Role of ABL Family Kinases in Breast Cancer

by

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Duke University

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Xiao-Fan Wang

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pharmacology and Cancer Biology in the Graduate School of Duke University

2016
ABSTRACT

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Abstract

The ABL family of non-receptor tyrosine kinases, ABL1 (also known as c-ABL) and ABL2 (also known as Arg), links diverse extracellular stimuli to signaling pathways that control cell growth, survival, adhesion, migration and invasion. ABL tyrosine kinases play an oncogenic role in human leukemias. However, the role of ABL kinases in solid tumors including breast cancer progression and metastasis is just emerging.

To evaluate whether ABL family kinases are involved in breast cancer development and metastasis, we first analyzed genomic data from large-scale screen of breast cancer patients. We found that ABL kinases are up-regulated in invasive breast cancer patients and high expression of ABL kinases correlates with poor prognosis and early metastasis. Using xenograft mouse models combined with genetic and pharmacological approaches, we demonstrated that ABL kinases are required for regulating breast cancer progression and metastasis to the bone. Using next generation sequencing and bioinformatics analysis, we uncovered a critical role for ABL kinases in promoting multiple oncogenic pathways including TAZ and STAT5 signaling networks and the epithelial to mesenchymal transition (EMT). These findings revealed a role for ABL kinases in regulating breast cancer tumorigenesis and bone metastasis and provide a rationale for targeting breast tumors with ABL-specific inhibitors.
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<tbody>
<tr>
<td>ABL</td>
<td>Abelson kinase</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute lymphocytic leukemia</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>BCR</td>
<td>Breakpoint cluster region</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic myeloid leukemia</td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer stem cell</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>RANKL</td>
<td>Tumor necrosis factor ligand superfamily, member 11</td>
</tr>
<tr>
<td>WWTR1</td>
<td>WW domain-containing transcription regulator protein 1</td>
</tr>
</tbody>
</table>
1. Introduction

1.1 Breast cancer

1.1.1 Breast cancer overview

Breast cancer is the most common cancer in women and the second most common cause of cancer mortality. According to data from American Cancer Society, there were about 1.7 million newly diagnosed breast cancer cases in 2012 worldwide and more than 200,000 patients are diagnosed with breast cancer each year in the United States. The death rate for breast cancer has been decreasing over the past couple decades because of treatment advance, early detection and increased awareness.

Approximately 95% of breast cancers are carcinomas, which arise from breast epithelial cells. There are two major types of breast cancer: in situ carcinomas and invasive carcinomas. The in situ carcinomas arise in ductal or lobular epithelium and remain confined there. The risk of developing metastases is very low for this type of breast cancer. However for invasive carcinomas, there is extension of the malignancy beyond the basement membrane and the potential for metastases and mortality is relatively higher than in situ carcinomas.

Breast cancers are divided into four major molecular subtypes: Luminal A, Luminal B, HER2 positive, and Triple negative/basal-like (Table 1). The luminal A subtype is characterized by positive estrogen receptor (ER) and/or progesterone receptor (PR) expression, negative HER2 expression and low level of Ki67. The luminal B subtype
is ER-positive and/or PR-positive, and HER2-positive or HER2-negative with high Ki67 expression. The HER2 subtype is ER-, PR-negative and HER2-positive, and triple negative/basal-like is ER-, PR-, and HER2-negative. A subset of triple negative breast cancer cases are basal-like (1).

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Characterization</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminal A</td>
<td>• ER-positive and/or PR-positive        • HER2-negative        • Low Ki67</td>
<td>30%-70%</td>
</tr>
<tr>
<td>Luminal B</td>
<td>• ER-positive and/or PR-positive        • HER2-positive (or HER2-negative with high Ki67)</td>
<td>10%-20%</td>
</tr>
<tr>
<td>HER2</td>
<td>• ER-negative        • PR-negative        • HER2-positive</td>
<td>5%-15%</td>
</tr>
<tr>
<td>Triple negative</td>
<td>• ER-negative        • PR-negative        • HER2-negative</td>
<td>15%-20%</td>
</tr>
</tbody>
</table>

1.1.2 Breast cancer bone metastasis

Metastasis of cancer cells from the primary tumor to secondary organ sites such as lung, liver, brain, and bone accounts for more than 90% of cancer mortality (2). Bone metastases are one of the most frequent complications of cancer, occurring in up to 70% of patients with advanced breast or prostate cancer and in approximately 15% to 30% of patients with carcinoma of the lung, colon, stomach, bladder, or kidney (3). Once tumors
metastasize to bone, they are usually incurable: the median survival time after detection of these metastases is approximately two years, and only 20 percent of patients with breast cancer are still alive five years after the discovery of bone metastases (4). Furthermore, bone metastases can cause severe pain, pathologic fractures, life-threatening hypercalcemia, spinal cord compression, and other nerve-compression syndromes (5). For all these reasons, bone metastasis is a serious complication of cancer. The mechanisms and signaling pathways involved in the initiation and progression of bone metastasis have remained largely unknown.

Bone metastases have been characterized as (1) osteolytic, which are caused by osteoclast-activating factors released by tumor cells; and (2) osteoblastic, which are caused by osteoblast proliferation, differentiation and bone formation. For most patients with breast cancer, bone metastasis is predominantly osteolytic (Fig.1). Central to the control for osteolytic metastasis is the modulation of osteoclast activity by the TNF family member RANKL (6). RANKL is expressed in both membrane-bound and soluble forms by osteoblasts, while its cognate receptor RANK is expressed on the surface of osteoclasts and controls the signaling pathway essential for osteoclast differentiation. OPG, a soluble decoy receptor of RANKL, is also produced by osteoblasts to antagonize the activity of RANKL (7).

Drugs, such as bisphosphonates or RANKL antibodies, that target osteoclastogenesis can decrease the incidence of skeletal complications and are the
current standard of care for breast cancer patients with bone metastases (8). However, 30-50% of patients on such therapies still develop new bone metastases, skeletal complications and disease progression (9), emphasizing the need for new therapies and a better understanding of the interactions between breast cancer cells and bone microenvironment.

Figure 1: Tumor and bone interactions promote breast cancer bone metastasis. When tumor cells invade into the bone, they secrete factors such as granulocyte macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) to directly induce osteoclast activation. Alternatively tumor cells induce osteoclast activation indirectly through osteoblast. Activated osteoclasts resorb the bone and release the growth factors such as transforming growth factor beta 1 (TGF-beta) and insulin-like growth factor (IGF) to promote tumor growth.

1.2 The ABL family of nonreceptor tyrosine kinases

The Abelson tyrosine kinases were initially identified as drivers of leukemia in mice and humans. The ABL family kinases, ABL1 and ABL2, regulate diverse cellular
processes during development and normal homeostasis, and their functions are subverted during inflammation, cancer and other pathologies.

The ABL kinase was first identified from studies of the Abelson murine lymphosarcoma virus \((A-MuLV)\) that induced transformation of murine fibroblasts and lymphoid cells \(in vitro\) and lymphomas \(in vivo\) \((10-12)\). Subsequent studies demonstrated that chromosomal translocation of human ABL1 to the Breakpoint Cluster Region (BCR) gene sequences results in production of the BCR-ABL1 fusion protein in patients with Philadelphia (Ph) chromosome-positive human leukemia \((11)\). The BCR-ABL1 chimeric protein exhibits elevated tyrosine kinase and transforming activities and has been identified in distinct human leukemias, including chronic myelogenous leukemia (CML) and acute lymphocytic leukemia (ALL). Sequencing studies identified ABL2 (also known as ABL-related gene or Arg) as a paralog of ABL1 \((13)\). Oncogenic forms of both ABL1 and ABL2 have been identified in some forms of T-cell acute lymphoblastic leukemia (T-ALL) and acute myeloid leukemia (AML) as a result of chromosomal translocation of the \(ETV6 (TEL)\) gene to either \(ABL1\) or \(ABL2\) \((reviewed in (14, 15))\).

The development of small molecule ATP competitive inhibitors targeting BCR-ABL1 for the treatment of CML was a major breakthrough that opened the door to the era of targeted therapies \((16, 17)\). Treatment with imatinib or other FDA-approved ATP-competitive inhibitors was shown to induce durable remissions and progression-free survival in the majority of chronic-phase Ph-positive CML patients.
While persistent activation of the ABL kinases is linked to the emergence of human and murine leukemias, the activities of the endogenous ABL kinases are highly regulated by diverse stimuli ranging from growth factors, chemokines, DNA damage, oxidative stress, adhesion receptors, to microbial pathogens (14, 18). Once activated, the ABL kinases regulate signaling pathways implicated in cytoskeletal reorganization important for cellular protrusions, cell migration, morphogenesis, adhesion, endocytosis, and phagocytosis (18, 19). ABL kinases can also regulate cell survival and proliferation pathways depending on the cellular context (14). Emerging data support a role for abnormally activated ABL kinases in diverse pathologies including several solid tumors, inflammatory disorders, and neurodegenerative diseases. Moreover, accumulating reports have revealed that ABL family kinase function is subverted by numerous microbial pathogens to achieve entry, motility, release and/or survival in mammalian host cells (20). Thus, targeting the ABL kinases with small molecule inhibitors that were initially developed to treat CML patients might be employed to treat distinct pathologies with hyper-active ABL kinases.

### 1.2.1 ABL kinases in normal cell biology

Material from this Chapter was originally published in Journal of Cell Science (2016) 129, 9-16. Figure 3, 4, 6 are generated by Aaditya Khatri.
1.2.1.1 Structure, modular domains, and enzymatic regulation of the ABL kinases

ABL kinases have been identified in all metazoan and premetazoan unicellular organisms such as the choanoflagellate Monosiga bevicollis (18). The structural domains of mammalian ABL kinases are shown in Fig.2. Vertebrate metazoans express ABL1 (c-ABL) and ABL2 proteins that share a highly conserved SH3-SH2-SH1 cassette containing a tyrosine kinase Src homology 1 (SH1) domain and regulatory SH3 and SH2 domains. This cassette is preceded by an N-terminal cap region. Both kinases also have F-actin binding domains within their large carboxy (C)-terminal sequences. The DNA-binding domain, nuclear localization signals, and nuclear export signal in the ABL1 C-terminus are consistent with its nucleo-cytoplasmic localization, while the microtubule- and actin-binding domains in the ABL2 C-terminus are consistent with its localization at the cell periphery (21). Conserved proline-rich sequences in the C-terminal domain of the ABL kinases mediate protein-protein interactions (18). Alternative splicing of the first exons produces various isoforms of ABL1 and ABL2 that harbor distinct N-terminal sequences. The 1b isoforms of both ABL kinases contain an N-terminal glycine that is myristoylated. Additional ABL2 isoforms have been reported but little is known regarding their function (22).

The activity of the ABL kinases is tightly regulated by intra-molecular and inter-molecular interactions that result in alternative conformations of the kinase domain corresponding to auto-inhibited and active states (23, 24). These include the binding of
the SH3 domain to the polyproline-containing sequence connecting the SH2 and SH1 domains as well as interactions of the SH2 domain with the SH1 domain, leading to the formation of a SH3-SH2-SH1 clamp structure (25). The auto-inhibited conformation is further stabilized by an N-terminal cap and binding of the N-terminal myristoylated residue to a hydrophobic pocket within kinase domain (Fig. 3). Interestingly, the *M. brevicollis* ABL2 lacks the N-terminal myristoylation and cap sequences and was reported to be constitutively active when expressed in mammalian cells (26).

**Figure 2**: ABL kinases structures and mechanisms for activation of ABL kinases in Leukemia and solid tumors. Schematic representation of ABL1, ABL2, and the various ABL1 and ABL2 fusion proteins that arise as a consequence of chromosome
translocations in leukemias. In solid tumors, ABL kinases are up-regulated through various mechanisms including amplification, increased mRNA expression, enhanced protein expression, and/or hyper-activation of catalytic activity. In leukemias, ABL kinases are activated mainly through chromosomal translocation events. Various N-terminal fusion partners generate chimeric proteins that retain both the SH3 and SH2 domains, or just the SH2 domain of ABL1 and ABL2 as indicated. The distinct partner sequences fused to the N terminus of the ABL kinases promote enhanced kinase and transforming activities by disrupting inhibitory intramolecular interactions, providing sequences that facilitate oligomerization, enhancing tyrosine phosphorylation and or by recruiting the chimeric kinases to distinct subcellular sites and protein complexes.

The ATP-competitive inhibitors of the ABL kinases can be sub-classified into type 1 inhibitors targeting the active conformation of the kinase domain (dasatinib,
bosutinib) and type 2 inhibitors targeting the inactive conformation of the kinase domain (imatinib, nilotinib, ponatinib). A third class includes allosteric inhibitors that do not target the ATP-binding pocket, but instead bind to regulatory domains to inhibit kinase activity. Among the allosteric inhibitors are GNF2 and GNF5, which bind to the myristoyl-binding pocket in the C-lobe of the ABL kinase domain (17). In contrast to ATP-competitive inhibitors that target multiple kinases, the allosteric inhibitors are highly selective for the ABL kinases (Table 2). Crystal structures of ABL kinases in complexes with several inhibitors have permitted visualization of their inactive and active conformations. The crystal structure of the ABL1 SH2-SH1 domains bound to dasatinib revealed an extended conformation that included a contact interface between the SH2 domain and the N-lobe of the kinase domain (27). Interestingly, a crystal structure of the isolated ABL2 kinase domain with imatinib revealed the presence of two imatinib molecules with one bound to the ATP-binding site and the second one to the myristate binding site (28). These and other studies have shown that binding of inhibitors to the SH1 domain can capture multiple conformational states of the ABL kinases.

The enzymatic activity of ABL kinases can also be regulated by inter-molecular interactions with distinct binding partners that negatively or positively regulate their activity, and by autophosphorylation or phosphorylation by other kinases, such as Src and receptor tyrosine kinases (RTKs) (18). Phosphorylation of ABL1 Y412 (corresponds
to ABL2 Y439) in the activation loop of the kinase domain enhances catalytic activity while phosphorylation of Y245 in the SH2-kinase domain linker (corresponds to ABL2 Y272) further enhances catalytic activity by disrupting inhibitory intra-molecular interactions. Upon activation, ABL kinases phosphorylate and bind to multiple targets to regulate diverse processes.

Table 2: Selective and non-selective ABL kinases inhibitors.

<table>
<thead>
<tr>
<th>Name</th>
<th>Targets</th>
<th>Inhibitor type</th>
<th>Regulatory status</th>
<th>Year</th>
<th>Company</th>
</tr>
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<tr>
<td>Imatinib</td>
<td>ABL1, ABL2, BCR-ABL1, CSF1R, DDR1, DDR2, KIT, NQO2, PDGFR1</td>
<td>ATP-competitive</td>
<td>FDA approved for CML, Ph+ ALL, MDS/MPD, ASM, HES/CEL, DFSP, GIST</td>
<td>2001</td>
<td>Novartis</td>
</tr>
<tr>
<td>Dasatinib</td>
<td>ABL1, ABL2, BCR-ABL1, BLK, BTK, CSK, CSR1R, DDR1, DDR2, EGFR, ERBB2, FGR, FRK, FYN, GAK, GCK, HCK, ILK, KIT, LCK, LIMK1, LIMK2, LYN, MAP2K, MAP3K, MAP4K, PDGFR, RIPK2, SLK, SRC, SYK, TEC, TYK2, YES1,</td>
<td>ATP-competitive</td>
<td>FDA approved for CML, Ph+ ALL</td>
<td>2006</td>
<td>Bristol-Myers Squibb</td>
</tr>
<tr>
<td>Nilotinib</td>
<td>ABL1, ABL2, BCR-ABL1, CSF1R, DDR1, DDR2, KIT, NQO2, PDGFR</td>
<td>ATP-competitive</td>
<td>FDA approved for CML</td>
<td>2007</td>
<td>Novartis</td>
</tr>
<tr>
<td>Bosutinib</td>
<td>ABL1, ABL2, BCR-ABL1, CAMK2G, CDK2, HCK, LYN, MAPKK1, MAPKK2,</td>
<td>ATP-competitive</td>
<td>FDA approved for CML</td>
<td>2012</td>
<td>Pfizer Inc.</td>
</tr>
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### Table

<table>
<thead>
<tr>
<th>Compound</th>
<th>Targets</th>
<th>Mechanism</th>
<th>Approval Status</th>
<th>Company</th>
</tr>
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<tbody>
<tr>
<td>Ponatinib</td>
<td>ABL1, ABL2, BCR-ABL1, BLK, CSFR1, DDR1, DDR2, EPHRs, FGFR1, FGFR2, FGR, FLT3, FRK, FYN, HCK, LCK, LYN, RET, SRC, TEK, TIE2, TRKA, TRKB, TRKC, PDGFR, VEGFR1, VEGFR2, VEGFR3, YES1</td>
<td>ATP-competitive</td>
<td>FDA approved for CML, Ph+ ALL</td>
<td>Ariad Pharmaceuticals Inc.</td>
</tr>
<tr>
<td>Axitinib</td>
<td>BCR-ABL1 (T315I), KIT, PDGFR, VEGFR1, VEGFR2, VEGFR3</td>
<td>ATP-competitive</td>
<td>FDA approved for Renal Cell Carcinoma</td>
<td>Pfizer Inc.</td>
</tr>
<tr>
<td>Vandetanib</td>
<td>ABL1, EGFR, RET, VEGFR</td>
<td>ATP-competitive</td>
<td>Thyroid Cancer</td>
<td>AstraZeneca</td>
</tr>
<tr>
<td>GNF2, GNF5</td>
<td>ABL1, ABL2, BCR-ABL1</td>
<td>Allosteric</td>
<td>Not FDA approved</td>
<td>Novartis</td>
</tr>
<tr>
<td>ABL001</td>
<td>ABL1, ABL2, BCR-ABL1</td>
<td>Allosteric</td>
<td>Phase I Trial for CML and Ph+ ALL</td>
<td>Novartis</td>
</tr>
</tbody>
</table>

1.2.1.2 ABL kinases regulate cytoskeletal dynamics required for cellular protrusions, cell adhesion, polarity, and migration

The most evolutionarily conserved and well characterized function of the ABL kinases is their ability to regulate cytoskeletal dynamics (14, 19). ABL kinases are transiently activated by growth factors that stimulate RTKs and lead to actin cytoskeleton reorganization that is required for the formation of lamellipodial...
protrusions, membrane dorsal ruffles, cell migration, invasion, cell scattering, and/or tubulogenesis (Fig. 3) (29-32). ABL-dependent regulation of cytoskeletal dynamics is mediated by the phosphorylation of target proteins such as cortactin, which is required for platelet-derived growth factor (PDGF)-induced dorsal membrane ruffles (32), as well as actin nucleating-promoting factors, such as N-WASP and WAVE proteins required for membrane protrusions (21, 33-35). ABL kinases also transduce signals from cell surface receptors, such as integrins, cadherins, chemokine receptors, and the T cell receptor (TCR), to reorganize the cytoskeleton. Integrin engagement induced by plating fibroblasts on fibronectin promotes activation of the ABL kinases required for the formation of membrane protrusions and remodeling of focal adhesions and F-actin stress fibers (19). ABL2 is activated by direct binding to the beta1-integrin cytoplasmic tail, and the phosphorylated ABL2 in turn phosphorylates the beta1 tail (36). Integrin-dependent spindle orientation was shown to require ABL1-mediated phosphorylation of the NuMA microtubule-binding protein, a modification that is required for NuMA localization to the cortex and correct alignment of the spindles (37). ABL1 knockout mice exhibit spindle misorientation in basal skin cells, a phenotype that has been previously linked to beta1-integrin-mediated cell substratum adhesion (38).

