitutive activation of STAT3 disrupts this pathway, reduces c-Met activity, and inhibits invasion of human squamous cell-carcinoma cells [267]. c-Met signaling through c-Abl/Arg in gastric carcinoma cells also promotes anchorage-independent growth, survival and metastasis. All of this experimental evidence in gastric cancer cells suggests that this c-Met/c-Abl/STAT3 axis might also regulate melanoma invasion, survival and metastasis. Furthermore, in pancreatic cancer cells, c-Met regulates IGF-1R-mediated invasion, indicating the presence of a considerable amount of crosstalk between these two receptors [268]. Moreover, our lab has previously shown that IGF-1R activates c-Abl and Arg [54]. For these reasons, it would be interesting to examine whether HGF stimulation, which activates c-Met, promotes invasion and survival in a c-Abl- and STAT3-dependent manner. c-Met also has been shown to activate Src kinases [269] and Src kinases directly phosphorylate and activate c-Abl and Arg in a

manner independent of IGF-1R. Thus, it was would be interesting to test if c-Met activates c-Abl and Arg in a Src-dependent or independent manner. To test this hypothesis, first we will silence c-Met and investigate the activation status of c-Abl/Arg by *in vitro* kinase assay following HGF stimulation. IGF-1R and Src also activate c-Abl and Arg, so we will also silence IGF-1R and Src to rule out that activation of c-Abl and Arg occurs via IGF-1R or Src, respectively. To investigate the dependence on Src during c-Met-induced c-Abl and Arg activation, we will examine if constitutive activation of Src can rescue the block in c-Abl and Arg activation following c-Met inhibition.

If silencing c-Met in 435s/M14 cells inhibits invasion, and constitutive expression of c-Abl-PP rescues the block in invasion induced by c-Met inhibition, this would suggest that c-Met and c-Abl lie in the same pathway to promote invasion of melanoma cells. Further experimentation using STAT3 siRNAs or constitutively active forms of STAT3 will identify whether c-Met/c-Abl/STAT3 lie in the same pathway. Interestingly, HGF binding to c-Met also activates MMPs and uPA. Hence, it would also be interesting to test whether c-Met activates MMPs in an Abl kinase/STAT3-dependent manner. These studies involving STAT3 will identify how c-Abl activates STAT3 in melanoma cells. The studies designed will identify other regulators that can activate c-Abl to promote the transcriptional activity of STAT3. These studies are of prime significance as it will establish c-Abl/STAT3 as an essential pathway in melanoma progression.

In Chapter 2, we demonstrated that c-Abl promotes transcription and activation of MMP-1, whereas Arg promotes the transcription and activation of

MMP-1, -3 and -14. The level of promotion of MMP-14 transcription and activation by Arg is very dramatic (almost 90%). In Chapter 3, we reported that Arg promotes the degradation of NM23-H1; however, this pathway seems not to be dependent on cathepsins B or L, as Arg does not promote the processing of the cathepsin to its double-chain form. Therefore, future experiments will involve investigating whether Arg, NM23-H1, and MMP-14 lie in the same pathway. Bossian et al, reported that NM23-H1 loss increased both cellular degradation of gelatin as well as cell invasion on native type I collagen. They also reported that NM23-H1 silencing promoted MMP-14 expression. Therefore, we will silence NM23-H1, in 435s/M14 cells, in order to assess whether NM23-H1 loss can rescue the block in MMP-14 induced by silencing Arg. Silencing of NM23-H1 induces β-catenin-TCF/Lef-1 transactivation and MMP-14 is a direct target of β-catenin/TCF signaling [159]. Moreover our lab had shown that Arg promotes the nuclear localization and expression of β-catenin in 435s/M14 cells [W. Friend, unpublished data]. These data led us to hypothesize that Arg may promote MMP-14 expression via a NM23-H1/β-catenin dependent pathway. Future experiments will be designed to evaluate whether Arg can induce the transcriptional activation of β-catenin using the TOP/FOP flash reporter assay and also by determining whether inhibition of nuclear translocation of β-catenin inhibits MMP-14 expression. To test whether Arg mediates MMP-14 transcription in a β-catenin-dependent manner, we will investigate if transcriptionally active form of β-catenin (S374A) rescues the effects of silencing Arg on MMP-14 transcription. We will

also investigate if silencing of NM23-H1 can rescue the inhibitory effects of silencing Arg on MMP-14 transcription.