Remodeling of the cytoskeleton by ABL kinases downstream of diverse stimuli is mediated in part by the small GTPases, Rac1, RhoA, and Rap1. ABL kinases activate Rac1 downstream of growth factor-stimulated RTKs leading to formation of membrane
ruffles (39). ABL kinases also activate Rac1 in T cells in response to chemokine stimulation which is required for T cell polarization and F-actin polymerization (40). ABL kinases activated downstream of chemokine receptors promote Rap1 activation leading to T cell migration and polarization (40). In contrast, activation of ABL2 in epithelial cells grown on collagen inhibits beta1-integrin signaling by suppressing Rap1 activity, while

Figure 4: ABL kinases regulate cytoskeleton dynamics.
also inhibiting Rac1 and disrupting the Par3/Par6 polarity complex (41). These examples illustrate that regulation of Rac1 and Rap1 by ABL kinases is cell context- and stimuli-dependent. In response to integrin engagement, ABL2 inhibits RhoA in fibroblasts and dendritic spines by phosphorylating p190RhoGAP which in turn inhibits RhoA (19, 42). However, ABL kinase activation downstream of the ligand-stimulated MET RTK kinase promotes RhoA activation (31). These findings indicate that regulation of RhoA activity downstream of ABL kinases is also cell context-dependent.

ABL kinases are also activated in response to E-cadherin and N-cadherin (43, 44). Active ABL kinases are required for both the maintenance and remodeling of cadherin-dependent adherens junctions. Simultaneous inactivation of both ABL1 and ABL2 disrupts adherens junctions in fibroblasts and epithelial cells (41, 43). Recently, ABL kinases were shown to phosphorylate the actin-binding protein vinculin at epithelial cell-cell junctions and this modification was required to promote E-cadherin-mediated adhesion and force transmission (44). Growth factor-induced turnover of cadherin-based epithelial and endothelial cell-cell junctions also requires ABL kinases. Inactivation of both ABL kinases impairs HGF-mediated dissolution of E-cadherin-mediated cell-cell junctions (31). Similarly, inhibition of ABL kinases prevents dissolution of VE-cadherin-dependent intercellular junctions in endothelial cells that have been treated with VEGF, thrombin, or histamine (45). ABL kinases link signaling from the HGF receptor, MET, to RhoA activation, which leads to increased phosphorylation of myosin light chain (p-
MLC2) and actomyosin contractility, thereby disrupting epithelial cell-cell junctions (31). However, inhibition of the ABL kinases in endothelial cells treated with VEGF or thrombin suppresses p-MLC2 levels through a Rho-independent pathway (46).

Importantly, treatment of wild type mice with ABL-specific allosteric inhibitors or genetic inactivation of ABL1 in ABL2 heterozygous mice blocks VEGF-induced vascular permeability (46). The phenotypes of mice lacking global or tissue-specific knockouts of the ABL kinases support a role for these kinases in the regulation of cytoskeletal dynamics required for cell-cell adhesion and other cellular processes.

### 1.2.1.3 ABL-dependent regulation of membrane and organelle trafficking

Plasma membrane plasticity is regulated by forces that are generated by the actin and microtubule cytoskeletons. ABL kinases contain both actin- and microtubule-binding domains, and phosphorylate several proteins that regulate the actin and microtubule cytoskeletons (reviewed in (18)). Thus, ABL kinases have the potential to directly and/or indirectly regulate the actin and microtubule cytoskeletons, thereby promoting plasma membrane remodeling and receptor endocytosis (Fig. 5). Activated ABL1 was shown to inhibit ligand-induced EGFR endocytosis in part through phosphorylation of the EGFR, which results in decreased binding to the Cbl ubiquitin ligase, a protein that promotes ubiquitination and lysosomal degradation of the receptor (47). Additionally, active ABL1 disrupts the interaction of Cbl with Abi1, a binding partner and substrate of the ABL kinases, which is also a component of the WAVE
protein complex that promotes Arp2/3-dependent actin polymerization (48). EGFR endocytosis is also regulated by RAB5, a GTPase that promotes early endosome fusion and subsequent degradation of EGFR in the lysosome (49). The activity of RAB5 is regulated by RIN1, a RAS effector that binds and activates the ABL kinases and is also a guanine nucleotide exchange factor (GEF) for RAB5 (50, 51). Recent data suggest that binding of the RIN1 SH2 domain to tyrosine phosphorylated EGFR balances RIN1-RAB5-mediated EGFR endocytosis and lysosomal degradation with RIN1-ABL-dependent EGFR stabilization by blocking macropinocytosis of the receptor (51). While overexpression of active ABL1 inhibits EGFR internalization, endogenous ABL1 activity was reported to be required for antigen-induced endocytosis of the B cell receptor (52). ABL1 inactivation impaired capping of B cell receptors and delayed their internalization which correlated with decreased Rac activation (52). A role for ABL kinases in clustering of receptors at the plasma membrane was also shown at the postsynaptic membrane of the neuromuscular junction (NMJ) (53). Here, ABL kinase activity is required for agrin-induced clustering of the acetylcholine receptors (AChRs) on the postsynaptic membrane of the NMJ, which may be mediated through ABL-dependent regulation of cytoskeletal dynamics.
1.2.1.4 ABL-dependent regulation of cell proliferation and survival pathways in normal cells

ABL kinase-mediated signal transduction from diverse cell surface receptors also regulates cell proliferation and survival pathways during development and cellular homeostasis (Fig. 6) (54). Cytoplasmic ABL kinases promote mitogenic signaling downstream of the PDGFR. Mouse embryo fibroblasts deficient for ABL1 exhibit a delay in DNA synthesis following PDGF stimulation (29, 55). Activation of ABL kinases downstream of the PDGFR is mediated in part by Src kinases, leading to induction of Myc and increased DNA synthesis (55). PDGFR-Src-ABL signaling also increases mitogenesis through Rac activation, which in turn activates the Jun N-terminal kinase (JNK) and NADPH oxidase (Nox) pathways in fibroblasts (56). Analysis of osteoblasts
derived from ABL1-null mice showed that ABL1 is required for osteoblast proliferation, and its loss results in premature senescence (57). ABL1 also mediates cell proliferation downstream of the EphB2 receptor in the intestinal epithelium as shown by decreased proliferation in the small intestine and colon of ABL1 mutant mice (58). ABL1 is required for EphB signaling to cyclin D1, which promotes cell proliferation in the intestinal epithelium.

ABL kinases regulate cell proliferation downstream of the antigen receptors in T and B cells. ABL1 deficient mice have decreased pro-B, pre-B and peritoneal B-1 cells (59-61). ABL1-deficient B cells exhibit decreased proliferation in response to anti-IgM and have defective B cell receptor-induced activation and signaling (61, 62). ABL kinases are activated downstream of the T cell receptor (TCR) and are required for tyrosine phosphorylation of downstream kinases (ZAP70) and adaptor proteins (LAT and Shc) required for maximal T cell proliferation and IL-2 production (63, 64). Interestingly, murine ABL1 was recently shown to function downstream of ShcA in immature thymocytes to regulate proliferation, differentiation, and migration (65).

Recently, ABL kinases were shown to regulate Angiopoietin (Angpt1)-mediated endothelial cell survival by activation of the Tie2 receptor that is activated by binding to angiopoietin-1 (Angpt1) (46). Inactivation of both ABL1 and ABL2 in endothelial cells markedly decreased Angpt1 signaling mediated in part by inhibition of the pro-survival Akt pathway. Inactivation of the ABL kinases also decreased expression of Tie2 (46).
Interestingly, expression of exogenous Tie2 only partially rescued Angpt1-mediated survival, suggesting that the ABL kinases modulate Angpt1/Tie2 signaling through additional mechanisms, beyond regulating Tie2 receptor levels. ABL1 may also promote vascular development through neuropilin-1, a receptor for vascular endothelial growth factor A (VEGF-A) (66, 67). Endothelial deletion of ABL1 in ABL2-null mice resulted in focal loss of vasculature due to apoptosis, leading to localized tissue necrosis, with late-stage embryonic and perinatal lethality (46). The phenotypes of endothelial ABL knockout mice are consistent with a role for these kinases in the regulation of cell survival pathways.

Figure 6: ABL-dependent regulation of cell proliferation and survival pathways in normal cells.
1.2.1.5 Physiological roles of mammalian ABL kinases: insights from knockdown mice

Analysis of mice lacking one or both of the murine ABL kinases has shown that ABL1 and ABL2 exhibit overlapping as well as unique functions. ABL1 knockout mice are either viable or exhibit perinatal lethality depending on the strain and display phenotypes distinct from those detected in the viable ABL2 (Arg) global knockout mice (59, 60, 68-70). ABL1 knockout mice exhibit splenic and thymic atrophy, reduced numbers of B and T cells, cardiac abnormalities, and osteoporosis that has been linked to defective osteoblast proliferation and premature senescence (57, 59, 60, 68, 69). In contrast, ABL2 knockout mice are viable and exhibit neuronal defects that include age-related dendrite destabilization and regression (68, 70, 71). Functional overlap between ABL1 and ABL2 is supported by the embryonic lethality of ABL1+/ ABL2−/− mice by embryonic day 11 (71). Analysis of conditional knockout mice with tissue-specific deletion of the ABL kinases has shown unique and overlapping roles for these kinases in immune and endothelial cells. For example, ABL kinases have redundant roles in mature T cells, as deletion of both ABL1 and ABL2 was required to inhibit TCR-induced proliferation and cytokine production, as well as chemokine-induced T cell migration (64). In contrast, only ABL1 is required for thymocyte differentiation (65). Mice with endothelial deletion of ABL1 in ABL2-null mice exhibit late-stage embryonic and perinatal lethality due in part to increased apoptosis (46). The unique phenotypes that are induced by loss of ABL1 and ABL2 might be due to their differential expression in
various tissues and/or might reflect distinct cellular functions of ABL1 and ABL2 mediated by their unique domains, differential subcellular localization, and/or association with distinct protein complexes.

1.2.2 ABL kinases in cancer biology

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1.2.2.1 ABL activation in leukemias and development of targeted therapies

Chromosomal translocations are the hallmark of oncogenic activation of the ABL kinases in human leukemias (72). Disruption of inhibitory ABL1 intra-molecular interactions in Philadelphia-positive (Ph+) human leukemias occurs as a consequence of the t(9;22)(q34;q11) chromosome translocation that generates BCR-ABL1 fusion proteins with constitutive tyrosine kinase activity (Fig. 2). CML begins with a chronic phase (CP-CML) that is characterized by expansion of the myeloid lineage and retention of hematopoietic differentiation (II). This early phase can progress to a blastic phase (BP-CML) characterized by reduced cellular differentiation and displacement of mature cells with immature blasts. The majority of BP-CML patients harbor several genetic alterations in addition to BCR-ABL1. Three different BCR-ABL1 proteins have been identified that differ in the amount of BCR sequences retained in the fusion protein leading to distinct types of leukemia: P210 BCR-ABL1 is causal in chronic myelogenous leukemia (CML); P185 BCR-ABL1 is found in 20-30% of adult and 3-5% of childhood B-
cell acute lymphocytic leukemia (B-ALL); and P230 BCR-ABL1 is associated with neutrophilic-CML and rare cases of CML (73). Oncogenic activation of ABL1 in the BCR-ABL1 fusion protein is dependent on the presence of the BCR N-terminal coiled-coil (CC) oligomerization domain. Multiple signaling pathways have been identified that function to mediate the oncogenic activity of BCR-ABL1, and include the RAS, NF-kB, PI3K-AKT, JUN, β-catenin, and STAT signaling pathways (72).

The most successful example of molecular targeted therapy to date has been the development of tyrosine kinase inhibitors (TKIs) against BCR-ABL1 for the treatment of CML in chronic phase. The majority of CP-CML patients treated with the BCR-ABL1 inhibitor imatinib (Gleevec; STI571) as first-line therapy have durable remissions with five-year overall and progression-free survival rates approaching 90% (74). However, imatinib is less effective for the treatment of blast crisis CML and Ph+ B cell-ALL patients. Several second- and third-generation TKIs targeting BCR-ABL1 have been approved or are under development for CML patients who are resistant or intolerant to imatinib. Among these are dasatinib and nilotinib, which have been FDA-approved as both frontline and second-line therapies, and bosutinib and ponatinib FDA-approved for second-line therapy to treat Ph+ leukemia patients with BCR-ABL1 kinase domain mutations (16). Recently, axitinib, a VEGFR kinase inhibitor approved for second-line therapy of refractory renal cell carcinoma, was reported to potently inhibit the BCR-ABL1 (T315I) gate-keeper mutation, which confers resistance to imatinib,
dasatinib and nilotinib (75). Interestingly, axitinib preferentially inhibits the BCR-ABL1 (T315I) mutant over wild-type BCR-ABL1 (75). Thus, axitinib might be useful for the treatment of BCR-ABL1 (T315I)-driven CML and Ph+ C-cell ALL. The ATP-competitive kinase inhibitors imatinib, dasatinib, nilotinib, bosutinib and ponatinib have broad target specificity and inhibit multiple tyrosine kinases in addition to ABL kinases (Table 2). Axitinib has a more restricted target specificity compared to other FDA-approved ATP-competitive inhibitors, as it only targets KIT, PDGFR, and VEGFRs in addition to the BCR-ABL1 (T315I) mutant kinase.

ABL TKIs can be classified into three main classes based on their mechanism of action. The ATP-competitive inhibitors can be sub-classified into type 1 inhibitors targeting the active conformation of the kinase domain (dasatinib, bosutinib), and type 2 inhibitors targeting the inactive conformation of the kinase domain (imatinib, nilotinib, ponatinib). The third class includes the allosteric inhibitors, which do not target the ATP-binding pocket, but instead bind to regulatory domains to inhibit kinase activity. Among allosteric TKIs targeting ABL are GNF-2 and GNF-5, which bind to the myristoyl-binding pocket in the C-lobe of the ABL kinase domain (17). In contrast to ATP-competitive inhibitors that target multiple kinases, the allosteric inhibitors are highly selective for the ABL kinases (Table 2). These allosteric inhibitors were shown to inhibit BCR-ABL1-driven leukemogenesis in mice and sensitize mutant BCR-ABL1 to inhibition by ATP-competitive TKIs (17). A phase I, multi-center clinical trial with a
novel allosteric inhibitor of BCR-ABL1 (ABL001) that targets the myristoyl-binding pocket is currently ongoing for patients with refractory CML or Ph+ ALL (http://clinicaltrials.gov/show/NCT02081378).

Oncogenic activation of the ABL kinases via chromosomal translocations has also been shown to occur in Ph-negative human leukemias (76-78). ABL1 has been identified as a fusion partner with a number of genes in T cell acute lymphoblastic leukemia (T-ALL), B-ALL, AML and other leukemias. The ABL kinase fusions identified in a precursor B-ALL subtype lacking the BCR-ABL1 fusion (designated Ph-like ALL) are associated with poor outcome among children and adolescents (76). Similar to BCR-ABL1, several translocations retain the ABL1 SH3 and SH2 domains. Among these are the N-terminal fusion partners: ETV6 (TEL), EML1, NUP214, ZMIZ1, and SEPT9 (76-78). Other translocations fuse N-terminal sequences present in RCSD1, SFPQ, FOXP1 and SNX2 to the ABL1 SH2 domain and lack the SH3 domain. Chimeric fusions involving the ABL2 gene have also been identified in rare leukemias. ETV6 and ZC3HAV1 are fused to ABL2 sequences upstream of the SH3 and SH2 domains, while RCSD1 and PAG1 are fused to the ABL2 SH2 domain. Some fusion partners encode proteins that contain coiled-coil or helix-loop-helix motifs that promote oligomerization of the resulting chimeric proteins leading to enhanced ABL kinase activity. However, the NUP-ABL1 fusion requires localization to the nuclear pore complex rather than oligomerization for enhanced transforming activity (79). The effectiveness of the ABL
TKIs for the treatment of Ph-negative leukemias associated with multiple ABL fusion partners remains to be established.

1.2.2.2 ABL kinases activation in solid tumors

Activation of ABL kinases in solid tumors is not linked to chromosome translocation events as found in human leukemias, but rather is driven by enhanced ABL1 or ABL2 expression and/or activation due to amplification, increased gene expression, enhanced protein expression, and/or increased enzymatic activity in response to stimulation by oncogenic tyrosine kinases, chemokine receptors, oxidative stress, metabolic stress, and/or inactivation of negative regulatory proteins (80-86).