Another potential pathway by which Arg may promote MMP-14-mediated invasion is the PI3K-Akt pathway. c-Abl and Arg activate the Akt pathway during nutrient deprivation (S Ganguly, unpublished observation) and Arg promotes invasion via MMP-3 and MMP-1 in a STAT3-independent manner. Akt is constitutively activated in melanoma and promotes RGP to VGP conversion [270,271]. Moreover Akt also stimulates the transactivation potential of the p65 subunit of NF-κB [272]. Furthermore, our laboratory showed that c-Abl/Arg induces nuclear translocation of p65 [237]. A consensus binding site for p65 has been detected in the MMP-14 gene, and NF-κB has been shown to promote MMP-14 expression in murine melanoma cells [273-275]. This evidence suggests that Arg also could promote the activation of MMP-14 via a Akt/ NF-κB-mediated pathway. We will test this hypothesis by investigating if constitutive activation of NF-κB in 435s/M14 cells (transfecting a constitutive active form of IκB kinase, IKK-2 S177E/S181E) rescues the inhibition of MMP-14 transcription following silencing of Arg. We will also investigate whether constitutive activation of NF-κB rescues the inhibition of invasion induced by silencing Arg. If inhibition of MMP-14 following constitutive activation of NF-κB inhibits invasion in 435s/M14 cells and overexpression of MMP-14 rescues the block in invasion after Arg silencing, this would indicate that Arg/ NF-κB/ MMP-14 lie in the same pathway to promote invasion of melanoma cells. These studies proposed are of

prime significance as it will discover new targets in Arg-mediated melanoma invasion and metastasis.

Survival in the absence of nutrients is an important determinant for cancer cells to grow and proliferate in distant metastasis sites and form macrometastasis. Excessive proliferation and survival in the absence of nutrients are important for neoplastic development. We show that c-Abl and Arg promote survival in response to nutrient deprivation via STAT3-dependent and -independent pathways. Even though c-Abl and Arg do not promote AKT activation under serum conditions in 435s/M14 cells [55], they activate AKT under serum-free conditions (S. Ganguly, unpublished data) and BCR-ABL is known to induce the activation of the PI3K-AKT pathway to promote leukemic cell survival [276]. Furthermore, c-Abl and Arg promote the expression of AKT isoforms in serum-starved conditions (S. Ganguly, unpublished data), and AKT isoforms play a major role in the survival of cancer cells. Inhibition of c-Abl/Arg in 435s/M14 cells induces the downregulation of AKT3 and silencing AKT3 in 435s/M14 cells sensitizes the cells to cell to death following nutrient deprivation (S. Ganguly, unpublished data). These data indicate that c-Abl and Arg might promote survival via an AKT3-dependent pathway. Constitutive expression of a myristoylated form of AKT3 into 435s/M14 cells could be used to test whether activation of Akt3 rescues the apoptotic effects caused by inhibition of c-Abl and Arg. We also could investigate the mechanism by which c-Abl and Arg activate Akt. Recently our lab has shown that c-Abl/Arg activate Akt in response to doxorubicin-induced stress via a p38/HSP27-mediated pathway in melanoma

cells [237]. Alternatively, c-Abl and Arg also could activate Akt via the downregulation of PTEN, since PTEN mediates the dephosphorylation of phosphatidylinositol (3,4,5)-triphosphate back to phosphatidylinositol (4,5)-biphosphate and thus Akt activation is compromised. Future experiments will test this hypothesis by examining whether c-Abl and Arg can phosphorylate PTEN and keep it in an inactive closed conformation. We will establish a phosphorylation-resistant PTEN (substituting the serine and theonine residues in COOH-terminal domain to alanine) to investigate this can inhibit activation of Akt in the presence of constitutively active c-Abl/Arg-PP.

NM23-H1 is reported to have no effect on the proliferation of cancer cells either *in vivo* or *in vitro* [159], whereas silencing of NM23-H1 has been shown to increase anchorage-independent growth of human hepatocarcinoma cells [159]. Here, we report that silencing NM23-H1 promotes anchorage-independent growth of 435s/M14 cells and that c-Abl and Arg mediate anchorage-independent growth partially via NM23-H1 loss. Anchorage-independent growth is the ability of cancer cells to proliferate and survive in an anchorage-independent state. Since inhibition of NM23-H1 does not promote proliferation, silencing NM23-H1 might promote survival during anchorage –independent conditions. NM23-H1 silencing has been shown to induce resistance to apoptosis in hepatocarcinoma cells treated with chemotherapeutic reagents; however the mechanism was not investigated. NM23-H1 also has been shown to induce apoptosis in Burkitt's lymphoma cells via a p53-dependent pathway and upregulation of caspases 3, 9 and Bcl-xL [277]. In human ovarian cancer cells and in ovarian clear cell

adenocarcinomas, low levels of NM23-H1 expression were correlated with activation of Akt, indicating that loss of NM23-H1 may confer resistance to apoptosis via a Akt-dependent pathway [278], although no direct evidence for this mechanism was demonstrated. Taken together, all these results indicate that expression of NM23-H1 could reverse resistance to apoptosis and render cancer cells sensitive to chemotherapy-induced apoptosis or apoptosis induced by serum deprivation. Chemotherapy-resistance is associated with a poor prognosis in many types of cancer, and is a common problem encountered in the clinic. Our laboratory has shown that imatinib reverses resistance to chemotherapy via a STAT3-dependent Akt pathway in breast cancer and melanoma cells [237]. Here, we report that c-Abl and Arg promote melanoma cell survival and activate Akt in nutrient-deprived conditions. Taken together, all these results indicate that activated c-Abl and Arg may mediate resistance to apoptosis in serum-deprived conditions via down-regulation of NM23-H1. To test this hypothesis, we will investigate whether silencing NM23-H1 can rescue the apoptotic effects of imatinib following serum-starvation. To investigate the dependence of Akt in this pathway, we will test if constitutively activated, myristoylated Akt can rescue the apoptotic effects mediated by NM23-H1 overexpression in 435s/M14 cells. Reversal of chemotherapy resistance by imatinib could also be attributed to inhibition of Akt mediated by stabilization of NM23-H1-dependent. To test hypothesis, first, we will investigate if silencing NM23-H1 rescues the synergistic apoptotic effects observed following treating cells with imatinib and other chemotherapies [237]. We will also investigate if inhibition of Akt can prevent the

rescue induced by silencing NM23-H1. Proteins that promote survival of cancer cells in nutrient deprived conditions and in response to chemotherapy play a critical role in cancer progression. This study will identify proteins that promote survival in c-Abl and Arg-dependent manner and will shed light on how c-Abl/Arg promotes melanoma progression.

In Chapter Two, we demonstrated that c-Abl and Arg promote the expression of TIMP-1. It is important to further investigate the role that c-Abl and Arg play in mediating TIMP-1 activation during the survival of melanoma cells. Our results suggest that TIMP-1 exerts effects contrary to its anti-metalloproteinase activity (Figure 2.16). Various prior studies suggest that TIMP-1 enhances tumor growth and metastasis [135,136]. Our study largely supports the hypothesis that TIMP-1 promotes oncogenesis rather than having tumor-suppressive effects. Elevated expression of MMP-1 and MMP-13 along with TIMP-1 and -3 is associated with increased invasion of primary melanomas [122]. The Oncomine cancer microarray database suggests that TIMP-1 is augmented in cutaneous melanoma patients as compared to normal skin or benign nevi [279,280], indicating that TIMP-1 expression may be necessary for melanoma progression. Since the transcription and activation of TIMP-1 by c-Abl/Arg is dependent on STAT3 activation (Figure 2.17), it is likely that TIMP-1 overexpression would result in the same partial rescue of imatinib-induced apoptosis as observed with STAT3CA expression. However, this hypothesis needs to be tested by investigating whether overexpressing TIMP-1 in 435s/M14 cells rescues the block in apoptosis induced by imatinib. Furthermore, if silencing

TIMP-1 blocks the partial rescue in survival observed with 435s/M14-STAT3CA cells following expression of STATCA cells treated with imatinib, then we can conclude that c-Abl/Arg, STAT3, and TIMP-1 all lie in the same pathway and that together they promote survival of melanoma cells in response to serum-deprivation conditions.