The Cancer Genome Atlas (TCGA) and other large-scale sequencing projects report ABL amplification, somatic mutations and/or increased mRNA expression in multiple solid tumors (www.cbioportal.org). These genomic alterations are more common in ABL2 than ABL1, with ABL2 alterations observed in 24% of liver hepatocellular carcinomas, and to a lesser extent in uterine endometrioid carcinoma (20%), breast invasive carcinoma (19%), lung adenocarcinoma (15%), lung squamous cell carcinoma (12%), and kidney renal clear cell carcinoma (6%) (www.cbioportal.org).

These findings are consistent with reports of elevated ABL2 expression in advanced high-grade breast, colorectal, pancreatic, renal and gastric tumors (82, 85-88). While ABL2 amplification and increased mRNA levels are genomic alterations found in a subset of human cancers, somatic mutations of ABL1 and ABL2 in solid tumors are rare,
but have been reported in lung cancer and uterine corpus endometrioid carcinoma among other cancers (www.cbioportal.org). The role of these mutations in regulating ABL oncogenic activity remains to be determined.

Oncogenic activation of ABL proteins in tumors is due to increased ABL tyrosine kinase activity. Enhanced activation of the ABL kinases downstream of multiple receptor tyrosine kinases (RTKs) including the platelet-derived growth factor receptor (PDGFR), the EGF receptor (EGFR), and the hepatocyte growth factor receptor (MET) has been reported by multiple groups (14, 31, 89). Cancer cells expressing activated ERBB receptors exhibited rapid EGF-induced ABL kinase stimulation (90). Subsequent studies demonstrated that ABL kinases are tyrosine phosphorylated and activated in breast, lung, colorectal, gastric, renal, and prostate cancer cells as well as in melanoma (14, 81). The catalytic activity of the ABL kinases can be up-regulated by ligand-dependent and ligand-independent activation of RTKs in cancer cells. Activation of ABL kinases in breast cancer cells has been reported to occur downstream of the EGFR, Her2 (ERBB2), insulin-like growth factor receptor (IGFR), and the CXCR4 chemokine receptor (14, 81). ABL1 activation downstream of ligand-activated MET was shown in gastric carcinoma and hepatocellular carcinoma cells (91), and ABL1 activation in human anaplastic thyroid carcinoma cells was induced by a constitutively active form of RET (92).
The cellular consequences of ABL family kinase activation and signaling are diverse and include changes in cell proliferation, survival, migration, and invasion. Below we will provide examples of ABL-dependent regulation of these processes in solid tumors, focusing primarily on studies that use knockout/knockdown strategies or specific allosteric inhibitors targeting the ABL kinases, rather than relying on the use of ATP-competitive TKIs (imatinib, dasatinib, nilotinib, bosutinib, and ponatinib) which are known to inhibit multiple tyrosine kinases in addition to ABL1 and ABL2 (Table 2) (14). Several studies have reported inhibitory and, in some cases, stimulatory effects of imatinib, nilotinib, dasatinib and other TKIs, on cancer cell proliferation, survival and motility. However, the cellular responses to these compounds cannot be solely attributed to inhibition of the ABL kinases as these compounds target numerous kinases and some non-kinase enzymes. Therefore, ATP-competitive inhibitors cannot be used to evaluate whether ABL kinases have a role in the progression of solid tumors and regulation of cellular processes in these tumors.

1.2.2.3 ABL-dependent regulation of cancer cell proliferation

The EphB2 receptor tyrosine kinase can function as tumor promoter during adenoma development, and as tumor suppressor in the progression of invasive colorectal cancer (58, 93). Genetic studies with ABL1-null mice showed that ABL1 is required for EphB2-mediated proliferation in the small intestine and epithelium, as deletion of ABL1 reduced the number of proliferating cells in these tissues (58).
Inactivation of ABL1 in the Apc\textsuperscript{min/+} mouse model of intestinal adenoma impaired EphB2-mediated tumor promotion without affecting its tumor suppressor function (58, 94). Further, ABL1 inactivation inhibited tumor initiation by intestinal stem cells, decreased tumor load, and extended the life span of Apc\textsuperscript{min/+} mice (94). Interestingly, ABL1 knockdown or pharmacological inhibition in some human colon carcinoma cell lines expressing low levels of EphB2 resulted in decreased levels of cyclin D1 and impaired cell proliferation (Fig. 7). Thus, ABL activity and function may become dissociated from EphB2 signaling at later stages of adenocarcinoma progression.

### 1.2.2.4 ABL-mediated metabolism and oxidative stress in cancer

A recent breakthrough study revealed a critical role for ABL1 in an aggressive form of hereditary kidney cancer (95). Patients with germline mutation in the enzyme fumarate hydratase (FH) are susceptible to the development of hereditary leiomyomatosis and renal cell carcinoma (HLRCC). FH-deficient renal tumors are highly glycolytic, accumulate high levels of fumarate, lactate, and hypoxia-stimulated transcription factor (HIF1a), and have decreased activity of AMP-activated kinase (AMPK) (96). The ABL1 kinase was found to be hyperactive in FH-deficient renal cancer cells in response to high fumarate levels (Fig. 7). Mechanistically, activation of ABL1 in HLRCC functions to promote aerobic glycolysis through activation of the mTOR-HIF1a pathway and also induces nuclear localization of the antioxidant response transcription factor NRF2. Thus, high ABL1 activity enables these tumors to simultaneously meet their high energetic
needs and to neutralize the elevated levels of oxidative stress generated by excess
fumarate accumulation in HLRCC. Importantly, ABL1 knockdown or inhibition with
either imatinib or vandetanib (an inhibitor that also targets EGFR, RET and VEGFR) was
cytotoxic to FH-deficient HLRCC (95). The anti-tumor activity of vandetanib in these
cells was shown to be ABL1-dependent. Moreover, vandetanib was shown to potently
inhibit the ABL1 kinase (IC\textsubscript{50} = 15 nM) \textit{in vitro} and in cells. Vandetanib alone markedly
inhibited the growth of HLRCC xenografts, and combination of low-dose vandetanib
with the AMPK activator metformin induced complete regression of the HLRCC tumors
in 100% of the treated mice (95). ABL kinases have been shown to be activated in
response to oxidative stress and reactive oxygen species (ROS) (97). Elevated levels of
ROS are a feature characteristic of many solid tumors and are also an inevitable by-
product of cellular metabolism. Thus, the data on the role for ABL1 in HLRCC suggest
that ABL1 kinase inhibitors could be developed for the treatment of FH-deficient tumors
and other cancers with high levels of oxidative and metabolic stress.

\subsection{1.2.2.5 Role of ABL kinases in cancer cell invasion and metastasis}

The progression of solid tumors require invasion of primary tumor cells into the
surrounding tissue, followed by intravasation, migration, extravasation, and formation
of metastases at distant sites (98). The various steps in the metastatic cascade require
dynamic remodeling of the actin cytoskeleton. ABL kinases have been shown to engage
the actin polymerization machinery to promote formation of membrane protrusions,
morphological changes, altered cell adhesion, migration and invasion of diverse cell types (19). Among the various functions of the ABL kinases, regulation of cell motility has been shown to be a predominant and evolutionarily conserved role for these kinases. A requirement for ABL kinases in cancer cell motility and invasion was shown downstream of IGF-1, EGF, serum and chemokines (14). This requirement is consistent with the localization of ABL2 to invadopodia, which are actin-rich, protrusive membrane structures that promote remodeling of the extracellular matrix during tumor invasion (99, 100). ABL kinases promote maturation of invadopodia and are required for matrix degradation and invasion in some, but not all, breast cancer types (99-101).

Among the actin cytoskeleton-regulatory proteins targeted by ABL kinases at invadopodia are cortactin, N-WASP, WAVE, and the ABL interactor 1 (Abi1) adaptor protein (Fig. 7). Importantly, ABL kinases regulate the expression, localization and activity of MMPs during invadopodia maturation. Active ABL2 interacts with and promotes phosphorylation of the membrane type 1-matrix metalloproteinase (MT1-MMP, MMP14) and is required for its localization and function at invadopodia (99). Both ABL1 and ABL2 kinases were shown to regulate matrix metalloproteinase (MMP) expression through STAT3-dependent and independent pathways in melanoma cells (81). Knockdown of ABL2 alone decreased cancer cell invasion and intravasation following implantation of MDA-MB-231 cells in the mammary fat pad (102). A requirement for ABL kinases for invasion and metastasis of melanoma cells was also
shown, which may be mediated in part by the NM23-H1 metastasis suppressor (89). Active ABL kinases induced cathepsin-dependent lysosomal degradation of NM23-H1 in melanoma and breast cancer cells.

A recent report demonstrated a novel role for ABL kinases in promoting colorectal cancer cell invasion and metastasis by linking the activation of the NOTCH receptor to the phosphorylation of TRIO (pY2681) leading to enhanced TRIO Rho-GEF activity and corresponding increase of Rho-GTP levels (103). Activation of NOTCH by homozygous deletion of Aes (Amino-terminal enhancer of split) in the intestinal epithelium of Apc+/D716 polyposis mice resulted in enhanced RBPJ-mediated transcription leading to increased levels of DAB1, a substrate and activator of the ABL kinases. Activated ABL in colorectal cancer cells induced tyrosine phosphorylation of TRIO on Y2681, leading to enhanced TRIO Rho-GEF activity (Fig. 7). Rho activation in colorectal cancer cells promoted invasion, extravasation and metastasis. Importantly, inhibition of ABL kinases in Apc/Aes compound knockout mice dramatically suppressed both invasion and intravasation incidence without affecting tumor size. These findings suggest that ABL kinases may function to link activation of other cell surface receptors to Rho signaling in different tumors. In this regard, it has also recently been shown that ABL kinases link the ligand-activated MET receptor tyrosine kinase to Rho activation required for cell scattering, tubulogenesis, migration and invasion (31).
Figure 7: Kinase activation and signaling of ABL kinases in solid tumors.

1.2.2.6 Role for ABL kinases in cancer drug resistance

Enhanced activation of the ABL kinases has been reported in some cancers that have intrinsic or acquired resistance to chemotherapy. Hyper-activation of both ABL1 and the PDGFR was detected in aromatase inhibitor (AI)-resistant breast cancer patient...
specimens (104). ABL1 expression increased at the point of relapse in AI treated patients and correlated with increased expression of the Ki67 proliferation marker. In vitro studies showed that estrogen deprivation of MCF7 breast cancer cells, which became AI resistant, was accompanied by up-regulation of PDGFR/ABL1 signaling (104). Treatment of these cells with nilotinib, a PDGFR and ABL inhibitor, suppressed proliferation and estrogen receptor (ER)-mediated transcription, in part by destabilizing the ER protein. Down-regulation of ABL1 in some human breast cancer cell lines by RNA interference or imatinib treatment was reported to overcome resistance to fulvestrant, a compound that down-regulates ERα levels and activity (105). Furthermore, in vitro studies using breast cancer cells resistant to lapatinib, an EGFR and ErbB2 inhibitor, showed that imatinib treatment or ABL1 depletion restored lapatinib sensitivity to these breast cancer cells (106). While the mechanisms by which decreased ABL1 signaling sensitizes breast cancer cells to various compounds was not reported, these studies suggest that inhibition of the ABL kinases may be effective for overcoming cancer cell resistance to diverse therapeutic agents.

A role for ABL kinases in reversing resistance to doxorubicin in breast cancer (BT-549 and MDA-MB-468) and melanoma (WM3248) cell lines has been linked to at least two pathways (107). Imatinib blocked intrinsic resistance to doxorubicin by inhibiting STAT3-mediated cell survival and repressing NF-kB target gene expression. Additionally, it was reported that imatinib prevented acquired resistance by inhibiting
the increased expression of the ABCB1 drug transporter, which mediates efflux of chemotherapeutic compounds such as doxorubicin. Similar to imatinib, other ATP-competitive inhibitors (nilotinib and dasatinib) have been reported to sensitize cancer cells to cytotoxic chemotherapies and targeted TKI therapies. However, the majority of these studies did not evaluate whether these effects were mediated specifically by inactivation of the ABL1 and/or ABL2 kinases in the cancer cells or associated cells in the tumor microenvironment.

While the ATP-competitive inhibitors imatinib, nilotinib and dasatinib have shown success for the treatment of BCR-ABL1-induced leukemias, treatment of diverse solid tumors with these compounds has not achieved similar success (108, 109). The variable clinical responses to these TKIs may be due to the lack of the relevant oncogenic target, the presence of additional mutations driving the tumor, tumor heterogeneity, and/or dynamic reprogramming of signaling networks in response to TKI treatment (110, 111). An alternative mechanism that underlies the poor response to TKI therapy is the paradoxical activation of proliferative pathways as a result of unintended inhibition of other kinases. This was demonstrated by the activation of BRAF/ CRAF complexes leading to enhanced activation of the MEK-ERK pathway by nilotinib, imatinib and dasatinib in melanoma, lung, colorectal, pancreatic carcinoma cells and BCR-ABL1 TKI-resistant leukemic cells expressing activated RAS (112). In contrast to the ABL-targeted ATP-competitive TKIs, the ABL allostERIC inhibitors do not induce formation of
BRAF/CRAF dimers, and fail to elicit paradoxical activation of the MEK-ERK pathway. Thus, it is critical to identify those tumors that may benefit from therapies with selective ABL TKIs in combinations to prevent the emergence of therapy resistance.
2. Materials & Methods

2.1 Cell Culture

The human breast carcinoma cell line, MDA-MB-231 was purchased from ATCC. The 1833 (bone metastasis), 4175 (lung metastasis), and BrM2a (brain metastasis) sub-lines were derived from the parental cell line MDA-MB-231 (113) and were generous gifts from Dr. Joan Massague (Memorial Sloan-Kettering Cancer Center). The SCP28 (bone metastasis) sub-line was generously provided by Dr. Yibin Kang (Princeton University). MDA-MB-231, their derivative sub-lines and genetically modified versions were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies) supplemented with 10% fetal bovine serum (FBS, Life Technologies), antibiotics, and appropriate selection drugs for transfected plasmids. HEK293T cells, a packaging cell line for lentivirus production, and the pre-osteoclast cell line RAW264.7 (ATCC) were maintained in DMEM supplemented with 10% FBS, and antibiotics. The murine osteoblast cell line 7F2 (ATCC) was cultured in alpha-MEM with 10% FBS. The human mammary epithelial cell line (HuMEC) was maintained in Human Basal Serum Free Medium (Life Technologies) with HUMECE Kit (Life Technologies). The human breast cancer cell line SKBR3 was purchased from Duke University Cell Culture Facility, and was maintained in McCoy’s 5 medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS, Life Technologies) and antibiotics. MII cells were maintained in DMEM/F12 medium supplemented with 5% horse serum (Life Technologies), 20 ng/ml
human epidermal growth factor (Peprotech), 10 µg/ml insulin (Life Technologies), and 0.5 µg/ml hydrocortisone. MIV cells were maintained in DMEM/F12 with 5% horse serum. All cultures were maintained at 37 degree in humidified air containing 5% CO2.

### 2.2 Antibodies

Antibodies used for Western blotting included cleaved caspase-3, phospho-CrkL(Y207), phospho-Akt (Ser473), Akt, TAZ, YAP1, phospho-STAT5 (Tyr694), STAT5 and ERBB2 from Cell Signaling; β-tubulin and actin from Sigma-Aldrich; ABL2 (9H5) from Santa Cruz; ABL1(8E9) from BD Biosciences; IL6, TNC and phospho-YAP1(Y357) from Abcam; MMP1 from CALBIOCHEM. Antibodies used for Immunofluorescence staining included YAP1 from Cell Signaling and TAZ from BD Biosciences. Antibodies used for CHIP assays were TAZ (V386) and YAP1 (D8H1X) from Cell Signaling. All other antibodies used in immunoblotting were produced by Cell Signaling Technologies.

### 2.3 Tumor Xenografts and Analysis

Procedures involving mice were approved and performed following the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Duke University Division of Laboratory Animal Resources (DLAR). Age-matched female athymic NCr nu/nu mice (5-6 weeks) were used for xenograft experiments. For intra-cardiac injections, cells were harvested from subconfluent culture plates, washed with PBS, and resuspended at 10⁶/mL (1833) or 5x10⁶/mL (SCP28) in PBS; 0.1mL of the suspended cells were injected into the left cardiac ventricle using 30G needles. Mice
were anesthetized with isoflurane before injection and imaged by bioluminescence imaging. For intra-tibia injections, mice were anesthetized using a mixture of ketamine (100mg/kg) and xylazine (10mg/kg). The injection site was cleaned with 70% alcohol wipe. Single-cell suspensions (1x10^5 cells) in a final volume of 10ul were injected into the upper half of the tibia medullary cavity, as felt by a lack of resistance when pushing cells into the cavity. Bioluminescence imaging was used to confirm successful cancer cell inoculation and progression of metastatic bone lesions. The allosteric inhibitor GNF5 was synthesized by Duke University Small Molecule Synthesis Facility. For drug treatment of IC injected mice, mice were dosed with GNF5 in DMSO/Peanut Oil (1:9) at 50 mg/kg by intraperitoneal injection daily. For subcutaneous injections, cells were harvested from subconfluent culture plates, washed with PBS, and resuspended at 10^7/mL in PBS. The injection site was cleaned with 70% alcohol wipe. 0.1mL of the suspended cells were injected subcutaneously using 28G needles. Mice were anesthetized with isoflurane before injection. The tumors were measured every other day and the size was calculated using the formula: tumor size = 0.5 * width * width * length. The allosteric inhibitor GNF5 was synthesized by Duke University Small Molecule Synthesis Facility. For drug treatment, mice were dosed with GNF5 by oral gavage twice a day.
2.4 Bone µCT Analysis

Hindlimb bones were excised, fixed in 10% neutral-buffered formalin and imaged using a µCT scanner (Skyscam 1176, Bruker Corp) at 17 µm resolution and 180° scanning with a rotation step of 0.7° per image, 242ms exposure time, 55kV photon energy and 455 µA current. The images were reconstructed using NReconServer and bone volume was analyzed by CT Analysis Software (CTAn, Bruker Corp).