Our laboratory showed that c-Abl and Arg promote survival in response to nutrient deprivation in both breast cancer [54] and melanoma cells (Figure 2.7). A closely related survival phenomenon is anoikis resistance, which refers to the ability of cells to resist death in suspension as a result of loss of physical contact with extracellular matrix. Activation of Src kinases promote survival, invasion, resistance to anoikis, and metastasis in breast cancer and lung adenocarnima cells [281,282]. TIMP-1 overexpression also has been shown to promote anoikis resistance in murine melanocyte lineage melan-a cells [279]**.** Our laboratory showed that Src kinases activate c-Abl/Arg in 435s/M14 cells [54], and here we show c-Abl and Arg promote TIMP-1 expression in a STAT3-dependent manner. Hence, it is possible that activated c-Abl and Arg induces resistance to anoikis in melanoma cells. To test this hypothesis, we could examine whether silencing or inhibiting c-Abl/Arg promote cell death in the absence of matrix attachment. To investigate whether the pathway is dependent on STAT3, we will assess whether expression of constitutively active STAT3 (STAT3CA) can rescue imatinib-mediated cell death in the absence of matrix attachment. To test if c-Abl and Arg mediate anoikis resistance via a TIMP-1-dependnet pathway, we will investigate

if overexpression of TIMP-1 can rescue imatinib-mediated apoptosis in the absence of matrix attachment.

Most of the survival and cell growth effects of TIMP-1 on cancer cells may be attributed to the direct binding of TIMP-1 to a cell surface receptor [284,285]. Binding of TIMP-1 to CD63 activates integrin β1 in human breast cancer epithelial cells [284]. This complex can interact with PI3K to activate the AKT pathway, thus, preventing the activation of the caspase cascade in breast carcinoma cells [285]. Thus, future experiments will investigate whether inhibition of c-Abl and Arg can inhibit activation of this pathway. TIMP-1 interacts with the CD63/integrin β1 complex [284,285], so we will investigate if inhibition of c-Abl and Arg disrupts TIMP-1 binding within this complex using confocal microscopy and co-immunoprecipitation methods. Future experiments also will be designed to test if overexpression of CD63 can rescue the effects of imatinib on caspase-3 activity and apoptosis following nutrient deprivation. These results will also provide evidence that c-Abl and Arg can modulate an outside to inside cell signaling cascade via TIMP-1. Future experiments also will be designed to test whether c-Abl and Arg prevent caspase activation (Figure 2.7) in a TIMP-1-dependnet manner. It has been reported that Arg modulates integrin β1signaling and regulates epithelial cell polarity [286]. There also is contrasting evidence regarding the role of integrin β1 signaling in cancer progression as there is evidence to support both a pro-neoplastic function as well as a function in suppressing cancer progression. Given, the role of c-Abl and Arg on TIMP-1 expression and its potential role in modulating integrin β1 signaling, we

hypothesize that c-Abl and Arg regulate integrin β1signaling through TIMP-1, and thus promote survival and metastatic progression. Experiments proposed in this section will give additional evidence in the role of c-Abl and Arg to promote melanoma metastasis in a TIMP-1-dependent manner. Experiments designed in this section will also identify how c-Abl and Arg activate cell surface proteins in an outside to inside cell signaling cascade via TIMP-1.

Invasion of cancer cells through the ECM, intravasation, extravasation, survival in nutrient free conditions in the ectopic site and proliferation of cancer cells from micrometastasis to macrometastasis are important steps in cancer metastasis. Previous studies in this section were aimed to determine the mechanism that c-Abl and Arg may upregulate to promote various *in vitro* steps in cancer metastasis. Here in this section we will discuss how various *in vivo* steps in melanoma metastasis can be promoted by activated c-Abl and Arg. We have shown that activated c-Abl and Arg promote melanoma metastasis using two different cell lines and two different mouse models. It would be interesting to test whether inhibition of c-Abl/Arg enhances overall survival of nilotinib-treated animals in comparison to vehicle only-treated mice. In order to test this, we need to repeat the experimental *in vivo* metastasis assay without euthanizing mice on a specific day but rather extend the study until all of the vehicle- treated mice expire due to disease progression. These data would then be used to generate a Kaplan-Meier survival chart, which would be important in determining whether nilotinib might be useful in the clinic in enhancing survival of melanoma patients.

We have shown that nilotinib, which inhibits both c-Abl and Arg, inhibits late stages of melanoma metastasis. To specifically identify whether c-Abl and/or Arg drive the process of melanoma metastasis, we will use Tetracycline-inducible (Tet-on) systems. Commercially available shRNA against c-Abl and Arg, cloned in a Tet-on-inducible vector, will be stably transfected in 435s/M14/GFP cells, and the resulting cells injected into mice for both spontaneous and experimental metastasis assays. One group of animals will be given water supplemented with doxycycline, in order to induce the activation the c-Abl and/or Arg shRNAs, and extent of metastasis will be compared with mice that do not receive doxycycline. These experiments will identify whether activated c-Abl and/or Arg can drive melanoma metastasis.