2.5 In Vitro Osteoclastogenesis Assay

Bone marrow cells were flushed out from femora and tibia of 6-week-old C57BL/6 mice and plated in basal culture medium (α-MEM supplemented with 10% FBS and antibiotics) overnight. Osteoclastogenesis assay and TRAP staining were conducted as described previously (7). Tumor cells were plated at 2 x 10^5 per well in 12-well plates to obtain conditioned media for incubation with either bone marrow cells or RAW264.7 cells. RAW264.7 pre-osteoclast cells were plated at 4 x 10^5 per well in 24-well plates overnight. RAW264.7 medium was replaced by conditioned medium harvested from tumor cells and supplemented with recombinant murine sRANKL (50ng/mL). Medium was changed every 3 days, and TRAP staining was performed on day 6 per manufacturer’s instructions (Sigma).

2.6 RNAseq Analysis

For RNAseq analysis, 3x10^6 breast cancer cells were plated in 10-cm Petri dish in triplicate in complete medium for 24 hours. Cells were harvested and RNA was isolated
using the RNeasy kit (Qiagen); 1 µg total RNA input was used for each sample. The libraries were sequenced on an Illumina HiSeq 2000 sequencing system using 50-bp single-ended reads. RNAseq data was mapped to reference genome (HG19) using Bowtie/Tophat. Reads were counted and differential expression between distinct experimental groups was quantified using Cuffdiff. Significant genes were extracted using R CummeRbund.

2.7 Viral Transduction

Retroviral or lentiviral constructs were transfected into HEK293T cells with their packaging vectors indicated below using FuGENE6 reagent (Promega). pMX-puro-STAT5A* (kindly provided by Toshio Kitamura, University of Tokyo): pCMV-Gag-Pol and pCMV-VSV-G; Plenti-EF-FH-TAZ-S89A: psPAX2 and pVSV-G; PLKO-NS and shTAZ lentiviral construct (kindly provided by Corinne Linardic, Duke University): pCMV-Rev, pCVM-VSVG; pMDL. Retroviral or lentiviral supernatants were collected and filtered 24 h and 48 h post-transfection. 1833 cells were incubated 48 h with retroviruses or lentiviruses medium in the presence of 8µg/mL Polybrene. Cells were cultured at least 3 days in 3µg/mL Puromycin or 5µg/mL Blasticidin for selection. Lentiviral shRNA-mediated knockdown of ABL1/ABL2 and expression of mouse ABL1/ABL2 were conducted as described previously (45, 46, 99). Lentiviral shRNA-mediated knockdown of STAT5A and STAT5B was conducted per manufacturer’s instruction (Dharmacon RHS4531-EG6776, EG6777).
2.8 Immunoblotting

Cells were lysed in radio-immunoprecipitation assay (RIPA) buffer with protease and phosphatase inhibitors. Cell debris was removed by microcentrifugation, and protein was quantified. Alternatively, equal cell numbers were plated onto 6-well plates and 24 hrs later, conditioned medium was harvested and concentrated using Amicon Ultra centrifugal filters (Millipore). Equal amounts of protein or conditioned media were separated by SDS/PAGE and transferred to nitrocellulose membranes and probed with the indicated antibodies.

2.9 Real-Time RT-PCR

RNA was isolated from cancer cells using the RNeasy RNA isolation kit (Qiagen), and cDNA was synthesized using Oligo(dT) primer and M-MLV reverse transcriptase (Invitrogen). Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad). Primers used were as follows- mouse RANKL (forward): 5’-TTG CAC ACC TCA CCA TCA, (reverse): 5’-TAC GCT TCC CGA TGT TTC; mouse OPG (forward): 5’-CAC TCG AAC CTC ACC ACA, (reverse): 5’-CAA GTG CTT GAG GGC ATA; mouse GAPDH (forward): 5’-CTC ATG ACC ACA GTC CAT GC, (reverse): 5’-ACA CAT TGG GGG TAG GAA CA; human ABL1 (forward): 5’-GTC TGT GAG TAC CTT GCT GC, (reverse): 5’-GGC GCT CAT CTT CAT TCA GGC; human ABL2 (forward): 5’-CCA GCT ACT CCC GAG GCT G, (reverse): 5’-CTT GAT CCC ACA GGG TGA AG; human GAPDH (forward): 5’-GTC TCT CCA GAA CAT CAT CCC, (reverse):
5’-GGG TGT CGC TGT TGA AGT CAG; human MMP1 (forward): 5’-GGT CTC TGA GGG TCA AGC AG, (reverse): 5’-AGT TCA TGA GCA ACA CG; human IL6 (forward): AGA CAG CCA CTC ACC TCT TC, (reverse): 5’-TTT CAC CAG GCA AGT CTC CT; human TNC (forward): 5’-CCC TAC GGG TTC ACA GTT TC, (reverse): 5’-TTC CGG TTC GGC TTC TGT AAC; human AXL (forward): 5’-ATC AGA CCT TCG TGT CCC AG, (reverse): 5’-ATG TCT TGT TCA GCC CTG GA; human WWTR1 (forward): 5’-GGC TGG GAG ATG ACC TTC AC, (reverse): 5’ –AGG CAC TGG TGT GGA ACT GAC; human YAP1 (forward): 5’ –ATG AAC TCG GCT TCA GCC AT, (reverse): 5’ –ACC ATC CTG CTC CAG TGT TG. Analysis was performed using a Bio-Rad CFX384 real-time machine and CFX Manager software. PCR assays were performed in triplicate. Expression of each gene was normalized to that of the GAPDH gene.

2.10 Histological Analysis

Hindlimb bones were excised, fixed in 10% neutral-buffered formalin, decalcified, and embedded in paraffin for hematoxylin (H&E) staining (114).

2.11 TUNEL Staining

For TUNEL staining, 2x10^4 cells were seeded onto each single chamber of a 4-chamber side in complete medium. The next day, the medium was replaced with serum-free media containing TRAIL (2ng/mL). After 3 days, cells were fixed using 4% PFA and permeabilized with 0.1% Triton X-100. TUNEL staining was performed following the manufacturer’s protocol (Roche Applied Science).
2.12 ELISA and Cytokine Array

Conditioned medium was collected after 24 h incubation from confluent cells and was applied to the Human Cytokine Antibody Array C1000 (Raybiotech) or IL6 ELISA kit (R&D) following the manufacturer’s instructions.

2.13 Invasion Assay

Invasion was evaluated by plating 25,000 cells in the upper chambers of 8.0 µm pore size matrigel chambers (BD Biosciences) in serum-free medium. Cells were allowed to invade for up to 48 h in the presence of serum-containing medium in the bottom chamber. Afterwards, the remaining cells, medium, and matrigel were removed from the upper chambers, and cells on the undersurface of the membrane were fixed, stained with DiffQuik (Dade Behring), and quantified by microscopy.

2.14 In Vitro Cell Growth Assays

For 2D-cell growth, 3000 cells were seeded onto each well of a 96-well plate. Cell growth was measured daily from day 1 to 5 using CellTiter-Glo (Promega) following the manufacturer’s protocol. For 3D-cell growth, 75µl of matrigel (BD Bioscience) was plated onto each well of a 96-well plate. A single cell suspension (50µl) containing 1500 cells was mixed with matrigel 1:1 and plated on top of the matrigel base onto wells of a 96-well plate; 50µl of complete medium was added and cells were cultured for 14 days. Colonies were analyzed and counted using microscope.
2.15 CHIP-QPCR Analysis

CHIP-QPCR was performed using Cell Signaling SimpleChIP Plus Enzymatic Chromatin IP Kit (#9005) according to manufacturer’s instructions. SimpleChIP human CTGF promoter primers were from Cell Signaling (#14927). Validated TAZ/YAP primers, as well as previously validated primers for AJUBA, AMOTL2, and WTIP were used for QPCR analysis (115). Antibodies used for CHIP assays were anti-TAZ (V386) and anti-YAP1 (D8H1X) from Cell Signaling. Primers used were as follows: AXL (forward): 5’-CAG CCT CCT CCT CAC AGA CA; AXL (reverse): 5’-GAG CCC TGA TCA TTC CAC TG; AJUBA (forward): 5’-AAG GAA AGA GTG TGG GGG TAG G; AJUBA (reverse): 5’-ACG CTG GGA ACA AAG TCA CG; AMOTL2 (forward): 5’-TGC CAG GAA TGT GAG AGT TTC; AMOTL2 (reverse): 5’-AGG AGG AGG GAG CGG GAG AAG; WTIP (forward): 5’-GCA GCG CCG TCT CCT TCC T; WTIP (reverse): 5’-GCG GCG GAG GAA TGT AAG CTC.

2.16 Mutagenesis

ABL2 E505K mutagenesis was conducted on pBabe-puro-mABL2 construct using Q5 Site-Directed Mutagenesis Kit (NEB E0554S) according to the manufacturer’s instructions. Primers used were as follows: ABL2 (forward): 5’-CAT CTC TGA AAA GGT AGC TCA G; ABL2 (reverse): 5’-CTG GAG TCA TGG AAC ATT G.
2.17 Soft Agar Colony Formation

Cells were seeded in 0.35% agarose and overlaid on top of a layer of 0.4% agarose mixed with DMEM/F12 + 5% horse serum and antibiotics. Seven thousand cells were added to each well of a 6-well plate in duplicate, in a total of 1 ml agarose/medium mixture. Following solidification of the agarose, 1 ml of media for MII and MIV cells was then added to each well. GNF5 was included in the medium that was added to the top of the wells and was replenished every 48 hrs. After an 18-21 day incubation, colonies were counted in three randomly selected fields of view.

2.18 Mammosphere Formation

30,000 (MII) or 20,000 (MIV) cells were added to each well of a low attachment six-well plate (Sigma), in a total of 2 ml DMEM/F12 supplemented with 20ng/ml hEGF, 0.5µg/ml hydrocortisone, and 10 µg/ml insulin. Cells were incubated for 7-8 days, with 1 ml fresh medium added every 2 days. Total numbers of mammospheres were counted in each well. For secondary mammosphere formation, primary spheres were pelleted, trypsinized, and 20,000 viable cells were re-plated in low attachment plates and spheres were counted after another 7-8 day incubation.

2.19 Limiting Dilution Analysis

MII cells harboring the empty PK1 vector or expressing PK1-Abl2PP were transduced with pFU-luciferase-tomato lentiviral DNA (pFULT) to facilitate bioluminescent imaging of orthotopic tumors. Cells were diluted to suspensions of 1 X 10^7, 1 X10^6, and 1 X 10^5 cells/ml with 20% Growth Factor-Reduced Matrigel (Corning). 100 µl of each suspension was injected into the #4 mammary gland of 10-12 wk age-matched female athymic nu/nu mice using a 28-gauge needle. Altogether, mice were injected with 10^6, 10^5, or 10^4 of either MII (vector) or MII-Abl2PP cells. Tumors were imaged for BLI on an IVIS Lumina XR (Caliper Life Sciences) and measured using calipers at various time points following injections. Mice were sacrificed (grouped
according to number of cells injected) when the largest tumor in each cohort reached approximately 1.5 cm in diameter and tumors were weighed. Palpable tumors of any size were counted as a tumor initiating events. n=6 mice per condition.

2.20 Flow Cytometry

5X10^6 cells were harvested, washed in 1X PBS, and blocked in PBS + 2% BSA for 30 minutes. Cells were then stained with the indicated antibodies in the dark. Antibodies were used at 1:25 or 1:50 dilutions in FACS buffer (1X PBS + 2% BSA) for staining cells, washed in PBS, then analyzed on a FACSvantage flow cytometer (BD).

2.21 Statistical Analysis

Statistical analyses were performed using GraphPad Prism 6, JMP Pro and R 3.2. Comparisons of two groups were performed using Student’s t tests (two-tailed). Comparisons involving multiple groups were evaluated using one-way or two-way ANOVA, followed by Tukey’s HSD. For all tests, p<0.05 was considered statistically significant. For all figures, p value was calculated using Student’s t test unless otherwise indicated. Data shown represent averages ± SEM unless otherwise indicated.
3. ABL kinases are required for breast cancer bone metastasis

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3.1 Introduction

ABL tyrosine kinases play an oncogenic role in human leukemias (11, 14) and promote the progression of solid tumors (14, 108). ABL kinases elicit pro-tumorigenic or anti-tumorigenic effects in breast cancer cells and promote cancer cell invasion (102, 116-118). However, whether ABL kinases have a role in the regulation of cellular processes critical for metastasis, other than invasion, has not yet been evaluated. Here we uncovered a critical role for the ABL kinases in the regulation of breast cancer metastasis to bone.

Bone metastases occur in up to 70% of patients with advanced breast cancer and are associated with high mortality and morbidity (8, 119). While the mechanisms that drive tumor cell homing, invasion and colonization to the bone are poorly understood, it is increasingly apparent that bone metastasis requires interactions between tumor and stromal cells in the bone microenvironment (120). When breast cancer cells invade into the bone microenvironment, they produce molecules that activate osteoclastic bone resorption, leading to the release of growth factors stored in the bone matrix to promote tumor growth. Currently, there are no available therapies to cure breast cancer
metastasis. Thus, there is a need to identify molecules that could be targeted simultaneously in tumor and bone to disrupt the tumor-stromal cells interactions that drive metastasis.

Here we report that increased expression of ABL1 and ABL2 correlated with enhanced breast cancer metastasis and decreased metastasis-free survival. Using metastasis models that bypass invasion and intravasation, we uncovered roles for the ABL kinases in the regulation of breast cancer cell survival and colonization in the bone microenvironment. Further, we identified a role for ABL kinases in promoting the expression of multiple pro-bone-metastasis genes such as AXL (which encodes a receptor tyrosine kinase), IL6 (which encodes interleukin-6), MMP1 (which encodes matrix metalloproteinase 1) and TNC (which encodes tenascin-C) through TAZ- and STAT5-mediated signaling. Moreover, we found that treatment with a selective allosteric inhibitor of the ABL kinases or simultaneous depletion of both ABL kinases in breast cancer cells impaired breast cancer bone metastases and decreased osteoclast activation in vitro and osteolysis in vivo.

3.2 Results

3.2.1 Increased expression of ABL kinase-encoding genes correlates with breast cancer metastasis

To evaluate whether altered expression of the ABL genes is associated with breast cancer progression and metastasis we examined the expression of ABL1 and ABL2 in normal and invasive breast tumor specimens using published TCGA datasets (82, 121,
ABL2 DNA and RNA abundance was significantly increased in breast tumor specimens (Fig. 8, A and B). To further evaluate the importance of enhanced ABL abundance in the context of metastasis, we analyzed an integrative database assembled from 22 publicly available datasets containing information on metastasis-related relapse (123). We found that increased ABL2 mRNA abundance correlated with metastasis across all subtypes of breast cancer, primarily the basal type (Fig. 8, C and D), whereas high ABL1 mRNA abundance significantly correlated with metastasis in HER2-enriched breast cancer but not in other breast cancer subtypes (Fig. 8E). Furthermore, high ABL1 mRNA was associated with bone metastasis in a microarray dataset reporting organ-specific metastasis (Fig. 8F) (124). Collectively these findings support a link between increased expression of the ABL genes and increased breast cancer metastasis.

3.2.2 ABL family protein kinases are required for bone metastasis

To directly evaluate the relationship between ABL family kinases and metastasis, we analyzed ABL1 and ABL2 protein abundance in MDA-MB-231-derived breast cancer cell lines with different organ metastasis tropisms (113). The MDA-MB-231-derived 1833 cell line, which is characterized by enhanced bone-specific metastasis compared to the parental cell line or cell lines with increased tropism to lung and brain, showed increased abundance of ABL1 and ABL2 (Fig. 9A). To examine the functional role of ABL kinases in these cells, endogenous ABL kinases were depleted with previously characterized shRNAs specific against ABL1 and ABL2 (46). The lentiviral-encoded
shRNAs decreased the abundance of ABL1 and ABL2 by 80% at day 2 after viral transduction, but ABL1 abundance was slightly increased by day 21 after viral transduction (Fig. 9B). Depletion of ABL kinases did not affect cell growth in monolayers or colony formation in matrigel (Fig. 9, C, D and E), but decreased cell invasion in both 1833 triple-negative and HER2-positive SKBR3 breast cancer cells (Fig. 10, A, B, C, and D). Conversely, overexpression of constitutively active forms of ABL1 (ABL1PP) and ABL2 (ABL2PP) enhanced the invasiveness of the parental MDA-MB-231 cells (fig. 10, E, F, and G).

ABL kinases regulate cancer cell invasion (99), but it is unclear whether they play a role in the regulation of subsequent steps of the metastatic cascade. To investigate whether inhibition of ABL kinases interferes with metastatic processes other than invasion, we depleted ABL1 and ABL2 (ABL1/ABL2) in two bone metastatic breast cancer cell lines 1833 and SCP28 (125), and evaluated the metastatic potential of these cells following intra-cardiac injection into immune-deficient mice. This mouse model bypasses the initial invasion step and allows for analysis of subsequent steps in the metastatic cascade. The 1833 and SCP28 breast cancer cells were engineered to express reporters with luciferase and green fluorescent protein (GFP) to monitor metastatic progression by bioluminescence imaging (Fig. 11A). We found that ABL kinase knockdown increased the survival of tumor-bearing mice (Fig. 11B) and markedly inhibited bone metastases by 1833 and SCP28 breast cancer cells as measured by
bioluminescence imaging (Fig. 11, C, D, E and F) and haematoxylin and eosin (HE) staining (Fig. 11, G and H). Decreased metastasis by ABL-deficient breast cancer cells was accompanied by a significant reduction in the extent of hind-limb osteolytic lesions, as determined by X-ray and μCT imaging (Fig. 11, I and J).