We also found that inhibition of c-Abl/Arg does not affect primary tumor growth of two melanoma cell lines (435s/M14 and WM3248)(S Ganguly, unpublished observation). However, another study demonstrates that inhibition of Abl kinases reduces primary tumor growth of gastric hepatocellular carcinoma cells [58], indicating that the effect of c-Abl/Arg inhibitors in reduction of tumor size might be cancer type-specific. Our data suggest that reduction in the size of primary tumors does not predict the efficacy of a c-Abl/Arg inhibitor in melanoma settings, since we found that a c-Abl/Arg inhibitor prevented metastatic colonization using experimental metastasis assays. Unfortunately, due to technical difficulties, we were unable to evaluate the metastatic endpoint using spontaneous metastasis assays (metastasis form a primary tumor). Thus, more studies need to be performed in the future. However, we did show that c-Abl and

Arg promote experimental metastasis (late stages of metastasis). In this process, the cancer cells bypass the few initial steps of cancer metastasis cascade, such as primary tumor growth, invasion through the extracellular matrix, and intravasation into the blood stream. However, in an experimental metastasis assay, the cells still have to extrasavate out of the blood stream and invade the new organ site to form micrometastasis in the ectopic site, survive in nutrient free conditions, and proliferate to form macrometastasis. Our studies have identified that Abl kinases promote cancer cell invasion in a MMP-1 and MMP-3 dependent manner and invasion is a very important process of cancer metastasis. Cancer cells have to degrade the ECM around the primary tumor, invade though the ECM and further invade into the blood vessels (intravasation). Although c-Abl and Arg do not promote tumor growth of melanoma cells, they may affect the process of invasion of tumor cells from the primary tumor or intravasation. Tumor invasion is associated with a characteristic distorted edge of the primary tumor, where tumor cells actively invade the ECM surrounding the primary tumor. Tissue samples of primary tumors from cancer patients and animals have shown elevated expression of MMPs [105]. Thus, future experiments will involve performing immunohistochemisty to determine whether there is a decrease in MMP-1 expression at the invasive front of the tumors extracted from mice treated with nilotinib. Expression of MMPs is generally observed in higher levels around the invasive front of the tumor [287]. To investigate whether c-Abl and Arg promote the process of dissemination of tumor cells in a MMP-1 dependent process, 435s/M1/GFP cells will be injected in the flank region by subcutaneous

injection and the mice treated with vehicle or nilotinib. We will investigate if inhibition of c-Abl/Arg can inhibit the tumor cell interaction with adjacent ECM, and we will analyze local invasion the cells by analyzing with intravital multiphoton microscopy or Spinning (Nipkow) disk confocal microscopy [105, 288-290]. To track the tumor cells for several imaging sessions (several days), 435s/M14 cells can be photomarked with photoconvertable flurophores like Kaede and Dendra2 to mark regions of interest in a tumor for over several days and intravital microscopy along with 'mammary imaging window' can be performed [283]. Mammary imaging window allows to visualize the metastatic process for several days [283].To investigate if c-Abl and Arg promote cancer cell invasion around the primary tumor in a MMP-dependent manner, cells overexpressing MMPs and GFP will be utilized to test if MMP overexpression – GFP labeled cells rescues the block in local tumor invasion following c-Abl and Arg inhibition and aid in increased tumor cell dissemination.

Escape of cells from the primary tumor involves invasion and polarization of tumors cells towards the blood vessels. Tumors cells invade through the collagen layers of the blood vessels (intravasation) and remain in the circulation until they extravasate into a favorable ectopic site. Since c-Abl and Arg promote invasion in vitro, it is possible they may affect the intravasation process. Quantification of intravasation can be performed by periodic examination of blood samples from vehicle and nilotinib-treated mice from spontaneous metastasis assays, and analyzing the blood for specific tumor-cell markers by RNA-targeted PCR methods [105]. Cytokeratin-20, a carcinoembryonic antigen has been used