Figure 8: Increased expression of ABL genes in invasive breast cancer is associated with metastasis. (A) ABL2 copy number in 813 normal samples compared
with 789 invasive breast tumor samples in the TCGA database. (B) ABL2 mRNA abundance in 61 normal samples compared with 532 invasive breast tumor samples in the TCGA database. Results shown in (A) and (B) are based on data generated by the TCGA Research Network (http://cancergenome.nih.gov/); whiskers represent 1\textsuperscript{st} and 99\textsuperscript{th} percentile. (C-D) Kaplan-Meier representation of the probability of cumulative overall distant metastasis-free survival (DMFS) in 2830 breast cancer cases (C), or 482 basal breast cancer cases (D) according to ABL2 expression. (E) Kaplan-Meier representation of the probability of cumulative overall distant metastasis-free survival in 279 HER2 enriched breast cancer cases according to ABL1 expression. (F) Kaplan-Meier representation of the probability of cumulative bone metastasis-free survival (BMFS) in 42 breast cancer cases according to ABL1 expression. P values (log rank test) and hazard ratio (HR) are shown in the graph.

Figure 9: ABL protein levels are elevated in breast cancer cells with enhanced bone metastatic activity and ABL1/ABL2 depletion does not affect proliferation in vitro. (A) Immunoblots with the indicated antibodies were performed on whole-cell lysates from human mammary epithelial cells (HuMEC), MDA-MB-231 (parental) and MDA-MB-231 derived breast cancer cell lines: 4175 (lung metastasis), 1833 (bone
metastasis), and BrM2a (brain metastasis); N=3 blots. (B) Immunoblots with the indicated antibodies were performed on whole-cell lysates from 1833 control (Scr) and ABL1/ABL2 knockdown cells (shAA) at 2 or 21 days after knockdown; N=2 blots. (C) Control and knockdown 1833 breast cancer cells were seeded onto 96-well plates and cell proliferation and survival were analyzed daily using CellTiter-Glo (days 1-5). N=3 biological replicates. (D-E) Control and knockdown breast cancer cells were seeded in matrigel and colony formation was quantified (E) on day 21. N=3 biological replicates.

Figure 10: ABL kinases promote breast cancer cell invasion. (A-D) Control and ABL1/ABL2 knockdown 1833 (A, B) and SKBR3 (C, D) breast cancer cells were plated on the upper wells of matrigel chambers; cells on the under surface of the matrigel membrane were stained (A, C) and quantified (B, D). N=3 biological replicates. (E) Representative immunoblots of whole cell lysates from MDA-MB-231 cells transduced with retroviruses encoding control vector (V), constitutively active ABL1 (ABL1PP), and constitutively active ABL2 (ABL2PP); N=2 blots. (F, G) Indicated cells were plated on the upper wells of matrigel chambers; cells on the under surface of the matrigel membrane were stained (F) and quantified (G). Scale bar=100µM. N=3 biological replicates.
Figure 11: Knockdown of ABL kinases decreases breast cancer bone metastasis.

(A) The experimental design. (B) Survival of mice after intracardiac injection of 1833 (1X10⁵) breast cancer cells transduced with control shRNA (Scr) or shRNAs against ABL1 and ABL2 (shAA). N=10 mice/per group. (C-F) Bioluminescent images (C, E) of bone metastasis from representative mice at day 22 after inoculation with 1833 cells (N=10 mice/group) or day 35 after inoculation with SCP28 cells (N=8 mice/group). Quantification of bone metastases (D, F). (G-H) Representative HE staining (G) and quantification of HE-stained tumor area of bone lesions. Arrows indicate tumor. N=3 mice/group. Scale bar=200µM. Met, metastatic. (I-J) Representative Xray and µCT reconstruction (I) and quantification of bone volume/total volume from µCT analysis of the mouse tibias (J). N=3 mice/group. (K) Representative immunoblots of 1833 cells transfected with control shRNA (Scr), shRNA against ABL1 (shABL1), ABL2 (shABL2), and shRNA #2 against both ABL1 and ABL2 (shAA#2), and ABL1/ABL2 knockdown cells with overexpression of mouse ABL1/ABL2 (shAA+mABL1/ABL2). N=3 blots. p,
phosphorylated. (L) Bioluminescent images of bone metastases from representative mice at day 18 after inoculation. N=8 mice/group. (M) Quantification of (L). * p<0.05, calculated using One-Way ANOVA followed by Tukey’s HSD.

To evaluate whether ABL1 and ABL2 are individually responsible for promoting metastasis, we employed specific shRNAs to silence either ABL1 or ABL2 in breast cancer cells. We found that ~90% knockdown of ABL1 alone resulted in enhanced ABL2 expression, and did not produce a significant decrease in the phosphorylation of CrKL, a reporter for the activation state of the ABL kinases (Fig. 11K) and did not inhibit metastasis (Fig. 11, L and M). Double knockdown of ABL1 and ABL2 was required to decrease the phosphorylation of CrKL by more than 90%, which indicates inactivation of the endogenous ABL kinases (Fig. 11K). While knockdown of ABL2 alone decreased metastasis, knockdown of both ABL1 and ABL2 was required to significantly decrease metastasis (Fig. 11, L and M). To further validate that the decreased metastasis induced by double knockdown of ABL1 and ABL2 was not due to off target effects of the lentivirus-encoded shRNAs, we employed a second set of ABL shRNAs (shAA#2) and carried out rescue experiments by expressing mouse ABL1 and ABL2 constructs (mABL1/ABL2) that are resistant to shRNAs against human ABL1 and ABL2. We found that expression of murine ABL1 and ABL2 kinases in the knockdown cells reversed the decreased metastasis (Fig. 11, K, L, and M). Loss of ABL1 and ABL2 in the lung metastatic 4175 breast cancer cell line did not significantly reduce metastasis (Fig. 12).
These findings reveal a function for ABL kinases in the regulation of breast cancer bone metastasis and tumor-induced osteolysis in vivo.

Figure 12: Depletion of ABL kinases does not inhibit metastasis of 4175 breast cancer cells with tropism to lung. (A-B) Bioluminescent images (A) and quantification (B) of bone metastasis from representative mice in each group at day 22 after inoculation of control (Scr) and ABL1/ABL2 knockdown (shAA) 4175 breast cancer cells; N=8 mice. (C) Representative immunoblots of whole-cell lysates from 4175 cells transduced with control shRNA (Scr) and ABL1/ABL2 shRNAs (shAA) and blotted with the indicated antibodies; N=3 blots.

3.2.3 Allosteric inhibition of the ABL kinases impairs breast cancer bone metastasis

To date few studies have directly evaluated the biological consequences of targeting the ABL kinases with selective inhibitors in solid tumors, including breast cancer in vivo. The ATP-competitive kinase inhibitors imatinib (STI571; trade name Gleevec), dasatinib, and nilotinib inhibit multiple tyrosine kinases in addition to ABL1 and ABL2 (14). Moreover, these ATP-competitive inhibitors induce the formation of B-
RAF and C-RAF dimers, leading to ERK activation in diverse cancer cell types (112).

Therefore, we wished to use a different approach to evaluate whether ABL kinases play a role in breast cancer metastasis using the allosteric inhibitor GNF5, which targets the unique ABL myristate-binding site and functions as non-ATP-site and selective inhibitor of the ABL kinases (17, 126). Notably, GNF5 does not activate the RAF-ERK pathway in breast cancer cells (112) (Fig. 13).

![Figure 13](image)

**Figure 13: Treatment of breast cancer cells with imatinib but not GNF5 promotes ERK activation.** Triple-negative 1833 breast cancer cells were treated with vehicle (DMSO), 10uM imatinib (STI571), and 10uM GNF5 for 3 hours; western blots with antibodies against phospho-Erk (p-Erk), phospho-CrkL (p-CrkL) and tubulin (loading control) were performed on whole cell lysates; N=3 blots.

Treatment of tumor-bearing mice with GNF5 starting on day eight following intra-cardiac injection of breast cancer cells (Fig. 14A) resulted in a significant increase in survival (Fig. 14B) and a decrease in bone metastasis burden as measured by
bioluminescent imaging (Fig. 14, C and D). Similarly, histological analyses revealed a decrease in bone tumor burden in mice treated with the allosteric inhibitor of the ABL kinases (Fig. 14, E and F). Notably, we found that bone destruction was decreased and the ratio of bone volume to total volume was increased in tumor-bearing mice treated with GNF5 (Fig. 14, G and H). These results demonstrate that ABL kinase activity is required for osteolytic metastasis in breast cancer and suggest that pharmacological inhibition of the ABL kinases may be an effective treatment for bone metastasis.

3.2.4 ABL kinases are required for tumor cell survival and tumor-induced osteolysis in the bone microenvironment

To directly examine whether ABL kinases play a role in regulating the colonization and survival of breast cancer cells in the bone microenvironment, we injected control or ABL1/ABL2 knockdown breast cancer cells directly into the tibia of immune-deficient mice. Depletion of the ABL kinases reduced tumor expansion in the tibia as measured by both bioluminescent imaging (Fig. 15, A and B) and histological staining (Fig. 15, C and D). Moreover, 3D-µCT reconstruction of the tibia revealed that mice injected with control cells had a significantly higher degree of osteolysis with a decreased ratio of bone volume to total volume compared to mice injected with ABL1/ABL2-knockdown breast cancer cells (Fig. 15, E and F). Depletion of the ABL kinases did not affect breast cancer cell proliferation or colony formation in vitro (Fig. 9). Therefore, these findings suggest that ABL1/ABL2-dependent expansion of breast cancer cells is mediated by factors present in the bone microenvironment.
Figure 14: Allosteric inhibition of ABL kinases decreases breast cancer bone metastasis. (A) The experimental design. (B) Survival of mice after intracardiac injection of 1833 (1X10⁵) breast cancer cells and treatment with either DMSO control or the allosteric ABL inhibitor GNF5. N=10 mice/group. (C) Bioluminescent images of representative mice at day 22 after inoculation. (D) Quantification of bone metastases. N=10 mice/group. (E-F) Representative HE staining (E) and quantification (F) of HE-
stained tumor area of bone lesions. Arrows indicate tumor. N=3 mice/group. HE: Scale bar=200µM. (G-H) Representative X-ray and µCT reconstruction (G) of mouse tibias and quantification (H) of bone volume/total volume. N=3 mice/group.

**Figure 15:** ABL kinases are required for tumor survival and tumor-induced osteolysis in the bone microenvironment. (A-B) 1X10⁵ control or ABL1/ABL2 knockdown 1833 cells were injected directly into the tibias of the mice. Representative bioluminescent images (A) taken at day 21 after inoculation and quantification (B) of bone lesions are shown. N=5 mice/group. (C-D) Representative HE staining (C) and quantification (D) of HE-stained tumor area of mouse tibias from each group. N=3 mice/group. Scale bar=500µM. (E-F) Representative 3D µCT reconstruction of mouse tibias (E) and quantification (F) of bone volume/total volume (BV/TV) from µCT analysis. N=3 mice. (G-H) Representative images (G) of TUNEL staining of cells treated with TRAIL and the indicated shRNA and quantification of the percent of TUNEL-positive cells (H). N=3 biological replicates. Scale bar=100µM. (I) Immunobloting was performed using the indicated antibodies on whole-cell lysates from cells incubated or not with TRAIL. N=3 blots.
Bone metastasis requires reciprocal interactions between tumor cells, stromal cells, and bone cells (5, 119). Several soluble factors released by stromal cells within the bone microenvironment promote tumor growth and survival (127). These factors include Chemokine C-X-C Motif Ligand 12 (CXCL12), a chemokine produced by bone marrow mesenchymal cells that functions as a chemo-attractant and survival factor for cells bearing the Chemokine C-X-C Motif Receptor 4 (CXCR4), and the insulin-like growth factor 1 (IGF-1), a factor that is stored in the bone matrix and released during osteolysis (114). We and others have previously shown that ABL kinases are activated by the binding of CXCL12 and IGF-1 to their cognate receptors (99, 108). Thus, we evaluated whether loss of ABL kinases could affect activation of AKT-mediated survival by these factors. We found that CXCL12 and IGF-1 induced activation of AKT was independent of ABL1 and ABL2 in 1833 breast cancer cells (Fig. 16, A and B). In contrast, we observed that ABL kinases protected breast cancer cells from TRAIL-induced cell death (Fig. 15, G, H, and I). TRAIL is a pro-apoptotic member of the tumor necrosis factor family, that induces apoptosis by binding to cell death receptors DR4 and DR5 (128). TRAIL and DR5 are present in clinical breast cancer bone metastases specimens, and DR4 and DR5 are present in 80% of patient bone tumor biopsies (114, 129). TRAIL enhanced apoptosis as measured by cleavage of caspase-3 (Fig. 15I), and knockdown of ABL kinases increased the sensitivity of 1833 breast cancer cells to the pro-apoptotic effects of TRAIL (Fig. 15, G, H and I). These data suggest that ABL kinases promote
breast cancer metastasis to the bone in part by increasing tumor cell survival within the bone microenvironment.

**Figure 16:** CXCL12- and IGF1-mediated survival pathways are independent of ABL kinases. (A) Control and ABL1/ABL2 knockdown 1833 breast cancer cells were incubated with or without CXCL12 (300 ng/mL) for 30min; immunoblots using the indicated antibodies were performed on whole-cell lysates. (B) Control and ABL1/ABL2 knockdown 1833 cells were incubated with or without IGF1 (10 ng/mL) for 30min; immunoblots using the indicated antibodies were performed on whole-cell lysates. N=3 blots.

### 3.2.5 Depletion of ABL kinases impairs tumor-induced osteoclast activation in part by decreased IL6 secretion

Osteoclast activation plays a central role in the progression of breast cancer bone metastasis. To directly examine whether ABL kinases regulate tumor-induced osteoclast activation, we employed an in vitro osteoclastogenesis assay (Fig. 17A). Mouse primary bone marrow cells were treated with conditioned media from control or ABL1/ABL2
knockdown breast cancer cells, and then stained for TRAP (an osteoclast marker). Bone marrow cells cultured with conditioned medium derived from ABL1/ABL2 knockdown 1833 and SKBR3 breast cancer cells had decreased numbers of TRAP+ cells compared to the control groups (Fig. 17, B and C; and Fig. 18, A and B). These data suggest that inactivation of the ABL kinases in breast cancer cells may impair secretion of soluble factor(s) required for osteoclast activation. Because bone marrow contains a heterogeneous population of cells, factors secreted in an ABL1/ABL2-dependent manner might interact directly with osteoclast progenitors and promote their differentiation, or might instead function to regulate osteoclasts indirectly by modulating the activity of osteoblasts (125). To test the first possibility, we carried out the osteoclastogenesis assay using the RAW264.7 murine pre-osteoclast cell line. Conditioned media derived from ABL1/ABL2 knockdown 1833 breast cancer cells did not impair tumor-induced RAW264.7 pre-osteoclast differentiation (Fig. 17D). These findings suggest that ABL kinases regulate osteoclast maturation indirectly, possibly by modulating osteoblast function.

Osteoblasts modulate osteoclast activity through secretion of the TNF family member RANKL. Binding of RANKL to the RANK receptor on the surface of osteoclasts activates a pathway essential for osteoclast differentiation. Osteoprotegerin (OPG), a soluble decoy receptor for RANKL, is also produced by osteoblasts and antagonizes the activity of RANKL (130). Several tumor-derived bone metastasis factors can increase
Figure 17: Depletion of ABL kinases impairs tumor-induced osteoclast activation in part by decreasing IL6 secretion. (A) The in vitro osteoclastogenesis assay. (B) TRAP staining of bone marrow cells treated with conditioned medium (CM) from 1833 breast cancer cells. Scale bar=50µM. (C) Quantification of TRAP+ cells in (B). (D) Quantification of TRAP+ cells derived from RAW 264.7 cells; ns= non-significant. (E-F) RANKL (E) and OPG (F) expression was detected by RT-PCR of the osteoblast cell line 7F2 treated with CM harvested from the indicated 1833 cells. (G) Identification of differentially expressed cytokines in the CM of 1833 cells using a cytokine antibody array. N=2 biological replicates. (H) ELISA quantification of IL6 in CM of the 1833 cells. (I) Quantification of TRAP+ bone marrow-derived osteoclasts incubated with the indicated doses of IL6. * indicates significantly different from 0; p value was calculated using One-Way ANOVA followed by Tukey’s HSD. (J) TRAP staining of bone marrow treated with CM from 1833 cells with or without added IL6. (K) Quantification of TRAP+ osteoclasts in (J). * p<0.05; **p<0.01 p value was calculated using Two-Way ANOVA followed by Tukey’s HSD. N=3 biological replicates unless otherwise indicated.
Figure 18: Depletion of ABL kinases in SKBR3 breast cancer cells decreases tumor-induced osteoclast activation. (A) TRAP staining of bone marrow cells treated with conditioned medium harvested from control (Scr) and ABL1/ABL2 (shAA) knockdown SKBR3 breast cancer cells. Scale bar=200µM. (B) Quantification of TRAP+ bone marrow cells in (A). N=3 biological replicates.

RANKL production or decrease OPG secretion by osteoblasts, thereby promoting osteoclast differentiation and activation (7, 119). To evaluate whether ABL kinases might regulate the secretion of osteoblast-derived RANKL or OPG leading to osteoclast differentiation, we analyzed RANKL and OPG mRNA abundance in the murine osteoblast cell line 7F2 in response to conditioned medium from control and ABL1/ABL2 knockdown breast cancer cells. While conditioned medium from ABL1/ABL2-depleted breast cancer cells did not affect RANKL abundance in osteoblasts compared with the cells treated with control conditioned medium (Fig. 17E), we found that conditioned medium from breast cancer cells lacking ABL kinases increased OPG abundance in the osteoblast cell line (Fig. 17F). These findings suggest that ABL kinases promote tumor-induced osteoclast activation in part by increasing OPG abundance in osteoblasts.
To identify tumor-secreted cytokines regulated by the ABL kinases that promote breast cancer metastasis to the bone, we used a human cytokine antibody array to identify changes in cytokine concentrations in the conditioned medium from control and ABL1/ABL2 knockdown cells. We found that IL6 concentrations were decreased in the conditioned medium derived from ABL1/ABL2 knockdown cells compared to that from control cells (Fig. 17G), results that were validated by ELISA (Fig. 17H). IL6 is a multifunctional cytokine with pleiotropic functions that include inducing osteoclast activation, enhancing bone resorption and increasing metastasis (6, 131, 132). Inhibition of the IL6 receptor directly blocks osteoclast formation in vitro and in vivo (133). Thus, we evaluated whether addition of IL6 could rescue in part defective osteoclastogenesis induced by conditioned medium from breast cancer cells depleted of the ABL kinases. We added the optimal dose of IL6 required to promote maturation of osteoclasts (Fig. 17I) to conditioned medium derived from ABL1/ABL2 knockdown breast cancer cells. Addition of IL6 to reconstituted CM from ABL1/ABL2-depleted breast cancer cells partially restored osteoclast activation (Fig. 17, J and K). IL6 induced RANKL expression but suppressed OPG expression in the 7F2 osteoblast cell line (Fig. 19, A and B). Thus, depletion of the ABL kinases in breast cancer cells and the accompanying decrease of IL6 secretion results in enhanced OPG expression and reduced overall RANKL/OPG ratio, thereby decreasing osteoclast differentiation and bone destruction.
Figure 19: IL6 affects RANKL and OPG expression by osteoclasts. (A) Murine 7F2 osteoblasts were treated with or without IL6 (0.1 ng/mL) for 24h and RANKL mRNA expression levels were detected by RT-PCR. (B) 7F2 osteoblasts were treated with or without IL6 (0.1 ng/mL) for 24h and OPG mRNA expression levels were detected by RT-PCR. N=3 biological replicates. * p<0.05; **p<0.01; ***p<0.001.

3.2.6 Next generation sequencing reveals ABL1/ABL2-regulated genes in breast cancer cells

To gain insight into the signaling pathway(s) required for ABL1/ABL2-dependent bone metastasis, we evaluated the consequences of single or double inactivation of ABL1 and ABL2 on the transcriptome of breast cancer cells using next generation sequencing (RNAseq) analysis (Fig. 20 and Fig. 21). We found that 180 genes showed significantly decreased expression and 40 genes showed significantly increased expression in ABL1/ABL2 knockdown cells (Fig. 20B and Table 3). Principle component analysis revealed that transcripts altered in breast cancer cells depleted of ABL1, ABL2,
or both ABL1 and ABL2 were clustered and were distinct from the control group (Fig. 20C). This analysis indicated that the transcriptomes of single and double knockdown cells were similar to each other, but different from that of the control cells, supporting the quality and validity of the RNAseq analysis. Comparison of the transcripts revealed that breast cancer cells with knockdown for ABL2 alone shared a similar gene expression pattern with that of the ABL1/ABL2 double knockdown cells (Fig. 21A and Fig. 20D). Notably, analysis of cell lysates from ABL2-depleted and ABL1/ABL2-double knockdown breast cancer cells showed a greater reduction of the phosphorylation of CrkL compared to cells with knockdown for ABL1 alone (Fig. 11K). Thus, the altered gene expression profiles correlate with decreased ABL kinase activity in breast cancer cells.

3.2.7 ABL kinases signal to TAZ and STAT5 to promote breast cancer bone metastasis

To identify the pathways affected by inactivation of the ABL kinases in metastatic breast cancer cells, we carried out Gene Set Enrichment Analysis (GSEA) using multiple databases (134). In addition to the GSEA oncogenic signature database, we employed the KEGG database and published breast cancer metastasis datasets. We found that a gene signature consisting of 273 genes important for breast cancer bone metastasis showed decreased expression in ABL1/ABL2 knockdown cells (Fig. 21B) (135).
Figure 20: Quality control and global statistics of RNAseq analysis for transcriptome comparison of control versus ABL1/ABL2 knockdown breast cancer cells. (A) Distribution of expression levels of genes from indicated samples. (B) CummeRbund volcano plot reveals genes that differ significantly between control (Scr) and ABL1/ABL2 knockdown (shAA) samples. (C) Principal component analysis reveals that the control sample has a different gene expression pattern compared with single or double ABL1/ABL2 knockdown samples. (D) Scatter plots highlight general similarities and specific outliers between indicated conditions.
Figure 21: ABL kinases regulate the expression of genes in the JAK/STAT and Hippo pathway signatures in metastatic breast cancer cells. (A) CummeRbund Heatmap of genes that were differentially expressed in control and single and double ABL1 and ABL2 knockdown cells. (B) GSEA analysis of the indicated gene signatures in ABL1/ABL2 knockdown cells (shAA) compared with control cells (Scr). NES, Normalized Enrichment Score (C) Expression of the indicated genes in control, ABL1 or ABL2 single knockdown and ABL1/ABL2 double knockdown cells quantified using Cufflinks CuffDiff. * indicates significantly (p<0.05 after Benjamini-Hochberg Correction for multiple-testing) different from Scr. Error bars represent Standard Deviation (SD). N=3 biological replicates for (A) and (C).

Further, inactivation of the ABL kinases resulted in decreased expression of the genes in the Hippo, JAK/STAT, and Cytokine/Cytokine Receptor pathway signatures (Fig. 21B). To identify key molecular mediators of the ABL kinases implicated in the regulation of the ABL1/ABL2-dependent pathways, we analyzed the expression of individual genes
for transcripts altered by loss of the ABL kinases. Among transcripts that were decreased in ABL1/ABL2 knockdown cells were TAZ (also known as WWTR1, WW domain containing transcription regulator protein 1), which encodes a transcriptional co-activator in the Hippo pathway, and STAT5A, which encodes a transcription factor (Fig. 21C).

Table 3: Differentially expressed genes in Control (Scr) and ABL1/ABL2 knockdown (shAA) breast cancer cells.

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Figure 22: ABL kinases are required for TAZ and STAT5 signaling in breast cancer cells. (A-C) Immunoblots using the indicated antibodies were performed on whole-cell lysates of 1833 and SCP28 cells. (D) Immunoblots were performed on whole-cell lysates (pSTAT5, STAT5, tubulin), or conditioned medium (MMP1, IL6, TNC). (E) Immunoblots were performed on whole-cell lysates (pSTAT5, STAT5, tubulin), or conditioned medium (MMP1, IL6, TNC) of parental 1833 and SCP28 cells. (F) Immunoblotting using the indicated antibodies were performed on whole-cell lysates (pSTAT5, STAT5, tubulin), or conditioned medium (MMP1, IL6, TNC) of 1833 cells. For (A-F), N=3 blots. (G-H) Bioluminescent images (G) of representative mice at day 25 after intracardiac injection of 1833 cells. Quantification (H) of bone metastasis. N=5 mice/group. * p<0.05; **p<0.01; ***p<0.001. p value was calculated using One-Way ANOVA followed by Tukey’s HSD. (I-J) Bioluminescent images (I) and quantification (J) of bone metastasis from representative mice at day 25 after intracardiac injection of 1833 cells transfected with control (NS) or shRNAs against STAT5 and TAZ (shSTAT5/shTAZ). N=8 mice/group. (K) Immunoblots were performed on whole cell lysate. N=3 blots.
Figure 23: ABL kinases positively regulate TAZ protein abundance and STAT5 phosphorylation. (A-C). Immunoblots with indicated antibodies were performed on whole cell lysates of indicated breast cancer cells. (A, B) Depletion or pharmacological inactivation of the ABL kinases decreases TAZ levels and STAT5 phosphorylation (p-STAT5) in 1833 and SKBR3 breast cancer cells. (C) Activated ABL kinases increases TAZ protein and p-STAT5 levels in 1833 and MDA-MB-231 (MDA231) breast cancer cells. N=3 blots.

TAZ and the related YAP1 proteins are components of the Hippo pathway and have been implicated in breast cancer progression and metastasis (136, 137). We found that knockdown of the ABL kinases decreased the mRNA expression of TAZ (Fig. 21C), and reduced the protein abundance of TAZ and its downstream target AXL (Fig. 22A, Fig 23, A, B). AXL encodes a receptor tyrosine kinase that promotes breast cancer bone metastasis in mouse models (138). Knockdown of ABL2, but not of ABL1, reduced TAZ abundance to a similar extent as ABL1/ABL2 double knockdown (Fig. 23A), suggesting
that ABL2 has a predominant role in regulating TAZ abundance. Overexpression of ABL1 and ABL2 in both 1833 and parental MDA-MB-231 breast cancer cells increased TAZ abundance (Fig. 23C). Further, immunofluorescence staining analysis indicated that the TAZ protein predominantly (~90%) localized in the nuclei of 1833 breast cancer cells (Fig. 24A), an effect decreased by double-knockdown of ABL1 and ABL2 (Fig. 24B). Similarly, inhibiting ABL kinase activity with the allosteric inhibitor GNF5 decreased TAZ protein abundance (Fig. 23). TAZ protein abundance was not decreased by GNF5 treatment in breast cancer cells expressing murine ABL2-E505K, a mutant that is resistant to the GNF5 allosteric inhibitor (Fig. 25). Moreover, ABL2 mRNA expression positively correlated with TAZ mRNA expression in a TCGA dataset of 971 invasive breast cancer patients (Fig. 26). To evaluate whether loss of ABL kinases affected TAZ activity, we performed ChIP analysis using primers for TAZ targets identified by ChIP-Seq analysis (115). We found that depletion of the ABL kinases decreased TAZ binding to some of its target genes (Fig. 27). While ABL1 has been reported to phosphorylate YAP1 in response to DNA damage (139), we found that ABL1/ABL2 knockdown did not substantially alter YAP1 protein abundance (Fig. 22A), nuclear localization (Fig. 28, A and B), phosphorylation of YAP at Tyr357 (Fig. 28C) or binding to some of its downstream targets (Fig. 27). However, we cannot rule out the possibility that ABL kinases might regulate YAP1-mediated expression of other target genes in breast cancer cells. Regardless, our data support a role for ABL kinases in the regulation of TAZ.
protein abundance and activity in breast cancer cells. Moreover, we found that expression of a constitutively active TAZ S89A mutant in ABL1/ABL2 knockdown breast cancer cells (1833 and SCP28) restored the abundance of its target AXL (Fig. 22, B and C). Together these data revealed a functional link between the ABL kinases and TAZ signaling leading to increased AXL abundance in breast cancer cells, and identified a potentially druggable pathway for the treatment of breast cancer bone metastasis.

We found that inactivation of the ABL kinases in breast cancer cells also decreased STAT5A mRNA and downstream expression of STAT5 target genes, including Tenascin C (TNC) (Fig. 21C). STAT5 belongs to a family of transcription factors that regulate cytokine-induced gene expression and is constitutively activated in several human cancers including breast cancer, where it promotes expression of genes encoding

Figure 24: Depletion of ABL kinases reduces nuclear TAZ protein levels. (A) 1833 cells were transduced lentiviruses encoding control shRNA (Scr) or shRNAs against ABL1/ABL2 (shAA). TAZ protein abundance and subcellular localization were analyzed by immunofluorescence (IF) staining for endogenous TAZ (green); DAPI (blue)
was used to stain the cell nuclei. Scale bar=20µM. (B) Quantification of the percentage of nuclear TAZ-positive cells in (A). N=3 biological replicates.

Figure 25: Allosteric inhibition of ABL kinases activity decreases TAZ protein levels. Control 1833 cells or 1833 cells expressing murine ABL2-E505K mutant protein were plated (3 x 10^5 cells per group) in six well plates. Cells were then treated with vehicle (DMSO) or GNF5 (20 uM) for 24 hours and harvested for immunoblotting with the indicated antibodies. N=3 blots.

Figure 26: ABL2 mRNA levels positively correlate with TAZ mRNA levels among invasive breast cancer patients. Co-expression analysis was performed for the Breast Invasive Carcinoma dataset (N= 971 patients) (TCGA) using www.cbioportal.org.
Figure 27: Depletion of ABL kinases decreases TAZ binding to target genes.
Breast cancer cells (1833) were transduced with lentiviruses encoding control (Scr), ABL2 single (shABL2), or ABL1/ABL2 double (shAA) knockdown shRNAs, and cultured in 15cm culture dishes to 90% confluence; 4x10^6 cells were harvested for each ChIP sample using negative control IgG (NC), anti-TAZ and anti-YAP1 antibody. QPCR was formed with primers for the indicated, previously identified TAZ and YAP1 targets; y axis corresponds to the percent of total input chromatin DNA. * p<0.05, calculated using One-Way ANOVA followed by Tukey’s HSD. N=3 biological replicates.

...cell survival factors (140). STAT5 is also activated by the oncogenic BCR-ABL tyrosine kinase and contributes to the transformation of leukemia cells (141). STAT5 promotes metastasis of human prostate cancer cells (142), and has been implicated in the resistance of metastatic breast cancer cells to targeted therapies (143). Moreover, ablation of a STAT5A allele reduces tumor incidence in a mouse model of breast cancer in which
mammary epithelial cells express T antigen (144). We found that depletion of ABL kinases in breast cancer cells decreased STAT5A mRNA expression (Fig. 21C), without decreasing total STAT5 protein abundance as measured by western blotting with antibodies that detect both STAT5A and STAT5B (Fig. 22D, and Fig. 23, A and B). However, depletion of ABL kinases decreased the phosphorylation of STAT5 (Fig. 22D, and Fig. 23, A and B). Conversely, overexpression of ABL kinases, predominantly ABL1, in both 1833 and parental MDA-MB-231 breast cancer cells increased STAT5 phosphorylation (Fig. 23C). Further, we found that double-knockdown of ABL1 and ABL2 decreased the abundance of various secreted proteins, including IL6, TNC, and MMP1 (Fig. 22D). Both MMP1 and IL6 have been linked to the regulation of osteoclast activation (7, 131), and depletion of TNC decreases breast cancer metastasis (145). Expression of a constitutively active STAT5A mutant (STAT5A*) reversed the reduction in MMP1, IL6, and TNC abundance induced by depletion of both ABL kinases in breast cancer cells (Fig. 22, E and F and Fig. 28). These findings support a role for STAT5 in regulating the ABL1/ABL2-dependent secretome.

Figure 28: Active STAT5 expression increases mRNA levels of MMP1, IL6 and TNC. The mRNA levels of the indicated genes were detected using RT-PCR of control
cells and cells transfected with constitutively active STAT5A*; * p<0.05; **p<0.01; ***p<0.001. N=3 biological replicates.

To evaluate whether TAZ and STAT5 pathways promote breast cancer bone metastasis downstream of the ABL kinases, we expressed the constitutively active mutants of TAZ S89A and STAT5* in ABL1/ABL2 knockdown cells. Expression of either TAZ S89A or STAT5* alone in ABL1/ABL2-depleted breast cancer cells only partially rescued bone metastasis, and expression of both STAT5* and TAZ S89A was required to fully rescue the impaired bone metastasis by ABL1/ABL2-depleted cells (Fig. 22, G and H). To evaluate whether depletion of TAZ and STAT5 in breast cancer could phenocopy the reduced bone metastasis caused by depletion of ABL1 and ABL2, we performed intracardiac injection of control or TAZ/STAT5 double knockdown 1833 breast cancer cells, and found that cells depleted of TAZ and STAT5 exhibited markedly impaired metastasis that phenocopied the inhibitory effects of ABL1/ABL2 knockdown (Fig. 22, I, J and K). TAZ and STAT5 might regulate each other (Fig. 29). TAZ knockdown with two distinct shRNAs decreased the phosphorylation of STAT5 and to a lesser extent total STAT5 protein abundance (Fig. 29A). Moreover, knockdown of STAT5 with two different shRNAs slightly decreased TAZ protein abundance (Fig. 29B). However, these reciprocal decreases were much lower than those induced by knockdown of the ABL kinases (Fig. 22). Future studies will evaluate the pathways that mediate the crosstalk between the TAZ and STAT5 pathways. Together our findings suggest that ABL kinases
activate TAZ and STAT5 pathways and that co-activation of their downstream targets promote the bone metastasis of breast cancer cells in mouse models. Using a TCGA dataset with 971 invasive breast cancer patients, we found that patients with alterations in the expression of ABL2 and eight validated downstream targets (TAZ, AXL, CTGF, STAT5A, STAT5B, TNC, IL6, and MMP1) exhibited decreased disease-free survival (Fig. 30).

**Figure 29: Potential inter-dependence of TAZ and STAT5 in breast cancer cells.** (A) 1833 cells were transduced with lentiviruses encoding vector control (NS) or two different shRNAs against TAZ (shTAZ1, shTAZ2). Immunoblots were performed on whole cell lysates with the indicated antibodies. N=2 blots. (B) 1833 cells were transduced with retroviruses encoding control (NS) or two different shRNAs against STAT5 (shSTAT5_1, shSTAT5_2). Immunoblots were performed on whole cell lysates with the indicated antibodies. N=2 blots.
Figure 30: ABL kinases activate TAZ and STAT5 pathways to promote breast cancer bone metastasis. (A) Kaplan-Meier representation of the probability of cumulative overall disease free survival in TCGA dataset with 971 invasive breast cancer patients according to whether the ABL signature (ABL2, TAZ, AXL, CTGF, STAT5A, STAT5B, TNC, IL6, MMP1) was altered or not. P value was derived by log rank test. (B) Model for the role of ABL kinases in the regulation of breast cancer bone metastasis.