to identify colorectal circulating cells to quantify intravasation [105]. Blood samples from vehicle and nilotinib- treated mice from spontaneous metastasis assays can also be analyzed by radioactive or fluorescence-based PCR techniques to detect human-specific DNA sequences. This analysis also will give a quantitative estimation of how many human-derived tumor cells are present in the bloodstream and thus, will give an estimation of the differences in intravasation efficiency between vehicle- and nilotinib-treated mice. Intravasation can also be measured using by intravital multiphoton microscopy on live animals [105,288]. 435s/M14-GFP cells will be injected to form primary tumor, and mice treated with vehicle or nilotinib. The blood vessels will be labeled by injecting Texas red-Dextran in the carotid artery [291]. The presence of GFP- positive cells in the red fluorescent blood vessels will be monitored by intravital multiphoton microscopy in order to detect differences in intravasation following inhibition of c-Abl/Arg. Since blood vessels have collagen layers and MMP-1 and MMP-3 can efficiently degrade collagen, we hypothesize that c-Abl and Arg may mediate the process of intravasation via MMP-1 and -3-dependent pathways. We predict that overexpression of these MMPs will rescue the block in intravasation induced by nilotinib treatment.

Since we have demonstrated that c-Abl/Arg promote late stages of cancer metastasis and there is an increase in the metastatic burden in the lungs of vehicle-treated mice and significant decrease following nilotinib treatment, we can hypothesize that nilotinib may inhibit proliferation and survival of these cancer cells after they seed in the ectopic site. We can test this hypothesis by

staining the lung metastases with antibody to a proliferation index marker (Ki67), and use TUNEL stain to identify apoptotic lesions. Since c-Abl and Arg promote both proliferation and survival of melanoma cells, *in vitro*, nilotinib might exert its anti-metastatic effects by inhibiting both of these processes.

Metastasis is the main cause of death in cancer patients. Some patients who receive adjuvant therapy initially respond to the drugs, but come back later to the clinic with metastases. Most of the tumor cells that disseminate from the primary tumor and intravasate into the blood vessels do not progress to form clinically overt metastases [292, 293]. This process is called as metastatic insufficiency. Only a small fraction (2%) can grow to form micrometastasis and even a smaller fraction (0.02%) can survive to form macrometastasis, making this last step of conversion from micro metastasis to macro metastasis an important part step in inhibiting tumor dormancy [293]. Tumor dormancy can persist for months or even for years. These dormant cells stay quiescent in solitary state without proliferating and survive in nutrient deprived conditions. The property to survive in nutrient-free conditions helps them to maintain dormancy, and later, they revert back to a proliferative state and form metastases. Although relatively little is known regarding tumor dormancy several key pathways have been identified, including p21,p27,p38, uPAR, and cells that express very low levels of angiogenic markers or high levels of angiogenic inhibitors also can attain tumor dormancy [294,295].

In chapter 2, we showed that activated c-Abl and Arg promote survival of melanoma cells in nutrient deprived conditions and promote late stages of

metastasis, indicating that c-Abl and Arg can promote the survival of a small fraction of solitary cells after extravasation and lead to dormancy. Using *in vivo* video microscopy techniques, we could determine if activated Abl kinase promote dormancy of melanoma cells by examining whether some 435s/M14 cells injected intravenously attain tumor dormancy after successful extravasation from the blood vessels and if nilotinib is effective in eradicating these dormant cells. Using *in vivo* video microscopy at periodic intervals starting from 30 minutes after intravenous injections of 435s/M14-GFP cells to days after injection, we could ascertain the percentage of fluorescent-labeled 43s/M14 cells that successfully reach the lung and survive to form micrometastasis, and form macrometastasis, or remain solitary as dormant cells. This experimental procedure also will allow us to assess the percentage of cells that remain dormant following treatment with nilotinib. The inability to extravasate has been considered a rate-limiting step in cancer metastasis. We could also assess whether inhibition of activated c-Abl/Arg by nilotinib, can decrease the percentage of cells that can successfully extravasate as compared to vehicle-treated mice. The cells that attain tumor dormancy will be analyzed for Ki67, TUNEL via immunohistochemical methods, to assess their proliferative index and apoptotic nature. We can also stain these cells to detect the activation status of c-Abl and Arg and determine whether these dormant cells have activated c-Abl and Arg. The tumors that leave dormancy and form macrometastasis will be assessed for level of c-Abl/Arg activation, using immunohistochemistry, to investigate if increased c-Abl/Arg activation is associated with loss of dormancy. Future experiments will also identify if nilotinib

can exert its anti-metastatic capacity by promoting apoptosis of these dormant cells. We could also extract a subset of these dormant cells and grow them in culture to assess if these dormant tumor cells have upregulated the p38 pathway, which makes them growth arrested and dormant, or whether the Erk pathway is downregulated, so that the cells do not proliferate and remain dormant [294,295]. Alternatively we can stain the lung sections using specific antibodies against p38 or Erk. Dormant cells lack angiogenic signals and stay quiescent and survive in nutrient free conditions. p38 leads to downregulation of VEGF and upregualtion of anti-angiogenic factor like TSP [296,301], and c-Abl and Arg promote the activation of p38, so activated c-Abl/Arg may promote tumor dormancy via this pathway. To test this hypothesis we can perform immunohistochemistry on the lung sections of nilotinib and vehicle-treated mice to investigate if nilotinib can effectively inhibit p38. Inhibition of p38 by nilotinib will free the cells from dormancy and also make them sensitive to the anti-metastatic capacity of nilotinib. Ki67 and TUNEL staining will also be performed to investigate if nilotinib can promote apoptosis of these quiescent (Ki67-negative) dormant cells**.**

We also could study dormancy by labeling 435s/M14 cells expressing GFP cells (435s/M14-GFP) with fluorescent nanospheres, which will identify cells that have not divided [297]. As cells divide, nanospheres will be diluted to undetected levels in rapidly proliferating cells. 435s/M14-GFP cells with nanospheres will be injected intravenously and periodically imaged and later dormancy will be defined by detection of fluorescence nanosphere *in vivo* against a background of growing metastases. We will also investigate if nilotinib can

promote apoptosis of these dormant cells in the lungs. Lower levels to no detection of these nanospheres in the lungs after nilotinib treatment will indicate that nilotinib can effectively prevent cancer dormancy. As an alternate approach, these solitary cells in the lungs, can be stained for Ki67, negative Ki67 staining will define non-proliferating, quiescent cells.

The spontaneous and experimental mouse models describe above utilizes the use of injecting human tumor cells into immunocompromised mice. To recapitulate the clinical settings, genetically engineered mouse models (GEMMs) will be utilized to evaluate the dependence of c-Abl and Arg in melanoma metastasis. These GEMMs also are immune component, and the tumors that develop in the mice reflect the human counterpart, having stroma and vasculature of the same species [298]. Currently mouse models of melanoma are available, where oncogenes are ectopically expressed. Our lab has shown that c-Abl/Arg are highly activated in melanoma arising from chronic-sun-exposure, indicating that UV-induced model of mouse melanoma like MT::HGF could be utilized. c-Abl/Arg PP oncogene will be cloned under the control of metallothionein-1(MT promoter) in the melanocytes, generating a MT::Abl mouse model in C_{57}BL6 background. These neonatal mice will be UV-radiated, the incidence and latency of metastasis will be compared against control mice having β-galactosidase gene in place of c-Abl/Arg PP. To investigate if nilotinib is effective in treating metastasis arising from activated c-Abl/Arg-PP in the GEMM model, mice harboring the c-Abl/Arg-PP transgene will be treated with nilotinib and metastatic endpoint will be compared with vehicle-treated mice. Similarly the

tyrosinase distal regulatory element (DRE) or constructs in which SV40 large T antigen is under the control of the mouse tyrosinase promoter could be utilized to enhance transgenic expression of c-Abl/Arg-PP oncogene in melanocytes of C_{57}BL6 background [299]. The different animal experiments proposed in this section will identify in vivo processes that activated c-Abl/Arg upregulate to promote melanoma metastasis. These proposed experiments will also identify whether activated c-Abl/Arg can promote tumor dormancy and whether nilotinib can induce apoptosis of these dormant cells. These experiments are of prime significance as it will identify if c-Abl/Arg inhibitors can be used to treat patients with metastatic melanoma.

Abnormal vesicular trafficking plays a crucial role in cancer progression, as endocytosis permits the internalization of signaling receptors, and targets them to be destroyed in the acidic lysosomes [250]. In Chapter 3 we showed that c-Abl likely effects endosome maturation, which is independent of the acidification step. Endosomes are motile, and they move from the cytoplasm to the perinuclear region along the mircotubules, which is an important step in endosomal maturation, independent of endosomal acidification [248,249]. During endosome maturation, early endosomes migrate towards the nucleus along microtubules. Early endosomes which are Rab5-positive, are replaced with larger perinuclear-located endosomes (late endosomes), which express Rab7. This process is called Rab5/Rab7 switching, and it can be blocked by expression of a constitutively active from of Rab 5(Q79L) [248]. Our results indicate that silencing of c-Abl block endosome maturation, independent of acidification (chapter 3).

Although silencing c-Abl alters the distribution of early endosomes, it does not change the localization or induce perinuclear clumping of Rab7-positive late endosomes or LAMP1-positive lysosomes, indicating that c-Abl impairs the Rab5/Rab7 switching during endosome maturation. We will test this hypothesis by investigating if constitutive active Rab7 can rescue the block in endosome maturation observed after silencing c-Abl. Another possibility in the delay of endosome maturation might be due the presence of Rab5 on endosomes together with Rab7 (hybrid endosomes) [300]. Double staining of Rab5 or EEA 1 and Rab7 following silencing of c-Abl will answer the question whether some amount of EEA 1 positive vesicles are also positive for Rab7. We will also investigate whether silencing c-Abl delays the loss of Rab5 from early endosomes and thus, prevents endosomal maturation and cargo movement. Thus, inhibition of c-Abl may delay the removal of Rab5 from endosomes by keeping it in an active GTP bound state. To test this hypothesis, we will express a dominant-negative form of Rab5 (Rab5S34N) and test whether it can rescue the inhibitory effects of silencing c-Abl on endosome maturation. Using video-enhanced fluorescence microscopy in live cells transfected with c-Abl siRNA we can track if Rab5 persistently stays on Rab5/Rab7 hybrid vesicles and thus, delays endosome maturation.

Another possible mechanism in that may explain the defect in endosome maturation following inhibition/silencing of c-Abl is disruption of the endosomal sorting complex required for transport (ESCRT-0, -I, II, -III), which is present on the cytosolic surface of early endosomes [248]. The ESCRT complex drives the

process of sorting ubiquitinated-tagged cargo in the early endosomes and processes it to the intralumenal vesicles (ILVs) present in the late endosomes. Disruption of the ESCRT machinery leads to the accumulation of cargo in the early endosomes and subsequent decreased number of multivesicular bodies [245,302]. Thus, we will investigate whether inhibition of c-Abl deplete any of the ESCRT complexes by western blotting techniques. Immunoflouresence assays will be used to identify the depletion of the ESCRT components following silencing of c-Abl. These experiments proposed will specifically identify how inhibition/silencing of c-Abl blocks endosome maturation and thus prevents degradation of NM23-H1. In the case of a metastasis suppressor, endocytosis is considered an attenuator of signaling as degradation of the metastasis suppressor can lead to cancer progression

Metastatic melanoma is a death sentence as all known chemotherapeutic drugs have less than a 5% success rate and do not extend life expectancy beyond 10 months [1,3]. The emergence of vemurafenib, a selective inhibitor of the V600E activating mutation in BRAF, showed promise; however, the success has been dampened due to relapse, which is observed in a large number of patients within one year [6]. These data indicate a dire need to find new treatment options to increase survival rates and reduce relapse/recurrence. Our studies indicate that c-Abl and Arg are activated in primary melanomas and melanoma cells, and c-Abl promote invasion in a STAT3 and MMP-1 dependent manner, survival, proliferation and metastasis (Chapter2). We also demonstrate that c-Abl and Arg promote invasion and metastasis via downregulation of NM23-

H1 (Chapter 3). Melanoma cells resistant to BRAF inhibitors have upregulated EGFR-SFK-STAT3 signaling which promotes invasion and metastasis in these resistant cells [22]. Previous studies from our lab demonstrate that Src and EGFR activate c-Abl and Arg in cancer cells, indicating that nilotinib might be able to be used in a group of patients who do not respond to vemurafenib. Our studies indicate that nilotinib could be used to treat patients with metastatic melanoma; however, patients should be selected properly in order to increase the likelihood of a favorable response. Patients whose tumors have activated c-Abl and Arg should be pre-screened and preferentially selected for treatment over other patients.

All of the studies proposed here will strengthen the hypothesis that c-Abl and Arg are required for melanoma progression, will identify various effector targets of c-Abl and Arg-mediated melanoma progression, and will establish tumor expression signatures (STAT3, Akt, NF-κB, MMP and TIMP) that can be used to identify patients with metastatic melanoma who are likely to respond to c-Abl and Arg inhibition.

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