3.3 Discussion

In this work, we uncovered a role for the ABL kinases in promoting breast cancer bone metastasis through the regulation of distinct pathways required for tumor colonization and survival in the bone microenvironment. We showed that ABL kinase activity was required for osteolytic metastasis of breast cancer cells and that depletion or pharmacological inhibition of these kinases impaired breast cancer metastasis to bone. We demonstrated that ABL kinases promoted tumor-induced osteolysis in part through the osteoclast-activating cytokine IL6, increased serum concentrations of which are associated with poor clinical outcome in breast cancer patients (146). IL6 can induce
osteoclast activation indirectly by altering the expression of RANKL and OPG in osteoblasts (147). Depletion of ABL kinases in breast cancer cells decreased IL6 concentrations and was accompanied by increased OPG expression in osteoblasts. We found that the addition of IL6 partially enabled medium conditioned by ABL1/ABL2 knockdown breast cancer cells to activate osteoclasts, and suppressed OPG expression in the 7F2 osteoblast cell line. These findings support a role for ABL-mediated IL6 secretion by breast cancer cells in osteoclast activation through decreased OPG expression in osteoblasts. IL6 has pleiotropic roles during tumor progression and metastasis that include increased tumor cell survival, expansion of breast cancer stem cells and resistance to targeted therapies (148). IL6 may also function downstream of the ABL kinases to protect breast cancer cells from TRAIL-induced cell death.

Depletion of ABL kinases in breast cancer cells also decreased the abundance of MMP1, a protease that cleaves fibrillar collagens and promotes the proteolytic release of bound growth factors (7). High serum concentrations of MMP1 correlate with bone metastasis in breast cancer patients (7). Through its various targets, MMP1 promotes not only tumor invasion but also breast cancer colonization to the bone by mechanisms that include the release of membrane-bound EGF-like growth factors from tumor cells, leading to activation of EGFR signaling and suppression of OPG expression in osteoblasts, which in turn promotes the differentiation and activation of osteoclasts required for bone destruction and enhanced tumor growth in the bone
microenvironment (7). Thus, decreased osteoclast activation and colonization of breast cancer cells lacking ABL kinases may be mediated in part by reduced MMP1 abundance.

Mechanistically, we found that STAT5 was required for the production of the secreted factors MMP1 IL6, and TNC downstream of ABL kinases. Similar to IL6 and MMP1, TNC is abundant in some breast tumors and promotes metastasis in mouse models (145). We showed that inactivation of the ABL kinases in breast cancer cells resulted in decreased expression of genes in the JAK/STAT and Cytokine/Cytokine Receptor pathway signatures, which may be due to decreased STAT5A mRNA expression as well as reduced STAT5 phosphorylation in ABL1/ABL2-depleted breast cancer cells. Expression of a constitutively active version of STAT5A in ABL1/ABL2 knockdown cells restored the production of secreted factors (IL6, MMP1, and TNC) and partially rescued the ability of breast cancer cells to promote bone metastasis. These findings support a role for the ABL kinase-STAT5 signaling axis in breast cancer metastasis.

In addition to inhibiting STAT5 signaling, we found that depletion of ABL kinases decreased the expression of the Hippo pathway mediator TAZ and downstream target genes in triple-negative and HER2+ breast cancer cells. Inactivation of ABL kinases inhibited expression of the TAZ target gene AXL, which shows increased expression in several human cancers and correlates with poor prognosis, increased invasiveness and metastasis, and enhanced drug resistance (149, 150). AXL mediates
resistance to TRAIL-induced cell death in esophageal adenocarcinoma (151); it remains to be determined whether ABL-mediated protection from TRAIL-induced apoptosis in breast cancer cells is mediated by AXL or other targets. Co-expression of both activated TAZ and STAT5 pathways was required to fully rescue the bone metastatic phenotype of ABL1/ABL2 knockdown breast cancer cells. These data, together with the finding that knockdown of both TAZ and STAT5 phenocopied the decrease in breast cancer cell metastasis caused by ABL1/ABL2 depletion, supports a model for a requirement of ABL-dependent TAZ and STAT5 signaling networks in promoting breast cancer metastasis (Fig. 30B).

Our data raise the possibility that inhibition of ABL kinases can increase apoptosis of breast cancer cells and block osteoclast activation that is required for osteolytic metastasis. Some Phase I and II trials have been carried out using non-selective ATP-competitive inhibitors such as imatinib to treat patients with advanced breast cancer. However, patients enrolled in these trials were not evaluated for ABL abundance and/or activation (152, 153). The allosteric inhibitors specific for ABL kinases (which are currently in clinical trials) provide a potentially useful tool for selectively targeting ABL kinases in metastatic breast cancer types with an increase in the ABL pathway signature (154). We found that allosteric inhibition of the ABL kinases effectively impaired breast cancer bone metastasis and blocked tumor-induced osteolysis in mouse models. Future studies will test and compare the efficacy of imatinib
and allosteric compounds in mouse models of breast cancer. We will also test whether ABL-directed therapy can shrink established bone metastasis in the mouse models. Together, our data suggest that clinical studies may be warranted to evaluate the therapeutic potential of ABL allosteric inhibitors and to determine whether combination therapies that incorporate these compounds are effective in treating metastatic breast cancer. It is also striking that multiple downstream targets of the ABL kinases in breast cancer cells (TAZ, AXL, STAT5 and IL6) have been implicated in therapy resistance. It remains to be determined whether activated ABL kinases play a role in the development of intrinsic and/or acquired resistance in breast cancer cells.
4. ABL kinases promote breast cancer tumorigenesis and chemoresistance.

The work in this chapter was done collaboratively with Ryan Overcash. Figures 32-38 were generated by Ryan Overcash.

4.1 Introduction

While we found that the growth of metastatic breast cancer cells derived from the MDA-MB-231 triple negative cells was not affected by inactivation of the ABL kinases in vitro, others have reported that proliferation and/or survival of some breast cancer cells requires the activity of one or both ABL kinases (116, 118). Thus, regulation of proliferation and/or survival by ABL kinases may be dependent on the cell context and the tumor microenvironment. Therefore we examined whether ABL kinases are required for tumorigenesis in vivo. We found that ABL kinases promote breast cancer tumorigenicity through regulation of the cancer stem cell (CSC) phenotypes (stemness), tumor angiogenesis, myeloid cell recruitment and epithelial-mesenchymal transition.

The epithelial-mesenchymal transition (EMT) is a developmental pathway that is often hijacked by cancer cells upon disease progression and leads to increased invasive and migratory capabilities (155). Additionally, it was demonstrated that the EMT program confers a stem-cell phenotype to breast cancer cells (155). The promotion of tumor stem-cell characteristics may be a major contributing factor to metastatic progression and tumor cell resistance to standard-of-care chemotherapies (155). Therefore, strategies to target molecular drivers of the EMT and/or CSC program, likely
in combination with other agents that cause significant disease regression, are a promising approach to treat breast cancer patients and to achieve durable responses.

We found that ABL2 drives EMT in weakly tumorigenic breast cancer cells and promotes the transition of epithelial cells into a stem-like and highly tumorigenic state. ABL2 activation results in increased expression of a plethora of proteins with known roles in EMT and the CSC phenotypes. Functional analyses of cells with elevated ABL2 activation demonstrate a potent increase in tumorigenicity both in vitro and in xenotransplants. Genetic knockdown of ABL kinases in metastatic breast cancer cells impairs tumor growth and myeloid cell recruitment to the tumor, a process promoted by soluble factors associated with EMT (156). Transcriptomic profiling of aggressive breast cancer cells with ABL kinase loss-of-function revealed inhibition of EMT and other invasive programs, supporting a key role for ABL kinases in cellular processes required for metastasis. Moreover, treatment of breast cancer cells with a specific allosteric ABL kinase inhibitor impairs mesenchymal and stem-like traits of aggressive breast cancer cells and reduces expression of EMT and CSC markers. Importantly, we also demonstrate that ABL2 activation induces a potent survival advantage in breast cancer cells that including resistance to anoikis and resistance to chemotherapy.

Intrinsic and acquired resistance to chemotherapies are major limitations to combating cancer (157). Targeted therapies against activated receptor tyrosine kinases (RTKs) in breast cancer have also shown limited efficacy due to intrinsic or acquired
resistance (110, 111, 158, 159). Several mechanisms underlie the emergence of therapy-resistant breast cancer, which often leads to metastasis. Cancer cells treated with tyrosine kinase inhibitors (TKIs) can acquire resistance by increasing ligand-induced RTK signaling through autocrine or paracrine production of growth factors (160). Alternatively, inhibitor-treated cells undergo dynamic genomic alterations that include reprogramming of the kinome (110, 111, 158). Thus, targeting common downstream signaling mediators of multiple RTKs might be an effective strategy for overcoming intrinsic and acquired resistance. We and others have shown that ABL family kinases are activated downstream of several RTKs implicated in breast cancer including EGFR, MET, PDGFR, and FGFR. ABL1 is also highly activated in tumors with enhanced levels of metabolic and oxidative stress, and inactivation of ABL1 alone and in combination with other agents elicits profound and sustained inhibition of renal tumors in vitro and in mice (95). In vitro studies support the ABL pathway as a potential therapeutic target for overcoming resistance in some breast cancer subtypes (104). Enhanced ABL1 expression was reported in breast cancer patients that have acquired resistance to aromatase inhibitor therapy (161). Here we found that triple negative breast cancer cells were sensitized to cell death by the EGFR kinase inhibitor gefitinib, as well as to the antimitotic agent docetaxel.
4.2 Results

4.2.1 ABL2 is upregulated in breast cancer tissue and induces mesenchymal and stem-like features of breast cancer cells.

Recent results from The Cancer Genome Atlas revealed that ABL2 is amplified in approximately 17% of invasive breast tumors, a frequency comparable to that of Her2/Neu in the same data set (Fig. 31). ABL1 was only upregulated/amplified in a much smaller amount of patient samples. The high frequency of amplification of ABL2 suggests that it may play a critical role in the establishment and/or progression of breast cancer. To evaluate the role of ABL2 in breast cancer cells, we expressed a constitutively activated form of ABL2 (ABL2PP) in weakly tumorigenic MCF10ATK.cl2 (MII) cells. Expression of activated ABL2 in MII cells induced a striking change in cell morphology from epithelial to a morphology that resembles mesenchymal cells (Fig. 32). MII cells that were enriched for the stem cell population by FACS sorting for the CD44\textsuperscript{Hi}/CD24\textsuperscript{Lo} population displayed mesenchymal morphology (Fig. 32). Recent evidence has emerged that the acquisition of mesenchymal traits through the epithelial-mesenchymal transition (EMT) confers stem-cell traits (155). Interestingly, although MII cells contain a distinct stem-cell population and a mature population based on FACS sorting, expression of activated ABL2 shifts the entire population to the CD44\textsuperscript{Hi}/CD24\textsuperscript{Lo} stem-cell population (Fig. 33). MII and MII-ABL2PP cells were analyzed for expression of a panel of proteins with established roles in both EMT and/or the cancer stem cells (CSC) state. Nearly all of the EMT-associated proteins were upregulated in MII-ABL2PP cells, while
the epithelial marker E-Cadherin was down-regulated (Fig. 34). These results suggest that ABL2 activity may play a critical role in the regulation of EMT and stemness in breast cancer cells.

All Complete Tumors (971 samples)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Frequency</th>
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<tbody>
<tr>
<td>ABL1</td>
<td>9%</td>
</tr>
<tr>
<td>ABL2</td>
<td>17%</td>
</tr>
<tr>
<td>ERBB2</td>
<td>19%</td>
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Figure 31: ABL2 is up-regulated in invasive breast cancer samples. Data from The Cancer Genome Atlas showing frequencies of ABL1 and ABL2 alterations compared to HER2 in invasive breast cancer patient samples.

Figure 32: ABL2 overexpression induces mesenchymal morphology. Bright-field microscopic images of MII cells expression activated ABL2 (Abl2PP) showing

CD24$^{\text{Hi}}$/CD44$^{\text{Lo}}$

CD24$^{\text{Lo}}$/CD44$^{\text{Hi}}$
mesenchymal morphology (top panel) and compared with MII cells that were FACS sorted to isolate the CD44HI/CD24Lo stem cell population (bottom panel).

**Figure 33:** ABL2 overexpression induces stem cell population. CD44/CD24 FACS profile of MII cells harboring the empty vector or expressing activated ABL2 (Abl2PP).

**Figure 34:** ABL2 overexpression promotes the protein abundance of EMT and cancer stemness markers. MII cells expressing active Abl2PP display enhanced levels of mesenchymal protein markers and decreased E-cadherin levels.
4.2.2 Expression of activated ABL2 promotes stemness and tumorigenic properties of MII breast cancer cells

We next evaluated the functional consequences of activated ABL2 expression in MII breast cancer cells by employing a series of cell-based functional assays. These assays were designed to gauge the properties of cells that undergo EMT and have increased stemness compared with differentiated epithelial cells. Epithelial cells typically undergo a process of programmed cell death called anoikis when not attached to a solid or semi-solid substratum (162), however, by undergoing EMT cells acquire the ability to resist anoikis and survive without attachment. As expected, MII cells harboring the empty vector readily die when plated in suspension, while cells expressing ABL2PP are highly resistant to cell death, with about 90% still viable at 48 hrs without attachment to any substratum (Fig. 35). MII-ABL2PP cells also demonstrated a substantial increase in the capacity for anchorage-independent growth compared with control MII cells (Fig. 36). To directly assess the stem-cell phenotype of MII cells with and without ABL2PP expression, we evaluated primary and secondary mammosphere formation and observed a marked increase in the number of mammospheres that formed in cells that express activated ABL2 (Fig. 37). Together, these results demonstrate a role for ABL2 in driving a stem-like, tumorigenic phenotype concomitant with regulation of EMT in breast cancer cells.
Figure 35: ABL2 overexpression increases the resistance to anoikis. Survival of MII and ABL2PP cells in suspension for the indicated time points.

Figure 36: ABL2 overexpression promotes breast cancer colony formation. Colony formation of MII cells harboring the empty vector or expressing activated ABL2 (Abl2PP). Colonies were counted in three randomly selected fields of views at a 10X magnification and data are expression as mean± S.E.
Figure 37: ABL2 overexpression promotes primary and secondary mammosphere formation. MII cells expressing ABL2PP or vector control were analyzed for mammosphere formation.

4.2.3 Activated ABL2 promotes tumor initiation capacity and promotes growth of orthotopic mammary tumors in vivo.

Cancer stem cells are characterized by a striking ability to form tumors in xenotransplant tumor initiation studies. In fact, cell populations with robust stem-like properties are capable of forming tumors when injected into immunocompromised mice with as few as 100-1,000 cells, while unsorted tumor cell populations require orders of magnitude higher numbers of cells to initiate a tumor. We next sought to assess the influence of ABL2 on tumor initiating capabilities of MII cells and tumor growth in vivo. Expression of activated ABL2 conferred enhanced tumor forming ability by MII cells following orthotopic injection into nude mice (Fig. 38A) and increased the size of tumors (Fig. 38B, C). Injection of limiting dilution of MII and MII-ABL2PP cells demonstrated a
substantial increase in tumor initiation capacity in cells expressing activated ABL2, with at least 50% of injected mice forming tumors when injected with as few as 10,000 cells (Fig. 38D). These results reveal that ABL2 promotes tumor initiation and growth in vivo and supports a role for ABL2 in the establishment/maintenance of a stem-like state.

Figure 38: ABL2 overexpression promotes tumor initiation. (A) Bioluminescent imaging (BLI) of nude mice 21 days following orthotopic injection of $1 \times 10^5$ MII cells labeled with pFU-LT and expressing luciferase. (B) Quantification of masses of tumors that were isolated from mice in (A). (C) Image comparing sizes of tumors isolated from mice injected with $1 \times 10^6$ of the indicated cells. (D) Table summarizing the number of mice that developed tumors following orthotopic injections of the indicated number of cells.

4.2.4 ABL family protein kinases are required for breast cancer tumorigenesis.

To evaluate whether ABL kinases are required for breast cancer tumorigenesis, we depleted ABL1 and ABL2 (ABL1/ABL2) in a highly metastatic breast cancer cell line 1833, which is derived from triple negative breast cancer cell line MDA-MB-231. We
then evaluated the tumorigenic potential of these cells following subcutaneous injection into immune-deficient mice. We found that knockdown of the ABL kinases markedly inhibited tumorigenesis measured by both tumor size and tumor weight (Fig. 39, A, B and C). Decreased tumorigenesis by ABL-deficient breast cancer cells was accompanied by a significant reduction in tumor angiogenesis indicated by the decreased CD31+ staining in ABL1/ABL2 knockdown cells (Fig. 39D). 1833 cells were engineered to express green fluorescent protein (GFP). Flow cytometry analysis indicated that loss of ABL kinases decreased myeloid cells (CD11b+; GFP-) recruitment in the tumors (Fig. 39E). To gain insight into the signaling pathway(s) required for ABL1/ABL2-dependent tumorigenesis, we evaluated the consequences of loss of AB1/ABL2 on the transcriptome of breast cancer cells using next generation sequencing, and we found that Epithelial to Mesenchymal Transition (EMT) was one of the top biological processes affected by ABL1/ABL2 knockdown (Fig. 39F). Notably, it has been shown that EMT programs trigger the expression of soluble mediators in cancer cells that stimulate angiogenesis and myeloid cells recruitment. These findings reveal a function for ABL kinases in promoting breast cancer tumorigenesis, tumor angiogenesis and myeloid cells recruitment in vivo.
Figure 39: ABL family protein kinases are required for breast cancer tumorigenesis. (A) Representative images of the tumors isolated from mice injected with control (Scr) or ABL1/ABL2 knockdown (AA) 1833 cells. (B-C) Volume (B) and weight (C) of the tumors isolated from mice injected with control (Scr) or ABL1/ABL2 knockdown (AA) 1833 cells. (D) IHC staining of tumor sections using CD31 antibody. (E) Flow cytometry analysis of CD11b+, GFP- myeloid cells abundance in the tumors isolated from the mice. NC is negative control. (F) Cellular processed most affected by ABL1/ABL2 knockdown using Genego software.
4.2.5 Allosteric inhibitor of ABL kinases decreases mesenchymal markers and tumorigenesis.

ABL kinases are well-established therapy targets for the treatment of leukemia and there are many FDA approved ABL kinases inhibitors in the clinic such as imatinib, nilotinib and dasatinib. However, these inhibitors inhibit multiple tyrosine kinases in addition to ABL1 and ABL2 (154). Moreover, these ATP-competitive inhibitors induce the formation of B-RAF and C-RAF dimers, leading to ERK activation in diverse cancer cell types (112). Therefore, we used the allosteric inhibitor GNF5, which targets the unique ABL myristate-binding site of the ABL kinases. We found that GNF5 treatment led to a significant dose-dependent decrease in TGFβ-induced Snail protein levels, while induction of Slug protein expression was modestly inhibited by GNF5 treatment in MCF10A cells (Fig. 40A). GNF5 treatment resulted in dose-dependent inhibition of TGFβ-induced Snail mRNA expression and, to a lesser extent, decreased Slug mRNA levels (Fig. 40, B and C). Snail and Slug are EMT transcription factors that regulate distinct tumor initiating and mammary repopulation stem-cell programs in mammary epithelia (163). ABL kinase inhibition with GNF5 also markedly impaired colony and mammosphere formation capabilities of aggressive MCF10ACA1a (MIV) cells, a metastatic derivative of MII cells with high stem-cell potential (Fig. 40, D and E). In addition, treatment of tumor-bearing mice with GNF5 following subcutaneous injection of metastatic 1833 cells resulted in a significant decrease in tumor growth (Fig. 40, F and
These results demonstrate that ABL kinase activity is required for tumorigenesis and the expression of key EMT regulators Snail and Slug.

**Figure 40: Allosteric inhibitor of ABL kinases decreases mesenchymal markers and tumorigenesis.** (A) Confluent MCF-10A cells were treated with 5ng/mL recombinant human TGFβ1 for 6 hours in the presence of the indicated concentrations of the ABL kinase inhibitor GNF5 (UT = untreated). Levels of Snail and Slug proteins in total cell lysates were assessed; results are quantified in the panels at right. Values shown are mean protein levels +/- SEM (n=4) and are expressed relative to levels in cells treated with TGFβ (without GNF5 treatment). (B-C) Confluent MCF-10A cells were treated with rh-TGFβ1 (5ng/mL, 6 hours) +/- GNF5 prior to RNA isolation. Levels of Snail (B) and Slug (C) mRNA expression were quantified by real-time RT-PCR; values shown are means +/- SEM (n=3) and are expressed relative to mRNA levels in cells treated with TGFβ (without GNF5 treatment). *P<0.05; ***P<0.001 (one-way ANOVA, with
Bonferroni post-tests). (D) MIV cells were plated on agar plates and treated with DMSO control or GNF5 for 21 days and colony formation was evaluated. (E) MIV cells were treated with DMSO control or GNF5 and mammosphere formation was evaluated. (F) Representative images of the tumors isolated from tumor bearing mice treated with DMSO control (DMSO) or GNF5 (80mg/kg, twice a day). (G). Quantification of tumor volumes of control (DMSO) or GNF5 treated mice; values shown are means +/- SEM (n=8 mice). *P<0.05

4.2.6 ABL kinases inhibitor sensitizes breast cancer cells to chemotherapy.

Acquisition of breast cancer resistance to chemotherapy has been linked to the induction of the epithelial-to-mesenchymal transition (EMT). The acquisition of EMT phenotypes is regulated by a network of transcription factors including Snail (164) (165, 166). Some breast cancer cells that undergo EMT acquire properties characteristic of tumor initiating cells (TICs) and become chemoresistant (165, 167). In turn, EMT and stem-like phenotypes may be induced by chemotherapy and tyrosine kinases inhibitor (TKI) treatment leading to therapy resistance (168-172). We found that Snail and TAZ protein levels were markedly down-regulated by treatment with the ABL allosteric inhibitor GNF5 in tumorigenic MIV mammary epithelial cells. Therefore we evaluated whether ABL kinases inhibitors can sensitize breast cancer cells to chemotherapies and TKI. We found that the combination treatment of GNF5 and Docetaxel achieved a more significant inhibition of tumor cells growth in vitro and in vivo than single treatment of GNF5 or Docetaxel (Fig. 41 and 42). In addition, treatment of GNF5 or ABL kinases
knockdown made breast cancer cells more sensitive to the EGFR inhibitor gefitinib (Fig. 43 and 44).

**Figure 41:** Combination treatment of GNF5 and Docetaxel in vitro. Cell-titer Glo quantification of the *in vitro* growth of the 1833 cells treated with DMSO control; GNF5 single treatment (10uM); Docetaxel (5nM); or GNF5 and Docetaxel double treatment (Combination).

**Figure 42:** Combination treatment of GNF5 and Docetaxel in vivo. Quantification of xenograft (1833) from mice treated with DMSO control; GNF5 single treatment (50mg/kg twice a day); Docetaxel (10mg/kg twice a week); or GNF5 and
Docetaxel double treatment (Com); values shown are means +/- SEM (n=8 mice). *P<0.05; **P<0.01; ***P<0.001 (one-way ANOVA, with Bonferroni post-tests).

**Figure 43:** ABL kinases knockdown sensitizes breast cancer cells to gefitinib treatment. Cell-titer Glo quantification of the *in vitro* growth of the control 1833 cells (Scr) or ABL kinases knockdown cells (AA) treated with DMSO control or GNF5 (10uM).

**Figure 44:** ABL kinases inhibition sensitizes breast cancer cells to gefitinib treatment. Cell-titer Glo quantification of the *in vitro* growth of the 1833 cells treated with DMSO control; GNF5 single treatment (10uM); Gefitinib (10uM); or GNF5 and Gefitinib double treatment; values shown are means +/- SEM (n=3).
In this study, we uncovered a novel role of ABL kinases, especially ABL2, for the regulation of breast tumor progression by promoting cancer stemness and epithelial to mesenchymal transition. Expression of activated ABL2 in weakly tumorigenic breast cancer cells induces a striking change in morphology with enhanced mesenchymal features and increased tumorigenicity, accompanied by elevated expression of EMT and CSC markers including Snail, Zeb1, and CD44. Moreover, ABL kinase depletion or pharmacological inhibition in metastatic breast cancer cells suppressed tumorigenicity and decreased expression of mesenchymal markers. Consistent with a role in promoting mesenchymal and stem-like features, we demonstrate that ABL2 promotes tumor cell resistance to standard-of-care chemotherapies. Together our findings support a pro-tumorigenic role for ABL2 in breast cancer progression, and suggest that ABL-specific inhibitors might be developed in combination chemotherapy for the treatment of breast tumor types with hyperactive ABL signaling.
5. Concluding remarks and future directions

5.1 Summary

Metastases account for the vast majority of deaths due to breast cancer. In particular, bone metastases are a devastating complication associated with advanced breast cancer leading to morbidity and mortality (8). Breast cancer patients with bone metastases often experience severe pain, hypercalcemia, and bone fracture (8). Development of bone metastases requires reciprocal interactions between tumor cells and stromal cells in the bone microenvironment (BME) (120). Tumor cells disrupt the homeostatic balance of bone formation and remodeling maintained by osteoclasts and osteoblasts. Breast cancer bone metastases are characterized by osteolytic lesions, associated with increased bone turnover induced by tumor-derived osteoclast-activating factors. Currently, there are no therapies to cure breast cancer metastasis, and few effective therapies are available for treating bone metastasis. Thus, there is a need to identify molecules that could be targeted to disrupt the vicious cycle of tumor-stromal cell interactions driving bone metastasis.

We have uncovered novel signaling networks downstream of the ABL family kinases, ABL1 and ABL2, which are required to promote breast cancer metastasis to the bone. ABL kinases are druggable targets that have been well studied in the context of human leukemias (14). However, increased ABL tyrosine phosphorylation, indicative of kinase activation, has been detected in human tumors (14). In this regard, enhanced
ABL1 activity has been showed to be required for the growth of human renal cell carcinoma, which are glycolytically-dependent and oxidatively-stressed tumors (95). However, the identity of the upstream signals promoting activation of ABL kinases and their role in the initiation and progression of breast and other solid tumors are poorly understood. We have recently found that high-level ABL1 and ABL2 expression correlates with enhanced breast cancer metastasis and decreased metastasis-free survival. Using metastasis models that bypass invasion and intravasation, we uncovered novel roles for the ABL kinases in the regulation of breast cancer cell survival and colonization in the bone microenvironment. Further, we identified a previously unknown role for ABL kinases in the expression of multiple pro-bone-metastasis genes and transcriptional regulation of pathways required breast cancer metastasis to the bone. Importantly, treatment with an ABL-selective allosteric inhibitor or depletion of both ABL kinases in breast cancer cells impaired bone metastases and decreased osteoclast activation in vitro and osteolysis in vivo. Our findings have far-reaching implications for the treatment of metastatic breast cancer. We have revealed novel signaling networks critical for the emergence of metastatic and therapy-resistant breast cancer. These studies will expand the use of available ABL allosteric inhibitors (now in clinical trials for leukemia) to sensitize breast cancer subtypes to chemotherapies and/or targeted therapies, thereby generating novel drug combinations for the treatment of metastatic, therapy-refractory breast cancer.
5.2 Future directions

5.2.1 Can ABL inhibitors induce regression of established metastases?

We have demonstrated that ABL kinases inhibitor can decrease tumorigenesis and bone metastasis when the treatment was initiated shortly after tumor inoculation. However upon diagnosis in the clinic, many patients have already established micrometastases or even late stage metastases. Therefore whether the use of ABL inhibitors can reverse established metastases becomes a more clinically relevant question. In order to evaluate whether targeting ABL kinases might be effective for the treatment of established breast cancer bone metastasis, future studies will evaluate the effect of temporal inactivation of the ABL kinases with pharmacological inhibitors, or by doxycycline-inducible knockdown of ABL kinases (2-4 weeks after tumor inoculation) following formation of initial metastasis.

Future studies should evaluate whether the breast tumors expressing ABL activation signatures such as the signature we identified in 1833 triple negative breast cancer cells (Fig. 30) can be used to select patients that might benefit from treatment with ABL allosteric inhibitors likely in combination with other agents. Notably, clinical trials that have used imatinib to treat breast cancer patients have not selected for ABL1 and ABL2 alterations (Appendix A and B).
5.2.2 Does ABL inhibition affect the breast cancer metastasis to organs other than bone?

In this study, we found that ABL inhibition significantly reduces breast cancer bone metastasis using bone metastatic cell lines. In contrast, we did not find a significant effect of ABL inhibition on lung metastasis using lung metastatic cell line 4175. However, this finding does not rule out the possibility that ABL kinases regulate breast cancer metastasis to brain and other organs, even though we showed that ABL kinases play an important role in bone metastasis.

Bone is a unique and fertile environment for cancer metastasis. The blood vessels in bone marrow are more permeable than vessels in brain or lung, which makes it easier for tumor cells to extravasate into bone. Further, there are high levels of growth factors stored in the bone matrix that can be released to promote tumor growth upon bone degradation. Additionally, the interaction of unique cell populations, such as osteoclast, with tumor cells can further enhance tumor growth. We found that ABL kinases are critical for the interaction between tumor cells and the bone microenvironment and thus are important targets for bone metastasis. In addition, in a small clinical dataset with organ specific metastasis information (124), there was a significant correlation between high ABL1 expression and increased bone metastasis. In contrast we did not find a significant correlation between ABL expression and lung or brain metastasis.
In order to conclude that ABL kinases are required for bone-specific metastasis, future studies in a larger set of organ-specific metastatic cells are needed to test whether ABL kinases are involved in metastasis to lung, brain and other organs. Future experiments should examine whether overexpression of active ABL kinases in weakly metastatic cell lines can promote metastasis to various organs.

5.2.3 Potential role of ABL kinases in mammary tumor progression and metastasis using transgenic mouse model

To further validate the role of ABL kinases in breast cancer, we may use transgenic mouse models with either gain of function or loss of function of ABL kinases. Our laboratory has recently generated knock-in mice harboring a kinase-active Abl2-P269E/P276E allele to assess the consequences of activating ABL2 on tumor progression and metastasis. Moreover, we have generated knock-in mice harboring kinase-defective Abl2 for temporal and tissue-specific inactivation of the ABL kinases. Future studies will use inducible breast cancer mouse models, such as the inducible TetO-PyMT-IRES-Cre model (173), to study the role of ABL kinases in various steps of tumor progression and metastasis. An advantage of the model is that it couples the expression of PyMT oncogene to that of Cre-recombinase, thereby inducing tumors and inactivating ABL kinases in the same mammary tumor cells. Moreover, this model allows for temporal regulation of tumor induction as well as genetic inactivation of the floxed Abl alleles. Mice expressing TetO-PyMT-IRES-Cre can be crossed to the MMTV-
rtTA strain, and administration of doxycycline induces formation of mammary tumors as early as 4 days post-induction.

One limitation of using transgenic mouse models is that to date transgenic breast cancer models fail to form efficient bone metastasis. However in the clinic, more than 70% of advanced breast cancer patients develop bone metastases. Additional studies are needed to better characterize the biology of bone metastasis and to generate breast cancer mouse models than can better mimic human disease progression. One possibility is to promote inflammation to increase metastasis to bone in mammary tumor models (174).

5.2.4 Do the ABL kinases regulate the tumor microenvironment?

In our study, we focused primarily on studying the role of ABL kinases in tumor cells. It will be interesting to further characterize the role of ABL kinases in tumor stromal cells. Notably, previous studies in our lab have identified multiple roles of ABL kinases in various stromal cells populations including endothelial cells, myeloid cells, fibroblasts and immune cells (40, 45, 46, 175). We can use tissue specific knockout mice as recipient mice and transplant tumor cells or patient derived xenografts into the recipient mice and compare tumor growth and metastasis in mice lacking ABL kinases in different stromal cell populations.
5.2.5 More mechanism studies to better characterize the signaling networks regulation by ABL kinases

In addition to the functional studies discussed in the earlier sections, additional mechanistic studies are needed to better characterize the signaling networks regulated by ABL kinases. Remaining questions include but are not limited to: (1) Why is the regulation of TAZ more dependent on ABL2 compared to ABL1? (2) How does ABL2 regulate the expression of TAZ? (3) Do ABL kinases phosphorylate STAT5 directly or indirectly? (4) Does the ABL-dependent regulation of STAT5A and STAT5B have different roles in breast cancer progression? (5) Are there links between TAZ pathways and STAT5 pathways? (6) How do ABL kinases regulate EMT pathways?
## Appendix A

Clinical trials using ABL inhibitors for breast cancer

<table>
<thead>
<tr>
<th>#</th>
<th>Status</th>
<th>Study Description</th>
<th>Condition</th>
<th>Interventions</th>
<th>Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Completed</td>
<td>Docetaxel and Imatinib Mesylate in Treating Patients With Locally Advanced or Metastatic Breast Cancer</td>
<td>Breast Cancer</td>
<td>Drug: docetaxel; Drug: Imatinib mesylate</td>
<td>Phase I, ?</td>
</tr>
<tr>
<td>2</td>
<td>Active, not recruiting</td>
<td>Letrozole (Femara) Plus Imatinib Mesylate (Gleevec) for Breast Cancer Patients</td>
<td>Breast Cancer</td>
<td>Drug: Imatinib Mesylate; Drug: Letrozole</td>
<td>Phase II c-Kit or PDGFR+</td>
</tr>
<tr>
<td>3</td>
<td>Completed</td>
<td>Imatinib Mesylate in Treating Patients With Metastatic Breast Cancer</td>
<td>Male Breast Cancer; Recurrent Breast Cancer; Stage IV Breast Cancer</td>
<td>Drug: Imatinib mesylate; Other: laboratory biomarker analysis</td>
<td>Phase II PDGFR+ patients</td>
</tr>
<tr>
<td>4</td>
<td>Completed Has Results</td>
<td>Docetaxel Plus Imatinib Mesylate in Metastatic Breast Cancer</td>
<td>Breast Cancer</td>
<td>Drug: Imatinib; Drug: Docetaxel</td>
<td>Phase II Unselected</td>
</tr>
<tr>
<td>5</td>
<td>Completed Has Results</td>
<td>S0338, Imatinib Mesylate and Capecitabine in Treating Women With Progressive Stage IV Breast Cancer</td>
<td>Breast Cancer</td>
<td>Drug: Capecitabine; Drug: Imatinib mesylate</td>
<td>Phase II Unselected</td>
</tr>
<tr>
<td>6</td>
<td>Active, not recruiting</td>
<td>Gemcitabine + Imatinib Mesylate, Patients w/Previously Treated Metastatic Breast Cancer</td>
<td>Breast Cancer</td>
<td>Drug: gemcitabine hydrochloride; Drug: Imatinib mesylate</td>
<td>Phase IV c-Kit or PDGFR+, ?</td>
</tr>
<tr>
<td>7</td>
<td>Completed</td>
<td>Efficacy and Safety of Imatinib and Vinorelbine in Patients With Advanced Breast Cancer</td>
<td>Breast Cancer</td>
<td>Drug: Imatinib and Vinorelbine</td>
<td>Phase IV c-Kit or PDGFR+, ?</td>
</tr>
</tbody>
</table>
Appendix B

The expression of ABL, PDGFR, and c-Kit in breast cancer patient samples

All Complete Tumors (971 samples), TCGA 2015

<table>
<thead>
<tr>
<th>Gene</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABL1</td>
<td>9%</td>
</tr>
<tr>
<td>ABL2</td>
<td>17%</td>
</tr>
<tr>
<td>PDGFR A</td>
<td>5%</td>
</tr>
<tr>
<td>PDGFR B</td>
<td>5%</td>
</tr>
<tr>
<td>c-Kit</td>
<td>5%</td>
</tr>
</tbody>
</table>
References


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Biography

Jun Wang was born in Dalian China. She has a B.S. degree from Nankai University China and is expected to get her Ph.D. degree in Pharmacology & Cancer Biology from Duke University May 2016. Jun was awarded Duke Cancer Institute Scientific Retreat Featured Presenter Award, Duke University Chancellor’s Scholarship, and Duke Scholar in Molecular Medicine. She has published the following papers:


