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The Src- and Abl- Dependent Regulation of Net1A in Breast Cancer

Ashabari Sprenger

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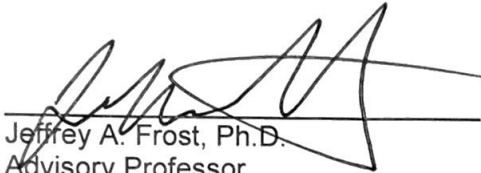
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The Src- and Abl- Dependent Regulation of Net1A in Breast Cancer

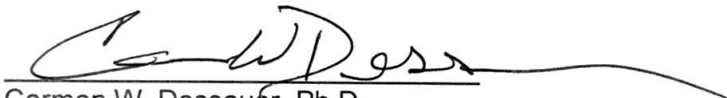
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The Src- and Abl- Dependent Regulation of Net1A in Breast Cancer

A

Dissertation

Presented to the Faculty of

The University of Texas

MD Anderson Cancer Center UTHealth

Graduate School of Biomedical Sciences

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

by

Ashabari Sprenger, B.S.

Houston, Texas

December 2021

Dedication

To my grandparents, Dr. Bharatendu Dev Sharma and Dr. Dipika Dev Sharma.

Acknowledgements

I would like to firstly thank my advisor, Dr. Jeff Frost, for his guidance, mentorship, and support through my training. Pursuing a PhD seems to be equal parts learning the science and learning resilience, and he has been a great resource for both. He has always been so patient through failed experiments and has spent many hours teaching me how to critically think about my hypotheses and design sound experiments. His training has been invaluable, and I will always be grateful for his mentorship. I would also like to thank my advisory committee, Dr. Carmen Dessauer, Dr. Jeffrey Chang, Dr. Swathi Arur, and Dr. Joya Chandra, for their guidance. I am very grateful they cared about me, and my project, enough to never shy away from asking tough questions. They pushed me to be better, and I will always appreciate that. I was so very lucky to have such excellent mentorship and training in graduate school.

I would also like to thank the MD Anderson UTHealth Graduate School of Biomedical Sciences faculty, staff, and Dean, for their support. Dr. Michael Blackburn: thank you for all that you do for our school and students. Dr. Bill Mattox: thank you for being there when students need someone to lean on for support. Dr. Barton: your work here was incredible, and I feel lucky to have had a fellow Illini welcome me to Houston. Thank you, Cheryl Spitzenberger and Dr. Natalie Sirisaengtaksin, for all your help and for always answering my questions with a smile.

I would like to thank the IBP department for creating an environment where fantastic research was a result of great, collaborative faculty and staff. Specifically, I'd like to thank my lab members, Dr. Yan (Susan) Zuo, Dina Montafur-Solis, Dr. Heather Carr, and (previous) Dr. Arzu Ulu. Thank you to Arzu and Susan for teaching me biochemistry from scratch. Your patience through failed IPs and IF staining was greatly appreciated. Dina, thank you for making the lab feel like a family. Your kindness and positivity made the late nights easier.

Heather, I sometimes doubt if I ever would have finished some of these experiments without your encyclopedic troubleshooting knowledge. You have been an amazing mentor at the bench and a great friend. Thank you all for shaping my last five years here.

We are so privileged to have an amazing BCB program which supports students. I'd like to thank Dr. Rebecca Berdeaux, Dr. Ilya Levental, and Amanda Williamson for shaping this program, and for facilitating amazing faculty and student involvement. Some of my favorite graduate school memories are our BCB retreats, and I was so honored to serve on student council with an amazing bunch! I'd also like to thank my BCB friends, and my friends who belong to less cool programs, for their friendship and support. Many have graduated, and some are not far behind! These friends have helped me troubleshoot experiments, reviewed grant proposals, sat through countless practice talks. They have also become some of my favorite lunch dates, my coffee and marg go-tos, and closest friends in life. Thank you, Dr. Tanya Baldwin, Dr. Samantha Berkey, Dr. Kelsey Maxwell-Acosta, Dr. Max Odem, Dr. Natalie Sirisaengtaksin, Dr. Autumn Marsden, Dr. Yasmin Chebaro, Dr. Brittany Jewell, Dr. Elia Lopez, Dr. Iman Sahnoune, Dr. Walaa Kattan, Dr. Ryan Durham, Rachel Tay and (soon to be Drs!!) Janani Subramanian, Vinay Nair, Tristen Tellman, Dominik Awad, Ileana Kretzer, and Alexis Mobley.

Most Importantly, I would like to thank my family. To my grandparents: Dr. Bharatendu Dev Sharma and Dr. Dipika Dev Sharma, for always telling me stories of their own research. They were so right in the amount of tenacity research takes and in the unparalleled joy successful experiments bring. To my parents, Subhasis and Lyra Mukherjee, for their unconditional love and support. Thank you for all the sacrifices you made to make this life possible and for instilling in me the value of education. My baby sister, Malabi, thank you for keeping me youthful. I'm proud of you and can't wait to see where life takes you. Thank you to my "American grandparents" Isabel Hansen, Gail, and Paul Rung for supporting me and

instilling in me a love of learning. Education is so much more than what we learn in classrooms, and I learned so much from you. To my husband, Brandon, thank you for being my life partner. You are my rock, my love, and my joy.

The Src- and Abl- Dependent Regulation of Net1A in Breast Cancer

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Abstract

The neuroepithelial cell transforming gene 1 (Net1) is a RhoA guanine nucleotide exchange factor that promotes cancer cell motility and metastasis. Two isoforms of Net1 exist, Net1 and Net1A, both of which are sequestered in the nucleus in quiescent cells to prevent aberrant RhoA activation. Many cell motility stimuli drive cytosolic relocalization of Net1A, but mechanisms controlling this event are not fully understood. Here we demonstrate that EGF stimulates Src- and Abl1-dependent phosphorylation of Net1A to promote its cytosolic localization. We find that Abl1 efficiently phosphorylates Net1A on Y373, and that phenylalanine substitution of Y373 prevents Net1A cytosolic localization. Aspartate substitution at Y373 is sufficient to promote Net1A cytosolic accumulation, and expression of Net1A Y373D potentiates EGF-stimulated RhoA activation, Myosin Light Chain 2 phosphorylation, and F-actin accumulation. Expression of Net1A Y373D in breast cancer cells significantly increases cell motility and Matrigel invasion. Moreover, Net1A is required for Abl1-stimulated cell motility, which is rescued by expression of Net1A Y373D, but not Net1A Y373F. This work demonstrates a novel mechanism controlling Net1A subcellular localization to regulate RhoA-dependent cell motility and invasion.

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Chapter 1: Introduction

1.1 The Role of Rho Family GTPases in Breast Cancer

1.1.1 Molecular Subtypes of Breast Cancer

1.1.1.1 Breast Cancer Subtype

Breast cancer is the most prevalent form of cancer specific to women and remains a leading cause of cancer death in the United States. Breast cancer is defined as the uncontrolled growth of breast tissue, usually causing a lump or a mass. The cells that proliferate generally originate from either the lobule or the duct connecting the lobule to the nipple. In a vast majority of cases, the primary cause of breast cancer death is not from the tumor itself, but from complications that arise when the cancer metastasizes to distant sites.

Breast cancer is classified by stage of invasiveness and histologic subtype. Molecular subtyping can be determined through gene expression analysis but is less common and cost prohibitive. Ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS) were the two originally identified types of breast cancer and refer to the tissue of origin. LCIS has more recently been associated with benign disease, but DCIS is more likely to become invasive and deadly. A tumor biopsy can be histologically stained for molecular markers of hormone receptor expression (estrogen receptor [ER], progesterone receptor [PR]) and growth factor receptor expression (human epidermal growth factor [HER2]). The four major breast cancer subtypes are Luminal A (ER+/PR+/HER2-), Luminal B (ER+/PR+/HER2+), Basal/Triple Negative Breast Cancer (TNBC) (ER-/PR-/HER2-), and HER2- enriched (ER-/PR-/HER2+). Using diagnostic imaging, usually a chest x-ray, CT scan, or possibly an MRI, the tumor is graded. Factors that guide grading include size, invasiveness, lymph node involvement, and metastasis to distant sites. Patients who have early-stage cancers, stage 0, I, and II typically undergo breast conserving surgery, or mastectomy if there is lymph node involvement. They may also receive adjuvant hormone therapy, chemotherapy, or radiation depending on their individual disease. Patients with Luminal A type breast cancers tend more responsive to

hormonal therapies than Luminal B. First line chemotherapy in pre-menopausal, Luminal subtype patients includes tamoxifen, a selective estrogen receptor modulator that blocks ER signaling. Post-menopausal women may receive aromatase inhibitors to lower levels of circulating estrogen. Basal/TNBC patients have poorer prognoses because they tend to have more metastatic disease and efficient targeted therapies have not yet been developed. Patients with metastatic stage III and stage IV breast tumors tend to require chemotherapy or targeted therapy. Common first line chemotherapeutic agents include anthracyclines, like doxorubicin, which intercalates into DNA and prevents topoisomerase activity. Taxanes, like paclitaxel, are also common and disrupt microtubule function during cell division. Chemotherapy is very cytotoxic and can have many severe side effects, due to their inhibition of the growth of any rapidly dividing cell, not just tumor cells. This results in patients having hair loss, GI complications, and brain fog. Finally, palliative therapies may be introduced in stage IV patients, including drugs to relieve symptoms of metastatic disease, such as pain medications or anti-emetics. The HER2-enriched subtype has historically had the worst prognosis; however, developments in targeted therapies for HER2+ tumors with trastuzumab have drastically improved overall survival rates by 37% in the last ten years (Perez et al., 2014). Unfortunately, no such extensive advances have been made in targeting triple negative breast tumors. (American Cancer Society, 2019). Finally, Inflammatory breast cancer (IBC) is the rarest and most aggressive form of invasive breast cancer, characterized by its rapid onset of symptoms and progression. It's often classified as being mixed in hormone receptor expression but can most often be classified as basal-like with amplified HER2 expression (Robertson et al., 2010).

1.1.1.2 Clinical Outcomes

The prognosis of breast cancer has improved dramatically over the last fifty years, due to advances in screening, detection, and development of targeted therapies. The prognosis

for early stage, localized breast cancer is currently quite promising, with a 5-year survival rate of 99%. This drops slightly in stage II and stage III cancers, where there is lymph involvement, but remains promising at a 5-year survival rate of 86%. The driving cause of death in breast cancer patients today is metastatic spread of the disease. Patients with stage IV breast cancer who have distant metastases have a far worse prognosis, with a 5-year survival rate of only 27%. For this reason, much of the work in the breast cancer field is focused on better understanding mechanisms of metastasis. The hope is that ongoing research will soon lead to the development of improved diagnostic tools and targeted therapies to improve the prognosis of patients with metastatic disease.

1.1.2 Contributions of Select Rho Family GTPases to breast cancer cell migration, invasion, tumorigenesis, and metastasis

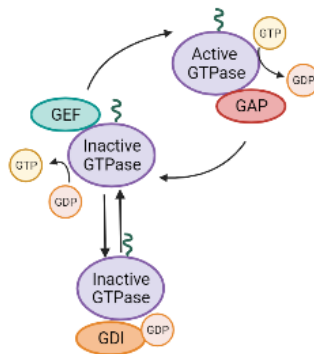


Figure 1. The Rho GTPase Cycle. Rho GTPases are active when bound to GTP (yellow) and inactive when bound to GDP (orange). Inactive Rho GTPases can be maintained inactive by guanine nucleotide dissociation inhibitors (GDI) association or activated by guanine nucleotide exchange factors (GEFs) that catalyze the exchange of GDP to GTP. GTPase activating proteins (GAPs) stimulate intrinsic GTPase activity, reverting the GTPase to its inactive, GDP-bound form.

Rho family GTPases are a subset of the Ras superfamily and are a highly conserved protein family found in nearly all eukaryotes. The primary role of these proteins is to control actin cytoskeletal organization in cells, contributing to processes such as cell cycle progression, morphogenesis, and migration (Jaffe & Hall, 2005). In humans, the Rho GTPase family is made of 20 proteins that are divided into 8 subfamilies (Haga & Ridley, 2016): (1) Rho, (2) Rnd, (3) RhoF/RhoD, (4) Rac/RhoG, (5) Cdc42/RhoQ/RhoJ, (6) RhoV/RhoU, (7) RhoH, (8) RhoBTB. Subfamilies 1, 3, 4, and 5 are considered typical because they act as molecular switches- cycling between the active, GTP-bound form and the inactive, GDP-bound form (**Fig. 1**). Additionally, they can be negatively regulated through interactions with guanine nucleotide dissociation inhibitors (GDIs) (Olofsson, 1999). On the other hand, atypical subfamilies are GTP-bound and are not regulated by guanine exchange proteins (GEFs) or GTPase activating proteins (GAPs) (Aspenström et al., 2007). Many aspects of GTPase signaling are often deregulated in metastatic breast cancer, including overexpression of GTPases and overexpression of their regulators, GEFs or GAPs. This body of work focuses on the contributions of Neuroepithelial Cell Transforming 1A (Net1A) to breast cancer, and since Net1A is a Rho GEF, its contribution to cell signaling is entirely through typical GTPases. Thus, a brief overview of the roles of select typical Rho family GTPases RhoA, RhoB, RhoC, Rac1, and Cdc42 is necessary to understand how Net1A contributes to breast cancer cell migration, invasion, tumorigenesis, and metastasis.

Coordinated GTPase activation facilitates efficient cell movement

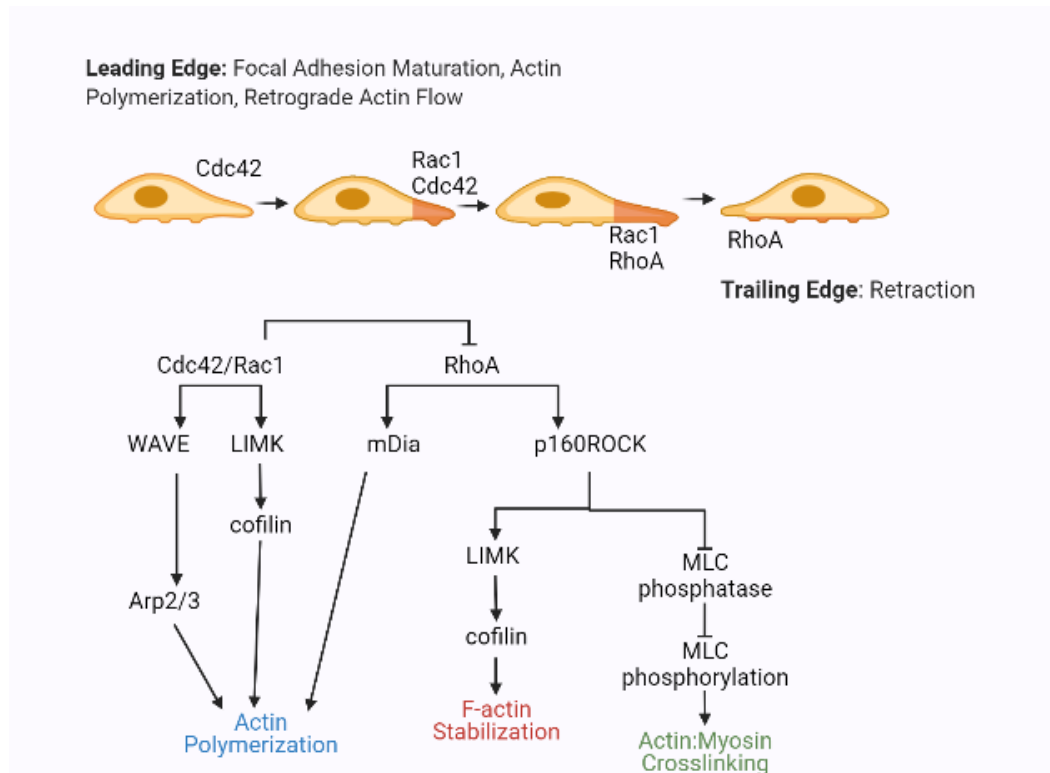


Figure 2. Coordinated GTPase activation regulates Cell Motility. Localized activation of RhoA, Rac1, and Cdc42 are required to regulate actin polymerization, F-actin stabilization, and actin: myosin crosslinking to regulate membrane protrusion, adhesion, and cell motility.

Mesenchymal cell motility requires (1) the establishment of a specific direction of movement, (2) extensions of the plasma membrane into the region of migration, (3) the formation of focal adhesions, (4) contraction, (5) Dissolution of previous adhesions and tail retraction (Raftopoulou & Hall, 2004).

The directional orientation of movement is regulated by the extracellular matrix (ECM) and extracellular stimuli by chemoattractants, such as growth factor ligands (Ware et al., 1998). At the leading edge of a moving cell, there are four different types of membrane

protrusions: lamellipodia, filopodia, blebs, and invadopodia. Lamellipodia and filopodia extend through the extracellular matrix; blebbing helps guide directional migration, especially during development; and invadopodia facilitate invasion through tissue by enhancing degradation of the ECM at focal adhesions. All four of these types of protrusions are characterized by carefully regulated actin polymerization – which is regulated by GTPases and several effector proteins (Ridley, 2011). The Rho GTPase Cdc42 controls cell polarity and the direction of migration in response to motility stimuli (Etienne-Manneville, Sandrine; Hall 2002). Early in migration, Cdc42 and Rac1 regulate actin polymerization through LIM Kinase and Arp2/3. LIM Kinase phosphorylates and inactivates cofilin, a protein which binds to actin to promote depolymerization. Cdc42 activity is required for the formation of filopodia as the cell begins extending its plasma membrane into a specific direction of movement. Rac1 activates Arp 2/3 to promote branching of actin filaments the leading edge, which facilitates a fanned actin formation to push the leading edge forward. Additionally, the Rac1 -dependent activation of the WASP-family verprolin homologous (WAVE) complex promotes actin polymerization and the formation of lamellipodia and membrane ruffles early in motility (Ridley, 2006).

The formation of stable actin filaments in plasma membrane protrusions is carefully regulated by RhoA activity. Monomeric Globular actin (G-actin) is nucleated and elongated by the addition of free actin to the (+) end to form Filamentous actin (F-actin). F-actin has inherent structural polarity which results in a (+) end elongating faster than the (-) end (Lodish et al. 2000). This process is mediated by GTPase activated Arp2/3 and formin proteins. GTP-bound RhoA interacts with the Rho Binding Domain (RBD) in formin proteins to enable unfolding. Now, formin proteins, such as mDia1, can add actin to the + end of a growing actin filament. Rho signaling also activates p160Rock kinase (ROCK), which activates LIMK to stabilize F-actin (Le Clainche and Carlier 2008; Schaks et al. 2019). The formation of stress fibers and focal adhesions requires careful spatiotemporal regulation of RhoA and Rac1. Rac1

is activated as the cell spreads and invadopodia are extended into the matrix (Ridley, 2011). As the spreading ends, RhoA activity is increased to form stress fibers and mature focal adhesions. To actually pull the cell body forward in the direction of movement, RhoA stimulated ROCK inhibits myosin light chain (MLC) phosphatase, facilitating increased phosphorylation of myosin light chain and actomyosin contractility for the retraction of the tail of the cell (Huveneers & Danen, 2009; Raftopoulou & Hall, 2004) (**Fig. 2**).

GTPase activation is regulated by Rho GEFs and Rho GAPs (**Fig. 1**). Guanine Exchange Factors, GEFs, facilitate the release of GDP so GTPases can bind GTP and become active. Rho GEFs are typically segregated into two classes: the DH/PH family of Rho GEFs or the DOCK family of Rho GEFs. The DH/PH Rho GEFs are named for their ~200 amino acid Dbl-homology (DH) domain, which is most always paired with the ~100 amino acid pleckstrin homology (PH) domain (Rossman et al., 2005). The first mammalian Rho GEF was identified in diffuse B-cell lymphoma cells, thus the designation Dbl (Eva, 1985), and it was shown to have activate both human Cdc42 and the yeast homolog. Currently, there are 72 identified human Dbl family Rho GEFs, and several have been implicated human cancers. The DOCK family Rho GEFs lack the DH domain but retain GEF catalytic activity through their Dock-homology-region (DHR) domains. This family is much smaller, consisting of only 10 Rho GEFs; however, there is evidence that dysregulated DOCK family Rho GEFs can also contribute to oncogenic transformation. GTPase Activating Proteins, GAPs, bind to GTPases to catalyze the hydrolysis of GTP, which reverts the GTPase to an inactive state. There are approximately 80 human Rho GAPs, all characterized by the ~150 residue GAP domain that mediates GTP hydrolysis. Within the context of cancers, overexpression of Rho GEFs is typically seen as tumorigenic, due to their GTPase activating role, whereas Rho GAP overexpression has typically been associated with tumor suppression. Additionally, it is far

easier to develop therapeutics towards an antagonist than an agonist, shifting the focus for therapeutic targeting more on GEFs than GAPs (Vigil et al., 2010).

The three best studied Rho GTPases -- RhoA, Rac1, and Cdc42 -- are integral regulators of actin cytoskeletal rearrangement and all of them have been implicated in breast cancer metastasis. Although there have been increased instances of identified mutations of RhoA and Rac1 in cancer, their contribution to oncogenesis is most often attributed to their overexpression (Liang et al., 2021; Porter et al., 2016). GTPase activity is increased as a consequence of overexpression, increased corresponding GEF activity, decreased GAP activity, deregulated degradation, or mislocalization (Alan & Lundquist, 2013; Phane Rihet et al., 2001). The dysregulation of GEFs and GAPs associated with breast cancer will be addressed later, so the next section will focus on expression levels, post translational modifications, and subcellular localization of Rho, Rac1, and Cdc42 that drive oncogenic transformation.

1.1.2.1 RhoA Subfamily

RhoA isoforms include RhoA, RhoB, and RhoC, which have greater than 90% sequence homology. Although they all regulate actin cytoskeletal organization, the slight difference in isoforms provides the basis for more specific subcellular localization and function. RhoA and RhoC overexpression have been documented in breast cancer and have been shown to drive metastatic spread. RhoC specifically has been implicated for its role in Inflammatory Breast Cancer (IBC). As IBC has the worst prognosis of any breast cancer diagnosis, much effort has been made to better understand how RhoC drives metastasis and to develop therapeutics. RhoB had previously been reported as a tumor suppressor; however, recent work provides evidence that it may also have pro-tumorigenic roles. Importantly, the overexpression of Rho isoforms in breast cancer, compounded with GEF/GAP dysregulation, makes them key players in transformation (Privat et al. 2020).

RhoA

Early on, RhoA overexpression was shown to have oncogenic effects in several cancers, including in breast cancer. (Fritz et al., 1999; M. Lin & van Golen, 2004; Moscow et al., 1994; Phane Rihet et al., 2001). RhoA expression is required for proliferation and invasiveness of the human breast cancer cell line, MDA-MB-231, *in vitro* and *in vivo*. siRNA knockdown of both RhoA and RhoC through intratumoral injections almost completely inhibited tumor growth and angiogenesis in a murine model of breast cancer (Pillé et al., 2005). RhoA mutations have only recently been identified in some cancers. For example, RhoA R5Q, RhoA G17E, and RhoA Y42C were identified in gastric cancer, colon cancer, and lymphoma, respectively (Bass, 2014; Porter et al., 2016); however, RhoA overexpression, hyperactivation, localization, and protein stability are still the main contributors to RhoA mediated oncogenic transformation. The ability of RhoA to drive proliferation and motility signaling relies heavily on its plasma membrane localization, which is carefully regulated. Several groups have identified RhoA S188 phosphorylation by Protein Kinase A and G (PKA and PKG) as negative regulators of RhoA signaling. RhoA pS188 binds more readily to RhoGDI and this interaction drives relocation of RhoA from the plasma membrane to the cytosol, inhibiting RhoA activity and protecting it from proteasome-mediated degradation (Ellerbroek et al., 2003; Lang et al., 1996; Rolli-Derkinderen et al., 2005, 2010; Tkachenko et al., 2011). Conversely, Protein Kinase C (PKC) interaction recruits RhoA to the plasma membrane, independent of its kinase activity (Su et al., 2013). Other groups have shown EGFR stimulated Extracellular signal-Related kinase (ERK) signaling drives phosphorylation of RhoA on S88 and T100, relocating RhoA from a nuclear pool to the plasma membrane. This promotes motility by increasing RhoA dependent stress fiber formation through enhanced interactions with ROCK1 and mDia (Tong et al., 2016). While some groups have shown ERK signaling to increase RhoA activity, phosphorylation by ERK2 can also enhance ubiquitination and degradation of RhoA by the

SCF^{FBXL19} E3 ligase (Wei et al., 2013) RhoA is a key player in Epithelial to Mesenchymal Transition (EMT), a process by which tumor cells become more plastic and invasive. One way in which epithelial cell polarity is maintained is through the degradation of RhoA. Regulators of epithelial cell polarity, such as Par6, interact with TGF β , and recruit E3 ubiquitin ligase Smurf1. Smurf1 targets RhoA for degradation by ubiquitination, maintaining the cells epithelial phenotype (Ozdamar et al., 2005). Overall, RhoA function and stability are very carefully regulated by localization and protein stability, as it is a key regulator of so many basic cellular processes. When dysregulated, RhoA contributes strongly to breast cancer cell motility, invasion, tumorigenesis, and metastasis, so many researchers have sought to better understand the mechanisms of its tight spatiotemporal regulation in the cell.

RhoB

While RhoB is overexpressed in breast cancer tissue, there has been much debate as to whether it functions as an oncogene or a tumor suppressor (Fritz et al., 1999; Ju & Gilkes, 2018). Unlike RhoA or RhoC, RhoB has a unique C-terminal region that can be farnesylated or geranylgeranylated (Delarue et al., 2007; Du et al., 1999). This differential prenylation state directs its altered subcellular localization. Farnesylated RhoB localizes to the plasma membrane and facilitates similar actin cytoskeletal remodeling as RhoA or RhoC. However, geranylgeranylated RhoB localizes to the endosomes and can induce apoptosis (Prendergast, 2001). Interestingly, *RhoB* expression can be upregulated by treatment with farnesyltransferase I inhibitors or geranylgeranyltransferase I inhibitors via the disassociation of HDAC1, HAT association, and histone acetylation of the *RhoB* promoter (Delarue et al., 2007; Du et al., 1999). This suggests cooperativity between mechanisms of RhoB localization and RhoB transcription.

Médale-Giamarchi *et al.* showed a positive crosstalk between RhoB and ER α expression, supporting the idea that RhoB is tumorigenic. An analysis of human breast cancer tissue showed a strong correlation between high RhoB expression and high hormone receptor expression, believed to be the result of RhoB upregulating transcription of ER α . Furthermore, RhoB was critical for proliferation in ER α expressing human breast cancer cells, like MCF-7 cells (Médale-Giamarchi *et al.*, 2013). Conversely, several groups have identified RhoB as a tumor suppressor. In HER2+ cancers, RhoB overexpression suppressed Rac1 translocation to the plasma membrane, decreasing cell proliferation (Nishiyama *et al.*, 2021). Additionally, an analysis of the TCGA database showed elevated expression of RhoB mRNA in lower malignancy breast cancer subtypes, such as luminal A (Ma *et al.*, 2019). Work by the Zhang and Benjamin groups both implicated AKT signaling as an important regulator of RhoB in breast cancer (Kazerounian *et al.*, 2013; Ma *et al.*, 2019). Ma *et al.* found that RhoB inhibits proliferation by inhibiting the PTEN/AKT pathway in breast tumor cells, and Kazerounian *et al.* showed that RhoB functions differentially in cancer vs. stromal cells. They further demonstrated a reduction of AKT signaling by RhoB reduces the amount of Epidermal Growth Factor Receptor (EGFR) at the cell surface in tumor cells. Additionally, overexpression of RhoB was observed to have minimal effect on tumor size, but decreased metastasis in the lung, liver, and lymph nodes in mice implanted with *RhoB*^{-/-} MDA-MB-231 (Ju *et al.*, 2019). Although there is data to support the role of RhoB as both a tumor suppressor and a driver of oncogenic transformation, more recent work has been focused on understanding the nuance of which cell types, which cancer subtypes, and what expression profiles characterize its function.

RhoC

Although RhoC is overexpressed in many subtypes of breast cancer, its overexpression is a driver primarily in inflammatory breast cancer (Kleer et al., 2003). One hypothesis for the increased metastatic potential of IBC is its hypersensitivity to tumor extrinsic factors, such as signaling from tumor associated macrophages. Allen *et. al.* show that IBC cell lines SUM190 and SUM149 overexpress RhoC when exposed to macrophage conditioned media, significantly more so than MCF10A cells, a normal breast cell line, or even MDA-MB-231 cells, an invasive, non-IBC cell line. This increase in RhoC expression is what drove increased migratory properties in cells exposed to conditioned media and showed increased activation of the Mitogen-activated protein kinases (MAPK) cascade in the IBC cell lines (Allen et al., 2016). Of note, MAPKs coordinate a myriad of cellular functions, including proliferation, differentiation, motility, and survival, and this cascade is often deregulated in cancers (Roux & Blenis, 2004). Additionally, IBC cell lines have increased expression of caveolin-1, which activates Akt. Akt1 phosphorylation of RhoC on serine 73 drives increased invasive behavior in IBC cells lines. siRNA knockdown of RhoC, Caveolin, AKT, or treatment with the Akt inhibitor LY294002 showed significantly decreased invasion, rescued only by expression of the gain of function mutant RhoC S73D (Kaushal et al., 2018; Lehman et al., 2012; Oglekar et al., 2015; X.-D. Xu et al., 2017). Overexpression of RhoC is not unique to IBC, in fact many non-IBC patient samples and cell lines overexpress the transcription factor ATOH8-V1, which can bind directly to the *RhoC* promoter and enhance expression (M. Xu et al., 2021); however, RhoC remains best studied for its driving role in IBC.

RhoC is also believed to contribute heavily to cancer stemness. Cancer stem cell theory describes the population of cells within a heterogenous tumor that act as stem cells and can sustain tumors, even after anti-tumor therapies. Cancer stem cells may transition between an epithelial or more mesenchymal phenotype, based on whether they are moving

through extracellular matrix or colonizing a new site of metastasis (Capp, 2019). Several groups have implicated RhoC as a regulator for the abundance of breast cancer stem cells, as well a key contributor to the EMT process (Rosenthal et al. 2012; Kawata et al. 2014; Thomas et al. 2019).

1.1.2.2 Rac1

Studies have shown elevated Rac1 expression levels in solid tumors in many cancers, including bladder/urinary tract, lung, melanoma, breast, colorectal, and endometrial (De et al., 2019). Although Rac1 dependent transformation is predominantly a result of overexpression, recently, a few Rac1 gain of function mutants have been identified in human cancers. Rac1 P29S and Rac1F28L are gain of function mutants originally identified in melanoma which drive rapid cycling of Rac1 activation. Rac1 P29S has been identified as the most commonly mutated GTPase in cancer, as it has been identified in 9% of sun exposed melanomas (Davis et al., 2012; A. Kumar et al., 2013). Other oncogenic mutations identified in Rac1 include Rac1 A159V, common in head and neck cancers, and Rac1 Q61R, primarily found in prostate cancer (Liang et al., 2021). Rac1b is a splice variant of Rac1 originally identified in breast tumor samples and also acts as a fast-cycling GTPase (Schnelzer et al., 2000). Additionally, evidence shows an increased ratio of Rac1b: Rac1 expression in more invasive breast cancer cell lines, suggesting Rac1b drives a more metastatic phenotype (Eiden & Ungefroren, 2021). Although these novel mutations and splice variant have been identified, the majority of Rac1 associated transformations in breast cancer remain a result of overexpression of wildtype Rac1 or dysregulation of key activating proteins, such as Rac GEFs.

Rac1 predominantly localizes to the plasma membrane or the nucleus. Activation of integrins, receptor tyrosine kinases, or scaffold proteins drives the localization of Rac1 to the

plasma membrane through the interaction of plasma membrane lipids PIP2/PIP3 with the polybasic sequence of Rac1 (Heo et al., 2006). Rac1 may be further modified, by prenylation, geranylgeranylation or palmitoylation to stabilize it at actin cytoskeleton- adhered membrane sections during migration and cell spreading. Palmitoylation specifically facilitates GTP loading, a key step required for Rac1 dependent PAK activation at the plasma membrane (Navarro-Lérida et al., 2012). In addition to plasma membrane targeting, nuclear sequestration is another way the cell regulates Rac1 activity. ERK signaling drives phosphorylation of Rac1 at T108, which drives nuclear localization of the GTPase (Tong et al., 2013). Sequestration of Rac1 away from the plasma membrane allows RhoA signaling to drive actomyosin contraction. In lung cancer, nuclear Rac1 complexes with its GEF, Ect2, and nucleolar transcription factor UBF1 to upregulate rRNA synthesis and has been shown necessary tumorigenesis (Justilien et al., 2017).

Rac1 is regulated by post translational modifications, in addition to its regulation by localization. Rac1 is ubiquitinated by several E3 ligases, including HACE, XIAP, and c-IAP1, FBXL19 and degraded in a proteosome dependent manner (Abdrabou & Wang, 2018). Additionally, when Rac1 is phosphorylated on S71 by AKT, its GTP binding activity is inhibited (Abdrabou & Wang, 2018). Downstream of EGFR signaling, Rac1 is also phosphorylated on Y74 by Focal Adhesion Kinase (FAK) and Src kinases to target it to focal adhesions and reduce binding to RhoGDI (Chang et al., 2007). Much like other Rho GTPases, Rac1 activity is tightly controlled through several mechanisms to ensure coordinated activation of specific pools of Rac1 in order for the cell to best respond to extracellular stimuli.

1.1.2.3 Cdc42

Cdc42 is required for oncogenic transformation, due to its central role in mediating cell cycle progression, cell polarity, and invasive behavior (Haga & Ridley, 2016; Jaffe & Hall, 2005; Mosaddeghzadeh & Ahmadian, 2021). Cdc42 localizes primarily to the plasma membrane, often at the leading edge- where it controls filopodia formation at membrane ruffles. It is often overexpressed in breast cancer and contributes to oncogenic transformation mainly through cell surface receptors, like EGFR. In mammary epithelial cells, Cdc42 can impair the activity of the E3 ligase c-Cbl, preventing the ubiquitination and degradation of EGFR, increasing aberrant growth factor receptor signaling (Hirsch & Wu, 2007). The G1/S transition in mammary epithelial cells is controlled by Cdc42 and cells that lack Cdc42 expression have a decreased proliferation rate. Interestingly, mammary epithelial cells that have hyperactive Cdc42 are not necessarily more proliferative but do have increased cell motility – likely induced through Cdc42 activated MAPK signaling (Zhang et al., 2019).

1.1.3 Therapeutic targeting of Rho family GTPases and downstream effectors in breast cancer

Inhibiting Rho GTPase activity has long been attempted, through the development of several small molecule inhibitors that target various aspects of the GTPase activation. (Y. Lin et al., 2015). Geranylgeranyltransferase inhibitors has been developed with two targeting mechanisms: either CAAL mimetic molecules (Bredel et al., 1998) or non-peptidomimetic molecules that compete with the protein substrates (Peterson et al., 2006). CAAL mimetic molecule tipifarnib was tested in two phase II clinical trials for metastatic breast cancer. Unfortunately, one study was withdrawn, and a second study determined there was no improvement in postmenopausal women with ER+ advanced breast cancer when tipifarnib was combined with letrozole therapy (Johnston et al., 2008).

Molecules directly targeting RhoA, Cdc42, or Rac have also been developed, with mechanisms including blocking GEF binding (Rhosin, NSC23766, EHop-016, CASIN, ZCL278, AZA1, AZA197), RhoA-dependent gene transcription (CCG-1423), or plasma membrane recruitment (CHS-111). Additionally, molecules locking Rac1/Rac2 in an inactive state (EHT-1864) or enhancing the interaction between Cdc42 and RhoGDI (Secramine) have also been developed (Y. Lin et al., 2015). While these inhibitors have been validated against their targets *in vitro*, none have successfully been entered in clinical trials for breast cancer patients.

The paradigm shift in targeting Rho GTPases was the development of ROCK inhibitors, the first being Y-27632. Uehata *et. al.* first published this small molecule as a potent inhibitor of smooth-muscle contraction by inhibiting Ca^{2+} sensitization. The use of Y-27632 was shown to inhibit Rho-induced stress fiber formation *in vitro* and correct hypertension in multiple rat models through the inhibition of Rho effector p160ROCK (Uehata et al., 1997). Several generations of ROCK inhibitors have been developed over the years, almost all have been ATP competitive inhibitors. Fasudil, and its derivative Dimethylfasudil, have been in clinical trials for several ocular diseases and for cardiovascular diseases (Ahmadiéh et al., 2019; Fava et al., 2012; Watanabe et al., 2013). Unfortunately, they have not successfully made it to clinical trials for breast cancer. The development of an ATP-competitive multi-ADC competitive kinase inhibitor AT13148 has meant that at least one small molecule inhibitor targeting ROCK has made it through phase I clinical trials. However, this drug also targets other commonly dysregulated kinases in cancer, including AKT and PKA (Sadok et al., 2015; Yap et al., 2012) and its effects have not been sufficient to shift the standard of care in breast cancer therapeutics.

1.2 The EGFR-Src-Abl signaling cascade in Breast Cancer

1.2.1 An Incredibly Brief Introduction to EGFR Signaling in Breast Cancer

EGFR was originally discovered in 1959 by Drs. Stanley Cohen and Rita Levi-Montalcini as a secreted factor from cancer cells that could drive cell proliferation of isolated nerve cells. Their work was revolutionary and would eventually earn them the Nobel prize (Cohen, 2004). In humans, the EGFR family is characterized as ErbB from the original discovery in avian erythroblastosis virus (v-ErbB), which was a mutant homolog of human EGFR (Downward et al., 1984; Ullrich et al., 1984). The human ErbB family includes EGFR/ErbB1/HER1, Neu/ErbB2/HER2, ErbB3/HER3, and ErbB4/HER4, which can hetero- and homodimerize with one another, transphosphorylate and activate downstream signaling effectors within the cell. The role of EGFR signaling as a driver of oncogenic transformation has been well studied, as HER positivity has become an important diagnostic and prognostic marker (Lemmon & Schlessinger, 2010). While EGFR dysregulation in breast cancer can occur from overexpression of the receptor, activation of downstream effectors, such as nonreceptor tyrosine kinases (NRTKs) amplify oncogenic signaling and have themselves in been implicated in cancers. Downstream of EGFR signaling, dysregulation of NRTKs, such as Src and Abl1, have been implicated for their role in driving breast cancer metastasis. This work focuses on this particular axis of EGFR signaling, as their activity converges to regulate Net1A localization.

1.2.2 Src- The Original Oncoprotein

1.2.1.1 Transforming capability of Src: c-Src and v-Src

Src was first identified as a transforming protein of the oncogenic retrovirus Rous sarcoma virus in 1977 (BRUGGE & ERIKSON, 1977). The mutant protein, v-Src, lacks a key residue in its c-terminus, specifically tyrosine 527. C-terminal Src Kinase (Csk)

phosphorylation of Src Y527 leads to an inactivating conformational change, which v-Src does not have (Sefton & Hunter, 1986; S. M. Thomas & Brugge, 1997). c-Src is the human homolog of this protein and its roles in actin-cytoskeletal rearrangement, cell motility and adhesion, and breast cancer tumorigenesis and metastasis are an enormous field within the study of human disease.

The largest family of nonreceptor tyrosine kinases is the Src family, which consists of Src, Lyn, Fyn, Lck, Gfr, Blk, Yrk, and Yes. - many of which have been well studied for their individual contributions to human disease. c-Src has several important domains, including the Src Homology 2 (SH2), and Src Homology 3 (SH3), and kinase domains. SH2 domains recognize and bind proteins at sites of tyrosine phosphorylation and SH3 domains facilitate interactions with proline-rich regions. Both domains are common in actin cytoskeleton rearrangement complexes, as they can be rapidly modified post translationally and regulate dynamic complex formation. Phosphorylation of Src Y527 allows it to interact with its own SH2 domain, changing the protein to a closed, inactive conformation, where its SH2 and SH3 domains are no longer available to other proteins to bind (Summy, 2003). Src is activated downstream of EGFR signaling and integrin stimulation. Ligand stimulation of EGFR promotes a conformational change in the receptor, exposing sites that are phosphorylated by Src. Once Src phosphorylates this residue, it uses its own SH2 domain to form stable active complex with the receptor at the plasma membrane (Sato, 2013).

1.2.2.2 Role of Src in Breast Cancer Cell Adhesion, Actin-Cytoskeleton Rearrangement, Migration, and Invasion

Src is a key integrator of receptor kinase signaling and integrin signaling, and the formation of intracellular signal amplifying complexes requires Src (Chen et al., 2018). During cell movement, integrin engagement and EGFR stimulation activate Src and Focal Adhesion Kinase (FAK) at the plasma membrane. These kinases phosphorylate a myriad of Rho GEFs,

Rho GAPs, and scaffold proteins to regulate the recruitment of GTPases and other actin remodeling proteins to drive focal adhesion formation and cell spreading. One such signaling cascade includes the recruitment of FAK and Src to the plasma membrane upon integrin stimulation. Increased FAK activity drives Src and the phosphorylation of the adapter protein, p130Cas, which recruits paxillin. Phosphorylated paxillin can interact with the adapter protein Crk and drive Rac1 signaling to promote focal adhesion formation and membrane protrusion. Rac1 activation of the WAVE/WASP complex also drives Arp2/3 mediated branched F-actin growth and increases membrane protrusions. In the cell body and retracting edge of the cell, Src drives phosphorylation of Rho GEFs and Rac GAPs in order to activate RhoA and ROCK signaling to inhibit cofilin and generate actomyosin contraction. This results in increased Arp2/3 mediated F-actin stabilization and also ROCK dependent myosin light chain phosphorylation, to drive cytoskeletal contractility. As mentioned, the regulation of motility, cell spreading, and invasion are hugely dependent on Src, and other non-receptor protein kinases, to locally activate and complex GEFs and GAPs for coordinated GTPase activation. (Brown et al., 2005; Huveneers & Danen, 2009; Iwasaki et al., 2002; S. Kumar et al., 2017; Lamorte et al., 2003; Mitra et al., 2005; Playford & Schaller, 2004).

1.2.2.3 Effects of Src expression on breast cancer progression

The role of Src in cancer has been long studied, including its role in driving breast cancer metastasis. Several groups have reported elevated Src expression in breast tumor tissue across several subtypes of breast cancer. (Elsberger et al., 2009; Finn, 2008; Summy, 2003; Tryfonopoulos et al., 2011). Additionally, c-Src plays an integral role in normal mammary gland development and c-Src knockout mice show diminished development of reproductive organs. Kim *et. al.* found mice that were c-Src deficient had ductal branching defects and significantly fewer terminal end buds, similar to ER knock out mice (Kim et al.,

2005). Elsberger *et al.* also correlated high levels of active Src in ER positive breast cancer patients with a reduced distant metastasis free survival, making the connection between the role of Src in ER regulated healthy mammary tissue growth and the role of Src in ER driven breast cancer progression. Interestingly, the same study showed an increase in metastasis free survival for TNBC patients who expressed high levels of Src pY215, a form of inactive Src. Inhibition of Src signaling using dasatinib significantly reduced invasion and migration of MDA-MB-231 cells, as well as proliferation in MDA-MB-231 and MDA-MB-468 cells, suggesting Src is a driver of oncogenesis *in vitro* (Tryfonopoulos et al., 2011). In an analysis of 150 breast cancer tumor samples, those overexpressing ErbB2 had significantly higher levels of PKC at the membrane. As such, direct activation through the ErbB2-Src axis results in elevated PKC activation, which then goes onto drive signaling that promotes transformation and metastasis (Tan et al., 2006). The role of Src in breast cancer has been extensively studied, as it is a crucial node of signaling amplification from commonly dysregulated receptors upstream and is often overexpressed itself. Amplification of growth, proliferation, and motility signaling at such a high level often leads to many different, often redundant, axes of signaling that drive oncogenic transformation.

1.2.3 Role of Abl in Breast Cancer Cell Adhesion, Actin-Cytoskeleton Rearrangement, Migration, and Invasion

Abelson nonreceptor kinase (Abl) is a family of nonreceptor tyrosine kinases consisting of Abl1(c-Abl) and Abl2 (Arg). This family has several conserved domains, including an N-terminus that can be myristoylated for plasma membrane localization, SH3 and SH2 domains utilized in complex formation, a kinase domain, and an F-actin binding domain. Abl1 and Abl2 also have three nuclear localization signals (NLS), and Abl1 has a nuclear export signal (NES), which allows it to shuttle between the nucleus and the cytosol in response to cell adhesion and other ECM signaling proteins (Wen et al. 1996; Taagepera et al. 1998;

Pendergast 2002). Abl1 also contains a DNA binding domain, not shared by Abl2. Abl family kinases regulate several cellular processes, including DNA damage repair, actin cytoskeletal rearrangement, ECM degradation, and proliferation.

Abl family kinases are regulated similarly to SFKs in that its SH2-kinase linker region adopts a conformation that interacts with its own SH3 domain. However, unlike Src, phosphorylation on a single site does not release autoinhibition to activate Abl. Instead, Abl family kinases can be phosphorylated by upstream kinases which leads to a conformational change (Furstoss et al. 2002). In this more open conformation, Abl1 is activated through interactions between its SH2 and SH3 domains and activator proteins like Crk and Abi. Activated Abl1 not only interacts with Crk and Abi family proteins, but also phosphorylates them, which facilitates a more stable interaction through Abl SH2 domain; this can modulate Abl function and supports the idea that Abl1 activation is very dependent on its ability to utilize its SH2 domain for activating interactions (Shishido et al. 2001; Jodi M. Smith 1 et al. 2002). The SH2 domain of c-Abl is critical not only for activation, but it is also required for Abl to function as processive kinase (Grebien et al. 2011; Hossain et al. 2012; Lamontanara et al. 2014; Lorenz et al. 2015). Eventually, activated Abl1 is eventually degraded in a ubiquitin dependent manner (Echarri and Pendergast 2001).

In response to extracellular stimuli, such as ligand stimulation or ECM engagement, Abl family kinases are key regulators of actin cytoskeletal rearrangement (Bradley and Koleske 2009). Downstream of EGFR signaling and Src activation, Arg is recruited to invadopodia to phosphorylate cortactin and enhance breast cancer cell invasion (Mader et al. 2011). Abl kinases are also required for efficient invadopodia maturation, invasion, and matrix degradation through their phosphorylation of cortactin and recruitment of MT1-MMP (Smith-Pearson et al. 2010). Loss of Abl1 function significantly reduces focal adhesions, F-actin stabilization, and p-MLC staining at the periphery of spreading fibroblasts, but loss of Arg function disrupts the distribution of contractile and adhesion elements. Abl1 dependent

phosphorylation of p190Rho GAP promotes its interaction with p120RasGAP to drive a Rho GTPase inhibitory complex and this complex is mislocalized in *Abl1* knockout cells. Abl1 and Arg have different spatiotemporal regulations of actomyosin contractility and focal adhesion in moving cells (Justin G. Peacock, Brian A. Couch 2010), but both are critical mediators of cadherin mediated cell-cell adhesion. This cellular process also requires intact Crk expression. Inhibition of Abl kinases has been shown to mislocalize active RhoA, driving aberrant actomyosin contractility, pulling the cells apart (Zandy et al. 2007).

Several Abl1 fusion proteins have been implicated in human cancers, including Bcr-Abl1, NUP214-Abl1, EML1-Abl1, ETV6-Abl1, ETV6-Ab1, ZMIZ1-Abl1, RCSD1-Abl1, and SFPQ-Abl1 (Hantschel 2012). Most of these fusion proteins have been best studied in leukemia, with the most work done to understand how Bcr-Abl1 drives leukemia progression. In breast cancer, there are rare reports of Abl1 fusion proteins, but they are not very prevalent – and thus do not drive research in this field (Edwards and Howarth 2012)

Abl family kinases have been heavily implicated in several cancers, including solid tumor cancers such as breast cancer. Increased expression of *Abl1* and *Abl2* is seen in breast cancers. *Abl2* mRNA levels correlated with metastasis across all breast cancer subtypes, and high *Abl1* expression correlated with metastasis in breast cancer subtypes with elevated HER2 positivity (Wang et al. 2016). This association with metastatic phenotype likely has to do with the indispensable role of Abl kinase activation in processes regulating invasive behavior. Blocking Abl activity in invasive cell lines, such as MDA-MB-435 cells, significantly reduces growth in soft-agar, whereas inhibiting Abl2, but not Abl1, significantly decreases proliferation (Srinivasan et al. 2008). Additionally, Abl kinase activity promotes breast cancer metastasis to the bone through its activation of STAT5 and TAZ. STAT5 drives transcriptional upregulation of IL-6, MMP1, and TNC and TAZ upregulates AXL. the resulting gene expression signature correlates with poor outcome. Mechanistically, this signaling drives osteoclast activation and facilitates interactions between breast tumor cells and osteoclasts

to promote bone metastasis formation (Wang et al. 2016). More recently, simply showing overexpression has not been enough, as Abl1 is activated by multiple regulatory steps, including phosphorylation by SFKs and interactions with SH2 domain containing proteins, such as Crk. In breast cancers with ErbB2 or EGFR deregulation, there are increased levels of Abl family kinase activation. Invasive breast cancer cell lines, such as MDA-MB-231, BT-549, and MDA-MB-468, have increased levels of Abl activity, demonstrated by increased levels of phosphorylation of Abl substrate CrkL (p-CrkL); this increase in p-CrkL levels is also seen downstream of constitutive Src signaling. (Srinivasan and Plattner 2006a). Interestingly, recent work has also shown p-CrkL is secreted in invasive breast cancer cell lines such as MDA-MB-231 cells and SUM159 cells. These cells have increased levels of p-CrkL in their culture media in comparison to MCF7 cells, which still has elevated levels compared to normal mammary epithelial MCF10A cells. This group also detected increased levels of p-CrkL in breast cancer patients in comparison to healthy patients and found that patients who received no treatment at the time of blood draw had 1.6X higher levels of p-CrkL than patients who had received some treatment. This suggests that this is a minimally invasive method of detecting progress in breast cancer patients receiving therapy (Srinivasan and Godin 2019a)

1.2.4. Effects of Src and Abl Overexpression on Breast Cancer Metastasis, Patient Survival and Therapeutic Targeting

In breast cancer, elevated Src or Abl activity, is correlated with more invasive disease and poor prognosis (Summy 2003; Srinivasan and Plattner 2006b; Elsberger et al. 2009; Wang et al. 2016; Srinivasan and Godin 2019b). In the MMTV-PyMT mouse model of breast cancer metastasis, Src activity is required for tumor formation (Guy et al. 1992; Guy et al. 1994). Elevated Src expression is often found in breast cancer cell lines expressing higher levels of *HER1* and these cells had increased DNA synthesis, soft agar growth, and tumor formation (Maa et al. 1995; Biscardi et al. 1999). Src activity is increased in breast cancer cell

lines that are more invasive, a phenotype that is attenuated when treated with SFK inhibitors (Tan et al. 2006). EGFR endocytosis is also regulated by Abl1 activity, where Abl1 dependent phosphorylation of EGFR on Y1173 impairs EGFR internalization, suggesting EGFR and Abl signaling may cooperate in breast cancer (Tanos and Pendergast 2006). This early identified synergism between EGFR and nonreceptor tyrosine kinases has continued to be exploited therapeutically, as tyrosine kinase inhibitors are commonly given in combination to EGFR inhibitors (Simatou et al. 2020). Several generations of therapies targeting nonreceptor tyrosine kinases have been developed and ATP competitive molecules often target both Src and Abl. Imatinib was the first selective Bcr-Abl tyrosine kinase inhibitor in the late 1990s, marketed currently as Gleevec. Unfortunately, imatinib failed to thrive in the clinic due to mechanisms of resistance related to point mutations in Bcr-Abl that altered inhibitor binding affecting. In response, second generation inhibitors, bosutinib, nilotinib, and dasatinib were developed. Bosutinib was originally developed from a leading Src inhibitor compound (2,4-dichlorophenyl) amino]-6,7-di-methoxy-3-quinolinecarbonitrile), so it varies slightly in structure from nilotinib and dasatinib. The most recent, third generation inhibitor developed is ponatinib – a dual Src/Abl mutation designed to overcome binding issues with the T315I mutation (Rossari et al. 2018). Sarcatinib is another tyrosine kinase inhibitor, originally designed to have stronger affinity to Src, but also inhibits both Src and Abl (Schenone et al. 2010). Interestingly, most of these ATP competitive small molecules tend to inhibit both Src and Abl, but to varying degrees. To increase targeting specificity, several allosteric inhibitors have also been developed, with more specificity to either Src, such as KX2-391 which inhibits Src and tubulin polymerization, or Abl, such as GNF-2 and GNF-5 which inhibit kinase activity by preventing myristoylation (Choi et al. 2009; MP et al. 2018).

Recently, Dasatinib successfully cleared a Phase II trial in which it was shown that Dasatinib sensitized HER2+ breast cancer patients to trastuzumab and paclitaxel, common front line breast cancer therapeutics (Ocana et al. 2019). It has also cleared Phase I/II testing

to assess maximal tolerated dosage in combination with the chemotherapy ixabepilone (Simatou et al. 2020). Nilotinib has been in Phase I trials and is currently being evaluated for its role in reducing paclitaxel induced peripheral neuropathy in patients with stage I, II, or III breast cancer (TB et al. 2021). Additionally, the role of Bosutinib, in combination with aromatase inhibitor therapy, has been evaluated in postmenopausal women who with hormone positive, but HER2 negative metastatic disease. The study was terminated early, so no conclusions were drawn from this Phase II clinical trial (Moy et al. 2014). The combination of bosutinib and capecitabine also reported minimal efficacy (Isakoff et al. 2014). Sarcatinib has been in several Phase I and Phase II clinical trials but have also shown minimal success in studies that assessed hormone receptor negative patients (Gucalp et al. 2011). Allosteric inhibitors of Src, specifically KX2-391, have recently been evaluated in a smaller trial in Korea, but the results are not yet available (NCT01764087). GNF-2 and GNF5 have yet to be evaluated in clinical trials. Although clinical data is not yet available from these allosteric inhibitors, I believe they may be better tolerated, due to their increased specificity.

1.3 Rho GEFs and Rho GAPs in breast cancer

1.3.1 Rho GEFs Associated with Tumorigenesis and Metastasis in Breast Cancer

Downstream of extracellular adhesion, motility, and growth factor stimulation, NRTKs regulate GEFs and GAPs to coordinate GTPase activity within the cell. As GEFs and GAPs have more tissue specific expression than GTPases, it allows researchers to narrow the focus to key players contributing to tumorigenesis and metastasis in breast tissue, specifically. Several Rho GEFs have been evaluated for their roles in breast cancer, including: Tiam1, Vav, Trio, and P-Rex1, β -PIX, ARHGEF 7, ARHGEF 10, GEF-H1, RILP, GEP100, Dbl, Rap1, MyoGEF, Ect2, and, of course, Net1. In the case of some Rho GEFs, such as MyoGEF, RILP, GEP100, their contribution has mainly been demonstrated in *in vitro* studies of breast cancer cell adhesion, motility, and invasion. Others, such as Tiam1, Vav1, P-Rex1, and Net1 have

been implicated through analyses of human patient samples or larger gene set analyses. This chapter will mainly focus on Rho GEFs, except Net1, which have been implicated in *in vivo* models or have been shown to be dysregulated in human breast cancer samples.

Tiam1, a Rac1 specific GEF, is often overexpressed in breast cancer and is a marker for poor prognosis (Lane et al. 2008). The addition of serum or heregulin to NIH 3T3 cells or MCF7 cells recruits Tiam1 to the plasma membrane and mediate efficient membrane ruffle formation. Downstream of ligand stimulation, PKC phosphorylation of Tiam1 recruits it to the plasma membrane. Once there, it also activates c-Jun N-terminal kinase (JNK) to promote proliferation signaling. (Michiels et al. 1997; Adam et al. 2001). Additionally, an analysis of several breast cancer cell lines showed a correlation between increased Tiam1 expression and increased invasiveness (Minard et al. 2004). Tiam1 expression not only drives increased GTPase activity in cancer cells, but it is also expressed in fibroblasts in the tumor microenvironment. Cancer cells do not exist in isolation, rather there is a complicated bidirectional cross talk between tumor cells and the surrounding tumor microenvironment. Silencing of Tiam1 in mouse mammary fibroblasts significantly increased invasiveness of breast tumors, even though tumorigenesis was not affected (Xu et al. 2010). Additionally, Tiam1 expression in mammary fibroblasts modulates cancer stem cell-like populations and Tiam1 silencing in these fibroblasts leads to less migration of breast tumor cells (Xu et al. 2016).

Vav proteins are Rho/Rac/Cdc42 family GEFs, and three isoforms are expressed in humans: Vav1, Vav2, and Vav3. Vav1 overexpression in breast tumor tissue was shown to correlate with estrogen receptor expression and be a marker for poor prognosis for ER+ breast cancer patients (Lane et al. 2008; Farago et al. 2020; Ojala et al. 2020). In ErbB4 positive breast cancers, Vav3 is directly phosphorylated by the receptor and drives GTPase activity (Ojala et al. 2020). In addition to its aberrant GEF function, Vav2 and Vav3 play a GEF independent role in upregulating gene expression to affect a cell's transition from EMT

(Bustelo et al. 1992; Rodríguez-Fdez and Bustelo 2019). Citterio *et. al.* demonstrated Vav2 and Vav3 overexpression upregulated Rac1-dependent and Rac1-independent expression of several proteins required for lung-specific metastasis, such as Ilk, Lng β A, Tacstd2, and Cox2 (Citterio et al. 2012). Like other Rho GEFs, Vav activity is dependent on phosphorylation to modulate its signaling potential; Vav1 is phosphorylated by Src family kinases on several sites in its regulatory acidic, zinc finger, and C-terminal SH3 domains. Interestingly, Bcr-Abl, the fusion gene implicated in leukemia, has also been shown to phosphorylate and complex with Vav1 and drive Rac-1 dependent signaling (Bassermann et al. 2002).

The Rac GEF P-Rex1 is highly overexpressed in luminal subtype breast cancers, specifically those that are ErbB2+ and ER+. It acts as a mediator of cell motility and integrates signaling from both growth hormone receptors and GPCRs, specifically by the PI3K product PIP3 and the G β γ subunit (Wertheimer et al. 2012). The activation of P-Rex1 can occur upon ligand stimulation of HER/ErbB Receptors, via the transactivation of CXCR4 through HEF/ErbB receptors, or sustained stimulation of HER3/ErbB3 via activation of HIF-1 α dependent transcription of more CXCR4 (Kazanietz et al. 2016). Montero *et. al.*, showed the mechanism by which FGF and IGF-1 can activate P-Rex1 in MCF7 cells is by upregulation of P-Rex1 phosphorylation on serine 1169 and activated P-Rex1 drives Rac signaling and associated breast cancer cell proliferation (Montero et al. 2013).

The overexpression of Dbl, a Rac1/Cdc42 GEF, in wildtype MCF-10A cells drives an EMT phenotype, including the upregulation of mesenchymal markers such as α -SMA, MMP-12, and CCL2, and the downregulation of E-Cadherin. This alters their morphology, drives increased anchorage independent growth – providing a mechanistic basis for the role of Dbl in breast cancer (Vanni et al. 2015). MyoGEF is a RhoA/ RhoC specific GEF best known for its roles in cytokinesis and breast cancer cell invasion. MyoGEF is highly expressed in invasive breast cancer cells and tissues, and depletion decreases RhoA/RhoC dependent invasion in MDA-MB-231 cells (Wu et al. 2009). It is also unique in that phosphorylation on

threonine 544 by Aurora B kinase creates a docking site for Plk1, facilitating sequential phosphorylation that localizes MyoGEF to the central spindle. Interestingly, these intramolecular interactions facilitate an interaction between MyoGEF's own DH domain and c-terminus- creating a novel autoinhibitory regulation. Clearly, the regulation of Rho GEF activity is tightly controlled, through phosphorylation or complex formation, so this new mode of regulation in creating its own autoinhibition speaks to the tight spatiotemporal regulation of Rho GEFs and what they contribute to cancer cell division (Wu et al. 2014). Similar to MyoGEF, Ect2, a RhoA GEF, also has roles in cytokinesis and transformation (Cook et al. 2011). Higher *ECT2* mRNA expression can be found in ER+, basal-like, and triple negative breast cancer and is associated with poorer outcomes in ER+ breast cancer patients (Xiu et al. 2019). Ect2 is one of only two GEFs that is regulated by nuclear sequestration, the other being Net1. Both Ect2 and Net1 are required for RhoB mediated cell death after DNA damage and the dysregulation of apoptosis by chemotherapeutic agents may contribute to the poor outcomes associated with increased GEF expression. More recently, Ect2 has been shown to activate Rac1 and form the Ect2, UBF1, NPM complex required for rRNA synthesis in lung cancer(Justilien et al. 2017b). This role has yet to be explored in breast cancer.

While there is ample evidence for the indispensable role of GEFs in metastatic disease, targeting these molecules therapeutically has had limited success. Brefeldin-A is a lactone antiviral that inhibits protein transport from the ER to the Golgi by inhibiting Arf family GEFs. This noncompetitive inhibitor prevents efficient protein trafficking (Niu et al. 2005). Treatment of MDA-MB-231 cells with Brefeldin A reduces anchorage-independent survival and migration, and even sensitizes these cells to antitumor drugs Actinomycin D and Vinblastine through ERK and AKT signaling (Tseng et al. 2014; Luchsinger et al. 2018). Unfortunately, Brefeldin-A has not yet been tested in clinical trials for breast cancer. A more specific aptamer that inhibits the Rho GEF Trio has been recently developed. TRIAP α is specific to the TrioGEFD2 and does not affect TrioGEFD1 – which is specific for RhoG

activation. This development has paved the way for the development of more specific GEF inhibitor molecules in the future (Schmidt et al. 2002) .

1.3.2 Rho GAPs Associated with Tumorigenesis and Metastasis in Breast Cancer

Some Rho GAPs have also been implicated in breast cancer, the best studied being those belonging to the IQGAP, RASA, and ARHGAP families. IQGAP1 is a RhoA/RhoC GAP that is activated downstream of EGF stimulation, similar to Net1, and is required for RhoC mediated migration in MDA-MB-231 cells (Casteel et al. 2012). IQGAP has a well-established role as a scaffold protein and is reported to bind directly to ER α . It is overexpressed in breast tumor tissue, in comparison to paired healthy tissue, and is also overexpressed in breast cancer cell lines when compared to the normal mammary epithelial cell line MCF10A. siRNA knockdown of IQGAP in breast cancer cells significantly reduces proliferation due to cell cycle arrest at the G₀/G₁ phase (Zeng et al. 2018). Hebert *et. al.* showed knockdown of IQGAP1 does not affect tumor size, but significantly reduces metastatic burden, strengthening the burden of evidence for its necessity in invadopodia formation and extravasation. IQGAP interacts with at least a hundred different proteins and is a signaling node in the MAPK/ERK pathway, MAPK B/C-Raf, Merk1/2, EGFR signaling, and YAP activation pathway. This made analysis of the order of IQGAP1 dependent metastatic steps very complex, but mutagenic analysis showed serine 1441 and serine 1443 to be required for metastasis. Interestingly, this seems to be GAP independent because mutants that were unable to bind to Rac1 and Cdc42 were all unable to rescue metastatic phenotype (Hebert et al. 2020). IQGAP3 is also overexpressed in breast tumor when compared to non-tumor tissue samples. siRNA knockdown of IQGAP3 also suppresses migration and invasion, similar to IQGAP1, and also acts in a GAP independent manner to contribute to metastasis through its association with many pro-metastatic proteins including MMP9, p53, Erk1/2, and Twist (Hu et al. 2016).

The ARHGAP family of proteins consists of at least 30 genes, and several have been implicated for their role in breast cancer. A recent analysis of the expression of ARHGAP family genes in breast cancer mined data from oncomine, Kaplan-Meier Plotter, bcGenExMiner and cBioPortal and reported decreased expression of ARHGAP 6,7,10,14,19, 23, and 24, and increased expression of ARHGAP 9,11,15,18, and 30 in patients with breast cancer (Chen et al. 2019). ARHGAP15 overexpression in breast cancer is corroborated by Takagi *et. al.* and they found it played a tumor suppressor role by reducing proliferation and migratory behavior. Additionally, ARHGAP15 expression can be induced by treating ER+ breast cancer cells with DHT and increased ARHGAP expression suppressed HRG-dependent Rac1 activation (Takagi et al. 2018). ARHGAP29 has also been shown to influence invasiveness of breast cancer cell lines and be required for efficient stress fiber formation in HCC1806, MCF7-EMT, T-47D-EMT breast cancer cell lines (Kolb et al. 2020). Although more work is necessary to understand the mechanisms by which ARHGAP family proteins influence breast cancer, it does seem to be predominantly GAP independent in function, suggesting their role as scaffold proteins in the cell is the driving force behind their role in oncogenic transformation.

The DLC family of GAPs has also been well studied in several cancers and been shown to be a tumor suppressor, including in breast cancer. DLC-1 expression is typically higher in normal tissue and in benign breast lesion specimens than that of breast carcinoma samples and early detection of DLC-1 has been posited as a diagnostic marker for early invasive breast cancer (Guan et al. 2012). Introduction of DLC-1 cDNA also significantly reduced tumorigenesis *in vivo* where MDA-MB-468, UACC 893, and MDA-MB-301 cells were implanted in nude mice and tumorigenesis was tracked over a three-month period (Yuan et al. 2003). Goodison *et. al.* showed that deletion of DLC-1 in the metastatic M4A4 cell lines to levels similar to nonmetastatic cell lines significantly reduced the ability of M4A4 cells to migrate and invade *in vitro*. These cells also were less efficient in colonizing the lung and

forming metastases in athymic mice (Goodison et al. 2005). Whether or not the role of DLC1 as a tumor suppressor is dependent on its GAP activity remains unclear.

p190RhoGAP is preferentially expressed in terminal end buds during mammary gland development and overexpression of *p190B* in normal human mammary epithelial cells disrupts actin cytoskeletal rearrangement (Chakravarty et al. 2003). p190Rho GAP expression is frequently decreased in breast tumor tissue and this reduced expression is correlated with breast cancer progression and reduced distant metastasis free survival (Liu et al. 2015). Tumors are very often hypoxic environments, and hypoxia has been shown to increase expression of RASAL1, or p120RasGAP, in MCF7 and MDA-MB-231 cells. This increased expression inhibits cell proliferation and invasion by decreasing HIF and intracellular ROS (Huang et al. 2017). On the other hand, RASAL2 is overexpressed in TNBC and ER- tumors and promotes GTPase signaling through binding to ARhGAP24 and Rac1.

While GAP deregulation is mechanistically important in breast cancer progression, it is lacking in several key characteristics required for therapeutic exploitation. Firstly, as the molecule that reverts the GTPase “molecular switch” to the off position, inhibiting its activity could result in unintended consequences of hyperactive GTPase activity. Furthermore, there is a growing body of evidence showing GAP independent functions are associated with driving cancers.

1.4 Established Roles of Rho GEF Net1

1.4.1 Net1 History and Discovery

Neuroepithelial transforming gene 1 (Net1) was originally characterized for its ability to cause oncogenic transformation in NIH 3T3 fibroblasts. It was isolated as a cDNA from a human neuroepithelioma cell line. NIH3T3 cells transfected with Net1 exhibited increased growth properties *in vitro* and were found to be tumorigenic in nude mice (Chan et al. 1996).

Subsequent work by Alberts *et. al.* demonstrated Net1 to have GEF activity towards RhoA, but not Cdc42 or Rac1, and showed that Net1 Δ N overexpression could drive stress fiber formation in NIH 3T3 cells. This seminal work also showed that deletion of the N-terminus was required for oncogenic transformation. The Hall group shortly followed up with the incredible discovery that the function of Net1 was regulated by its nuclear sequestration, which is unusual considering its target, RhoA, would be in the cytosol – more specifically the plasma membrane. While several Rho GEFs may localize to the nucleus transiently, only two are predominantly localized in the nucleus: Ect2 and Net1. This work also showed the necessity and sufficiency of the PH domain of Net1 for CRM1 dependent nuclear export (Schmidt and Hall 2002). Qin *et. al.* demonstrated that the amino terminus was not an autoinhibitory domain and instead showed two separate domains as nuclear localization sequences. Furthermore, this work showed that the DH and PH domains of Net1 were required for catalytic activity and the newly identified PDZ domain binding site was required for oncogenic transformation. This was the first paper that demonstrated a requirement for Net1A relocalization from the nucleus to the cytosol for transformation (Qin et al. 2005). The mechanisms for these observations would later be better defined by work from the Hall group, the Frost group, and others.

1.4.2 The Role of Net1 In Vivo and on Tumorigenesis

Net1 overexpression has been reported in several human cancers, including breast cancers, hepatocellular carcinoma, gastric adenocarcinoma, and gliomas (Leyden et al. 2006; Chen et al. 2007; Shen et al. 2008; Y et al. 2010). This correlates with increased tumorigenesis, invasiveness, and metastasis, suggesting an integral role for Net1 and Net1A signaling in metastatic breast cancer. *Net1* signaling is upregulated in 10% of human breast cancers, especially in patients with basal subtype breast cancer. This is correlated with increased activation of the PI3K pathway and breast cancer patients with high *Net1* gene expression typically have shorter distant metastasis free survival (Zuo et al. 2018a).

Many aspects of Epithelial to Mesenchymal Transition (EMT) mimic stages in development and cancer cells often exploit existing signaling networks to make cells more cancer cell stem like. Mesenchymal characteristics are often exhibited by cells during gastrulation and dysregulation of basally localized RhoA activity during the EMT process in gastrulation can lead to a breakdown of the basement membrane. In cancerous cells, extravasation occurs likely with a similar mechanism (Nakaya et al. 2008). Also, during development, RhoA mediated cell migration is important during limb formation and *Net1* expression is required for muscle cell migration. Sonic hedgehog (Shh) signaling promotes the expression of *Net1* early in myogenic differentiation and loss of Shh mediated *Net1* signaling impairs directional migration (Hu et al. 2012). Recent work has also shown that deletion of *Net1* significantly impairs macrophage motility due to impaired RhoA activation, F-actin accumulation, and adhesion *in vitro* (Zuo et al. 2020). *Net1* deletion also contributed to delays in tumorigenesis and reduced cell proliferation and angiogenesis, significantly decreases metastatic burden in PyMT-expressing mice (Zuo et al. 2018b). As the role of *Net1* expression in promoting breast cancer metastasis becomes better established, it is imperative to understand mechanisms that regulate Net1 localization and activity.

1.4.3 Transcriptional Regulation of *Net1*

There are two isoforms of the *Net1* gene, Net1 and Net1A. Net1A does not contain the first three exons of Net1 but instead contains a different first exon than Net1. Early work by the Wang group identified *Net1* expression as one of many actin cytoskeletal associated proteins to be induced by TGF- β . They demonstrated the requirement for Net1 for RhoA-mediated stress fiber formation and hypothesized this increased *Net1* expression was a result of TGF- β activating two waves of JNK signaling, the second being Smad-dependent (Shen et al. 2001). Both Smad 2 and Smad3 have been shown to increase *Net1* expression downstream of TGF- β activation and this newly synthesized Net1 activates RhoA signaling

(Lee et al. 2010). This mechanism was further described by Papadimitriou *et. al.* in 2012 when they established that short term TGF- β treatment selectively induced the Net1A isoform through Smad signaling and MAPK/ERK signaling (Papadimitriou et al. 2012). There is evidence to suggest that this regulation is not unidirectional, as Wei *et. al.* showed that, in zebrafish mesendoderm, Net1 associates with Smad2 and promotes Smad2 transcriptional activity, independent of Net1 GEF activity (Wei et al. 2017). *Net1* mRNA and protein are detectable as early as one hour after TGF- β stimulation and protein levels peak at after three hours. While mRNA can be detected up to 48 hours after TGF- β is present, the rapid decline of protein levels points to regulation through microRNAs, translational regulation, and protein degradation (Papadimitriou et al. 2012). Other transcriptional activators of the *Net1* gene include the progesterone and estrogen receptors. The *Net1* gene contains a PR binding site, identified by Chip-Seq, and PR signaling increases *Net1* transcription (Kougioumtzi et al. 2014). A more recent genome-wide RNA-Seq study determined that increased estrogen signaling in mice can also upregulate *Net1* expression (Yang et al., 2017). Interestingly, treatment with estradiol in MCF7 cells selectively increases transcription of Net1 over Net1A, due to alternative promoter activation (Dutertre et al. 2010). The Net1 isoform does not relocate to the cytosol as readily, so its contribution to oncogenic transformation is likely through its nuclear role, which includes promoting the transition of G₁ to S in the cell cycle and cell proliferation (Dutertre et al. 2010).

1.4.4 Translational Regulation of Net1 in breast cancer

mRNA transcripts must be processed prior to their export to the cytosol for ribosome mediated translation. Alternative poly-adenylation to the 3' untranslated region (UTR) of a transcript has been shown to affect mRNA stability, transcriptional efficiency, and localization of transcription isoforms. Recently, the Lai group demonstrated that metastatic gastric cancer

cells alternatively polyadenylate the 3' UTR of the *Net1* isoform and this shortened transcript stabilizes the mRNA and promotes migration and invasion (Lai et al. 2015).

The presence of *Net1* mRNA throughout the body, but a lack of ubiquitous protein, can be partially explained by regulation by microRNAs (miRNA). To date, two miRNAs have been identified that bind to the 3' UTR of *Net1A*: miR-24 and miR-22. miR-24 is activated by TGF- β signaling, specifically downstream of SMAD3. In breast cancer cells, miR-24 does not achieve the same level of *Net1A* downregulation as proteasome mediated degradation, so it is thought to target a small pool of localized *Net1A* (Papadimitriou et al. 2012). In Chronic Myeloid Leukemia and in non-small cell lung cancer, miR-22 was recently identified as a negative regulator of *Net1* translation, although no work has been done to show its presence or effects in breast cancer yet (Ahmad et al. 2014; Ding et al. 2020). Interestingly, the long noncoding RNA *lncRNA CTC-497E21.4* forms *lnc-RNA-miRNA-mRNA* complex with miR-22 and leads to increased *Net1* translation. As a result, in gastric cancer cells, there is a dynamic relationship between these three types of RNA, and it leads to malignancy through *Net1*-dependent RhoA signaling (Zong et al. 2020).

1.4.5 Post-Translational Regulation of Net1 in breast cancer

The Rho target CNK1 is a scaffold protein that binds *Net1* or p115-Rho GEF as well as MLK2 and MKK7, two kinases in the JNK/MAPK pathway. The formation of this protein complex facilitates GEF mediated JNK/MAPK activation and Jaffe posits that this is a mechanism by which Rho effector activation is compartmentalized within the cell (Jaffe et al. 2005). For example, the complex described here would likely form at the leading edge to activate RhoA mediated F-actin polymerization and stability while a complex comprised of other Rho effectors would function in the trailing edge to execute actomyosin contraction of stress fibers for movement. This is also likely controlled temporally by complexes containing both upstream activators and downstream effectors for rapid modulation, as needed.

Net1A cytosolic localization is also regulated by post-translational modification, and this is often studied in how it affects RhoA and FAK mediated cellular activity. In osteosarcoma, AMPK phosphorylation of Net1A promotes its interaction with Focal Adhesion Kinase (FAK) and phosphorylated Net1A modulates FAK activity in regulating invadopodia and focal adhesion formation (Schaffer et al. 2015). Additionally, Net1A is required for amoeboid invasion and loss of Net1A expression shifts the cell to a more mesenchymal phenotype (β -integrin high, MT1-MMP1 high). This occurs through Net1A controlling FAK activation to drive planar cell movement during ECM invasion (Carr et al. 2013). Downstream of constitutive Rac1 signaling, PAK1 is capable of phosphorylating Net1A on S152, which downregulates Net1A nucleotide exchange activity, providing a mechanism for the classical dogma in motility of Rac1 activation inhibits RhoA (Alberts et al. 2005). However, Rac1 also stimulates JNK phosphorylation of Net1A on S52 (Ulu et al. 2018), which promotes cytosolic localization of Net1A and subsequent RhoA activation, supporting the idea that both Rac1 and RhoA are active in the leading edge of a cell, but their activity is tightly spatiotemporally regulated.

Net1A protein is eventually degraded downstream of both TGF- β and Rac1 activation (Papadimitriou et al. 2012; Song et al. 2015), as constitutive RhoA activation is not conducive to healthy cell motility. Although the ubiquitylation sites are unknown, both acetylation of the N-terminus of Net1A and phosphorylation on serine 52 seem to protect Net1A from proteasome mediated degradation (Song et al. 2015; Ulu et al. 2018). The interaction of Dlg1 and Net1, through its PDZ domain, protects Net1 from proteasome mediated degradation and maximally stimulates RhoA activation, dependent on cell-cell contact (Carr et al. 2009).

The Net1 isoform seems to play a role in cell proliferation, playing a GEF independent role in mitotic progression. Cdk1 directly phosphorylates Net1 on S131 and T146 and inhibits Net1 activation. Inhibition of Cdk1 causes increased Net1 activity, leading to plasma membrane localization and F-actin accumulation in the cytosol. Net1 overexpression has

been shown to cause nuclear deformities and dysregulated mitosis, specifically through spindle polarity defects. The tight control of Net1 mediated RhoA activation appears to be important for maintaining plasma membrane stiffness. In cancer cells, the loss of this organization affects spindle positioning and could drive aneuploidy (Ulu et al. 2021). Although this regulation is downstream of active GTPases, catalytically-inactive Net1 rescued aberrant micronucleation seen in Net1 knockdown cells (Menon et al. 2013). Catalytically-inactive Net1 also binds Caspase Activation and Recruitment Domain Membrane-associated Guanylate Kinase-Like domain (CARMA) proteins to induce NF- κ B activation. NF- κ B plays an important role in cytokine production and cell survival. Stressors, such as ionizing radiation cause DNA damage, which also induces Net1 activation and Net1/RhoA-dependent signaling is required for rapid activation of p38 MAPK (Guerra et al. 2008; AD et al. 2011; Oh and Frost 2014). Although the role of Net1 has mainly been in mitosis, this may shed some light on other transformative properties of nuclear Net1 (Vessichelli et al. 2012).

1.5 Summary

Breast cancer metastasis remains a leading cause of cancer death among women and there is a wealth of evidence to suggest dysregulation of growth factor signaling and GTPase activity are key players of this metastatic spread. Although RhoA is overexpressed in breast cancer, its ubiquitous expression makes it difficult to target therapeutically. Importantly, GTPase overexpression does not confer hyperactivity, allowing us to focus on more activators of GTPase signaling, Rho GEFs, which are expressed in a more tissue specific manner. Net1A is one such Rho GEF whose expression is not only specific to limited tissues, including breast, but also has been implicated for its role in breast cancer. Net1A is one of only two Rho GEFs whose activity is regulated by nuclear sequestration. Downstream of specific stimuli, Net1A relocates and accumulates in the cytosol and promotes RhoA mediated motility and invasion. The unusual regulation of Net1A makes it a fascinating target in breast cancer

metastasis, and this work will provide evidence of a novel axis regulates Net1A subcellular localization and activity. We hypothesize that Src-dependent signaling regulates Net1A cytosolic accumulation to promote breast cancer cell motility and invasion.

Chapter 2: Materials and Methods

2.1 Cell Lines, plasmids, reagents, and pY373 Net1A antibody

MCF7 and MDA-MB-231 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose and glutamine or Dulbecco's modified Eagle's medium nutrient mixture F12 (DMEM/F12), respectively (Hyclone, Logan, UT, USA). All media contained 10% fetal bovine serum (FBS) (Invitrogen, Waltham, MA, USA), 100 µg/ml penicillin and 100 µg/ml streptomycin (HyClone). MCF7 cells were grown in 10% CO₂. MDA-MB-231 cells were grown in 5% CO₂. MCF7 cells were transfected using Lipofectamine Plus or Lipofectamine 2000 reagents (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Short interfering RNA (siRNA) transfection was performed using INTERFERin reagent (PolyPlus Transfection, New York, USA). After plasmid or siRNA transfection, cells were allowed to grow for an additional 48 hours or 72 hours, respectively. As indicated, cells were serum-starved overnight in DMEM plus 0.5% FBS and 1% penicillin-streptomycin. Recombinant human epidermal growth factor (EGF) (R&D Systems, Minneapolis, MN, USA) was used at 100 ng/ml for 15 min. Inhibitors of Abl1/2 (Imatinib, R&D Systems, Minneapolis, MN, USA); GNF5 (SelleckChem, Houston, TX, USA), Src (Bosutinib, R&D Systems), and FAK (PF-582271, Adipogen Life Sciences, San Diego, CA, USA) were dissolved in DMSO. Cells were treated with inhibitors overnight at 10 µM, while in starvation media.

Hemagglutinin (HA)-epitope tagged mouse Net1 and HA-Net1A was as previously described (Qin et al. 2005; Carr et al. 2013b). pcDNA3-Flag-Myc-Net1A 5YF

(Y126F/Y310F/Y311F/Y370F/Y373F), Y126F, Y310/311F, Y370/373F, Y373F, and Y373D were generated by site-directed mutagenesis using Phusion polymerase (Thermo Scientific). pCDNA3-Abl1-His-Flag (#52684) was purchased from Addgene (Deng et al. 2012). Abl1 K290R, P242E/P249E, I164E, and NLS1-3^{mut} (NLS1- K624-8A, NLS2 – K729/R730A, NLS3- R782A/K783A/R784A) were generated by site directed mutagenesis. cDNA inserts for all point mutants were fully sequenced to confirm correct DNA sequence. Wild type RhoA cDNA from pGEXKG-RhoA was PCR amplified and subcloned into the pCMV5M vector to generate pCMV5M-RhoA. A cDNA for constitutively active chicken c-Src was a kind gift of Jeffrey Rosen (Baylor College of Medicine). The active c-Src cDNA was subcloned into pCMV5. Control and pan-Net1 isoform targeting siRNAs were as described (Carr et al., 2013).

To produce a polyclonal antibody to phosphorylated Y373 of Net1A, a mouse pY373 peptide (RHSYQV-pY-RQPIPC) was conjugated to Keyhole limpet hemocyanin (KLH) and used to immunize two New Zealand white rabbits, according to the service provider's protocol (Pocono Rabbit Farm and Laboratory, Canadensis, PA, USA). Unpurified rabbit serum from one of the two rabbits was characterized for specificity towards pY373 Net1A and used for all western blotting experiments.

2.2 Immunofluorescence microscopy

MCF7 cells were plated on acid-washed glass coverslips 24 hours prior to transfection. For immunofluorescent detection of proteins, cells on coverslips were fixed with 4% paraformaldehyde in PBS for 5 minutes at room temperature (ThermoScientific, Waltham, MA, USA), washed with PBS, and transferred to a humidified chamber. Cells were permeabilized with 0.2% Triton-X 100 in PBS and blocked with 2.5% normal goat serum (NGS, Vector Labs, San Francisco, CA, USA) for 30 minutes at room temperature. Cells were

incubated with the relevant primary antibodies diluted in 2.5% NGS in PBS + 0.2% Tween 20 (PBST) for 1 hour at 37°C. For detecting pMLC2, cells were incubated with primary antibody diluted in 2.5% NGS in PBS (no Tween 20), overnight at 4°C. After washing three times in PBST, cells were incubated with secondary antibodies diluted in 2.5% NGS plus 4',6-diamidino-2-phenylindole (DAPI) (1 µg/ml) (Sigma-Aldrich, Burlington, MA, USA), and AlexaFluor 647-phalloidin (Invitrogen, Eugene, OR, USA) for 30 minutes at 37°C. Secondary antibodies were anti-mouse-IgG or anti-rabbit-IgG conjugated to AlexaFluor 488, and anti-mouse-IgG or anti-rabbit-IgG conjugated to AlexaFluor 594 (Invitrogen, Eugene, OR, USA). Cells were then washed three times with PBST, rinsed with water, and mounted on glass slides with Fluormount reagent (Invitrogen). Fluorescent staining was visualized using a Zeiss Axiophot epifluorescence microscope and image acquisition was performed using Axiovision software. To evaluate Net1A localization, cytosolic-to-nuclear ratios were calculated as previously described in detail (Song et al. 2015). Antibodies used for immunofluorescence were as follows: mouse anti-HA tag (1:100, sc-7392, Santa Cruz Biotechnology); rabbit anti-HA (1:1000, C29F4, Cell Signaling); mouse anti-Myc epitope 9E10 (1:100, National Cell Culture Center); rabbit anti-Myc epitope (1:100, 71D10, Cell Signaling), rabbit anti-pMLC2 (1:100, 3671, Cell Signaling); mouse anti-Flag M2 (1:250, F3165, Sigma).

2.3. Immunoprecipitation and western blotting

To determine if constitutively active Src stimulated tyrosine phosphorylation of either Net1 isoform (**Fig. 4A**), transfected cells were washed with PBS and harvested in Triton lysis buffer (0.5% Triton-X 100, 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 50 mM NaF, 80 mM β-glycerophosphate, 1 mM Na₂VO₃, 10 µg/mL pepstatin A, 10 µg/mL leupeptin, 2 µg/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride). To immunoprecipitate HA-epitope tagged Net1 and Net1A, lysates were incubated with 2 µg mouse anti-HA antibody and 40 µL

of a Protein A-Sepharose (Rockland, Philadelphia, PA, USA) slurry for 2 hours at 4°C. Sepharose beads were pelleted by centrifugation and washed with Wash buffer (20 mM Tris pH 8.0, 150 mM NaCl, 0.5% Triton-X 100). Beads were resuspended in equal volumes of water and 2X Laemmli sample buffer and boiled for 5 minutes in the presence of 1 mM dithiothreitol (DTT). Samples were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to 0.45 µm pore polyvinylidene difluoride (PVDF) membranes, and blocked in 5% nonfat dry milk, or 1% bovine serum albumin (BSA) for phospho-tyrosine blots, prior to incubation with relevant antibodies overnight at 4°C. Membranes were then washed three times with PBST, incubated with the relevant horseradish-peroxidase (HRP)-linked secondary antibodies for 30 minutes at room temperature, washed 3 times with PBST, and immunoreactive bands were detected by enhanced chemiluminescence (ECL) using either X-ray film or an Azure C280 Chemiluminescent Western Blot Imager, according to the manufacturer's instructions. The following antibodies were used: mouse anti-phospho-tyrosine (1:1000, 9411S, Cell Signaling), rabbit anti-HA (1:1000, C29F4, Cell Signaling).

To determine if Net1A was phosphorylated on Y373 (**Fig 5B, 9H, 10A**), transfected cells were washed with PBS and lysed in RIPA lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton-X 100, 0.5% Deocycholate, 0.1% SDS, 80 mM β-glycerophosphate, 1 mM Na₂VO₃, 10 µg/mL pepstatin A, 10 µg/mL leupeptin, 2 µg/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride, 25 mM N-ethyl malamide, and 10 µM MG132). Samples were passed 10 times through a 20-gauge needle and 10 times through a 27-gauge needle to shear DNA, and clarified by centrifugation at 16,100 x g for 10 minutes at 4°C. Immunoprecipitation of HA-tagged proteins and western blotting was done as described above. The following antibodies were used: rabbit anti-Flag (F2555, Sigma, 1:1000), mouse anti-Src B12 (SC-8056,

Santa Cruz, 1:1000), rabbit anti-HA (3724, Cell Signaling, 1:1000), rabbit anti- c-Abl1 (2862, Cell Signaling, 1:100), and rabbit anti-phosphotyrosine Y373 (pY373, described above).

For western blotting of whole cell lysates, samples were lysed in either SDS-Urea lysis buffer (Tris-HCl pH 6.8, 2% SDS, 20% glycerol, 6M Urea) (**Fig. 9 E-G, 17B, 18B**) or 2% SDS lysis buffer (2.0% SDS, 20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 80 mM β -glycerophosphate, 1 mM Na_2VO_3 , 20 mM NaF, 10 $\mu\text{g}/\text{mL}$ pepstatin A, 10 $\mu\text{g}/\text{mL}$ leupeptin, 2 $\mu\text{g}/\text{mL}$ aprotinin, 1 PMSF) for the detection of endogenous Net1 (**Fig. 15B**). Harvested cells were sonicated for 10-30 seconds and boiled for 5 minutes in Laemmli sample buffer plus 1 mM DTT. Western blotting was performed as described above. The following antibodies were used: mouse anti-phosphotyrosine (1:1000, 9411S, Cell Signaling), mouse anti-Flag M2 (1:100, F3165, Sigma), mouse anti-GAPDH (1:1000, sc-47724, Santa Cruz), mouse anti-Myc epitope 9E10 (1:100, National Cell Culture Center). All Western blots were quantified using ImageJ software.

2.4 Identification of Src stimulated tyrosine phosphorylation of Net1A

Ten, 10-cm plates of HeLa cells were co-transfected with HA-Net1A and constitutively active Src and allowed to grow for 48 hours. Cells were then lysed in RIPA buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 1 mM DTT, 1 mM EDTA, 50 mM NaF, 80 mM β -glycerophosphate, 1 mM sodium orthovanadate, 10 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ pepstatin A, 2 $\mu\text{g}/\text{ml}$ aprotinin, 1 mM PMSF) and sonicated for 15-30 seconds to fragment DNA. The insoluble portion was pelleted by centrifugation at 16,100 x g for 10 minutes at 4°C. The soluble fraction was pre-cleared by incubating with 2 μg normal IgG + 40 μL of a 50% slurry of Protein A-Sepharose at 4°C with gentle rocking for 30 minutes, followed by centrifugation to pellet beads. Once pelleted, the supernatant was transferred to a new tube and incubated with 2 μg of mouse anti-HA antibody (SC- 7392, SantaCruz

Biotechnology, CA, USA) at 4°C for 1 hour with gentle rocking, followed by the addition of Protein A-Sepharose beads and further incubation for an additional hour.. The beads were re-pelleted and the supernatant was aspirated, the pellet was washed three times with wash buffer (20 mM Tris pH 8, 250 mM NaCl, 0.5% Triton X-100). The beads were then suspended in 40 µl of 2x Laemmli Sample Buffer with DTT, boiled for 5 minutes, and resolved on a 10% SDS-PAGE gel. The appropriate band was excised from a Coomassie-stained gel and sent for mass spectrometry analysis (LC-MS/MS) at the Taplin Mass Spectrometry Facility (Harvard Medical School).

2.5 Subcellular Fractionation

MDA-MB-231 Cells were plated in 10 cm dishes and allowed to grow to 85% confluence and subsequently treated with either DMSO or GNF5 (10 µM) overnight. Cells were harvested and fractionated using the Subcellular Protein Fractionation Kit for Cultured Cells (Thermo Scientific, Rockford, IL, USA) according to manufacturer's instructions. The protein concentration of each sample was quantified using the Pierce BCA Protein Assay kit (ThermoFisher, Rockford, IL, USA) and equal amounts of protein for each fraction were resolved by SDS PAGE, transferred to PVDF, blocked with 5% milk, and probed with mouse anti Net1 (1:50, sc-271941, Santa Cruz), rabbit anti HSP90 (1:500, SPA-830, Enzo Life Sciences), mouse anti-Lamin A/C (1:1000, sc-376248, Santa Cruz).

2.6 *In vitro* kinase assays

Glutathione S-transferase (GST)-Net1A was expressed in BL21DE3 *E. coli* and affinity purified using glutathione–agarose (Pierce), as previously described (Qin et al. 2005). pGEXKG-Net1A plasmid was transformed into BL21(DE3) *Escherichia coli* (Stratagene, La

Jolla, CA, USA) and protein expression was induced by 400 μ M isopropyl 1-thio- β -D-galactopyranoside (IPTG) once the culture had reached an OD of $A_{600} = 0.8$. The culture was further incubated with IPTG for 4 hours at 37°C. Cells were pelleted by centrifugation, lysed by addition of lysozyme, and then sonicated. Insoluble proteins were pelleted by centrifugation at 20,000 x g and GST-Net1A in the soluble fraction was purified using glutathione-agarose affinity chromatography. Purified proteins were dialyzed in buffer A (20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 10% glycerol) and stored in aliquots at -80°C. *In vitro* kinase assays were performed with recombinant Abl1 (Fischer), Src (R&D Systems), and Focal Adhesion Kinase, catalytic domain (FAK) (R&D Systems) purified from Sf9 cells. For kinase reactions, GST-Net1A (2 μ g), GST (0.5 μ g), or purified cortactin (LSBio, Seattle, WA, USA) (0.5 μ g) was mixed with Src (0.8 μ g), Abl1 (0.2 μ g), or FAK (0.1 μ g) in the presence of 2 μ M ATP, 1 mM DTT, and Kinase Buffer (20 mM HEPES, pH 7.5, 100 mM NaCl, 0.05% NP40, 10 mM $MgCl_2$). Reactions were incubated for 30 min at 30°C and terminated by adding Laemlli Sample Buffer. Samples were resolved by SDS-PAGE, transferred to PVDF, blocked with 5% Bovine Serum Albumin (BSA), and probed with the rabbit anti-pY373 antibody, mouse anti-phospho-tyrosine (1:1000, 9411S, Cell Signaling), and rabbit anti Net1 antibody (1:500, HPA020068, Sigma).

2.7 GST-RBD pull down assays

MCF7 cells were transfected with plasmids expressing Flag-Myc-epitope tagged wild type Net1A, Net1A Y373D, or Net1A Y373F, along with pCMV5M-RhoA. 48 hours after transfection, cells were starved in serum-free media for 4 hours, lysed in RBD lysis buffer (50 mM Tris pH 7.5, 1% Triton-X 100, 0.5% deoxycholate (DOC), 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, 5 μ g/ml leupeptin, 5 μ g/ml pepstatin A, and 50 μ g/ml PMSF), and clarified by centrifugation at 16,100 x g at 4°C. The protein concentration of each sample was quantified

using the Pierce BCA Protein Assay kit. Equal amounts of lysate were incubated with 10 μ L of Rhotekin-RBD Beads (Cytoskeleton, Denver, CO, USA) at 4°C. After 1 hour, the beads were pelleted by centrifugation, the supernatant was removed, and beads were washed three times each with 1 mL of ice cold wash buffer (50 mM Tris pH 7.5, 1% Triton-X 100, 150 mM NaCl, 10 mM MgCl₂, 5 μ g/ml leupeptin, 5 μ g/ml pepstatin A, and 50 μ g/ml PMSF). Beads were resuspended in 45 μ L of water and 15 μ L 4 x Laemmli sample buffer. Samples were resolved by SDS-PAGE, transferred to PVDF, blocked in 5% milk and probed with mouse anti-Myc epitope 9E10 (1:100, National Cell Culture Center) and mouse anti-GST (1:1000, Santa Cruz).

2.8 Cell motility and invasion assays

MCF7 cells were transfected with empty pBabepuro and Myc-epitope-tagged β -Galactosidase, Flag-Myc-epitope tagged wild type Net1A, Net1A Y373F, or Net1A Y373D. Twenty-four hours later, the cells were treated with 10 μ g/mL puromycin overnight. The following morning, the selection media was replaced with full-serum media for 2 hours to allow for recovery. The cells were then trypsinized and 250,000 cells were seeded in 500 μ L DMEM without serum in the upper chamber of an 8- μ m pore transwell. For invasion assays, the transwells were coated with Matrigel by the manufacturer (Corning Life Sciences, Corning, NY, USA). The bottom well contained DMEM plus EGF (100 ng/mL). Cells were allowed to migrate for 6 hours for migration assays, and 20 hours for invasion assays. At the end of the incubation period, cells were removed from the upper surface of the membrane by scrubbing with a cotton-tipped swab, twice. Membranes were fixed with methanol for 5 minutes at room temperature, rinsed with distilled water, stained with 1 μ g/mL DAPI in PBST for 20 minutes at room temperature in the dark, rinsed with water, and inverted to dry in the dark. The membranes were carefully excised and mounted on a microscope slide with Fluormount

reagent. Cells were imaged at 20X magnification. 10 fields were imaged per membrane. The number of cells were counted in each field and averaged for quantification.

2.9 Statistical analysis

Comparisons among different treatment groups for western blot and immunofluorescence experiments were examined using unpaired Student's t-tests or non-parametric ANOVA, as appropriate. $P < 0.05$ was considered statistically significant. GraphPad Prism 5 software was employed for statistical analyses. All experiments were repeated at least three times.

Chapter 3. Abl-Dependent Phosphorylation of Net1A on Y373 is required for cytosolic accumulation

3.1 Introduction

Rho GTPases regulate many basic cellular functions that impact tumor progression, including cell migration, extra-cellular matrix (ECM) invasion, and cell proliferation (Lin and Van Golen 2004; Raftopoulou and Hall 2004; Wheeler and Ridley 2004; Pertz et al. 2006; Le Clainche and Carlier 2008; Narumiya and Thumkeo 2018). Within this gene family, Rac1, Cdc42, and RhoA have been the focus of the majority of studies. Like Rac1 and Cdc42, RhoA makes essential contributions to cell motility and invasion that are controlled by its tight spatiotemporal regulation. Importantly, dysregulated RhoA activation can promote oncogenic transformation. Although wild type RhoA is often overexpressed in cancers, including breast cancers, its overexpression alone does not confer increased activity, as wild type RhoA still requires activation by upstream guanine exchange factors (GEFs) (Fritz et al. 1999; Fritz et

al. 2002; Orgaz et al. 2014). These enzymes promote the release of GDP by Rho proteins, thereby allowing GTP binding (Rossman et al. 2005). Thus, it is important to identify RhoA GEFs that drive cancer progression.

The neuroepithelial transforming gene 1 (Net1) is a RhoA/RhoB-specific GEF that is overexpressed in multiple cancers, including breast cancer (Leyden et al. 2006; Shen et al. 2008; Dutertre et al. 2010a; Tu et al. 2010; Bennett et al. 2011; Kawata et al. 2012; Ahmad et al. 2014a). This is consequential, as overexpression of Net1, or expression of a Net1-dependent gene expression signature, is associated with reduced survival in breast cancer patients (Dutertre et al. 2010b; Zuo et al. 2018). Furthermore, co-expression of Net1 and $\beta 4$ Integrin protein is correlated with shorter distant metastasis-free survival and worse overall survival in treatment-resistant, estrogen receptor positive breast cancer patients (Gilcrease et al. 2009). Because Net1 overexpression is associated with more aggressive disease in breast cancer, understanding the mechanisms by which Net1 is regulated is important.

A critical function of Net1 is to control cancer cell motility. Our lab and others have shown that Net1 expression is required for cancer cell motility and invasion *in vitro* (Leyden et al. 2006; Carr et al. 2013b; Chen et al. 2021). Net1 is also important for metastasis in mouse models of disease, including breast cancer (Zuo et al. 2018). Two isoforms of Net1 exist in most cells, called Net1 and Net1A, which differ in their N-terminal regulatory domains (Qin et al. 2005). Both Net1 isoforms demonstrate unusual subcellular localization for Rho GEFs, as they are sequestered in the nucleus to prevent aberrant RhoA activation at the plasma membrane (Schmidt and Hall 2002; Qin et al. 2005). Importantly, we have shown that growth factors that stimulate breast cancer cell motility, such as LPA and EGF, as well as integrin engagement, cause relocalization of the Net1A isoform to the cytosol to drive RhoA-dependent signaling, actin cytoskeletal rearrangement, and cell motility (Carr et al., 2013a; Carr et al., 2013b; Song et al., 2015). Moreover, we have shown that EGF stimulation

promotes Net1A acetylation within one of its two nuclear localization signal sequences, as well as phosphorylation by the JNK family of MAPKs. Both of these modifications slow the rate of Net1A re-import into the nucleus, thereby causing cytoplasmic accumulation (Song et al. 2015; Ulu et al. 2018). We have also shown that proteasomal degradation of Net1A limits its cytosolic accumulation after integrin engagement (Carr et al. 2013a). However, it is unknown whether additional mechanisms exist to control the subcellular localization of Net1A.

Many cell motility stimuli activate the Src and Abl families of cytosolic tyrosine kinases. These kinases, in turn, regulate the spatial and temporal localization and activity of Rho GEFs and RhoGAPs, thus ensuring that the cell can precisely control the activation of different Rho family members (Timpson et al. 2001; Bradley and Koleske 2009; Wang 2014; Wang and Pendergast 2015). Both Src and Abl kinases function downstream of integrins and growth factor receptors and are required for efficient cell motility and invasion (Plattner et al., 1999; González et al., 2006; Peacock et al., 2010). Because they often function in the same signaling pathways, Src and Abl1/2 interact with many of the same molecules, including paxillin and FAK (Weng et al. 1993, Salgia et al. 1995, Cobb et al. 1994, Gotoh et al. 1995). Hyperactivation of either Src or Abl1 can result in oncogenic transformation and can increase cancer cell motility and invasion in breast cancer cells (Srinivasan and Plattner 2006; Finn 2008; Sen and Johnson 2011). Moreover, patients with elevated Src kinase activity have reduced distant metastasis free survival (RB and TJ 2000; Elsberger et al. 2009).

Because Src plays such a central role in regulating cancer cell motility, we examined whether it also controlled the subcellular localization and function of Net1 isoforms. We found that Src promotes Abl1-dependent phosphorylation of Net1A on Y373 to control Net1A cytosolic accumulation and signaling to RhoA. Additionally, substitution of this site with aspartate causes Net1A to accumulate in the cytosol without ligand stimulation and drives breast cancer cell motility and invasion. We also found that Net1A activity is necessary for

Abl1-dependent cell motility. These data indicate that Net1A function is precisely regulated by Src and Abl and suggest that targeting of Abl1 in Net1 overexpressing cancers would have therapeutic value.

3.2 Results

3.2.1. Src dependent phosphorylation relocates the Net1A isoform, not Net1

Src is well known to control RhoA activation following receptor tyrosine kinase activation and integrin engagement (Playford and Schaller 2004; Huveneers and Danen 2009). Because Net1A cytoplasmic accumulation is stimulated by both EGFR and integrin activation, we tested whether expression of constitutively active Src could stimulate relocation of Net1A to the cytoplasm. MCF7 cells were transfected with HA-epitope tagged, wild type Net1 or wild type Net1A, plus or minus constitutively active Src (Src*), and then serum-starved overnight. Epitope tagged Net1 isoforms were transfected because antibodies capable of distinguishing between Net1 isoforms do not exist (Ulu et al. 2018). We observed that co-expression of active Src was ineffective at driving cytoplasmic localization of the longer Net1 isoform (**Fig. 3A**), but significantly elevated the cytoplasmic localization of the Net1A isoform (**Fig. 3B**). To quantify this effect, we calculated the ratio of cytosolic to nuclear staining. Cells were co-stained with phalloidin to define the cell boundary and DAPI to define the nucleus. The fluorescence intensity of nuclear Net1 staining was subtracted from fluorescence intensity of Net1 staining in the whole cell to obtain the amount of Net1 staining in the cytosol. Net1 isoform localization was then quantified as the ratio of cytosolic to nuclear staining. We have shown previously that assessing Net1 isoform localization in this manner takes into account the inherent variation in transgene expression in transiently transfected cells and is linear over a wide range of expression (Song et al. 2015; Ulu et al. 2018). As

shown in Figure 1C, although there was significant variability between cells, expression of constitutively active Src caused a 4-fold increase in the median ratio of cytoplasmic to nuclear staining for Net1A. On the other hand, active Src did not significantly change the localization of the Net1 isoform. These observations are consistent with previous observations indicating that EGFR and integrin activation stimulates cytosolic localization of Net1A, but not Net1 (Carr et al. 2013a; Ulu et al. 2018).

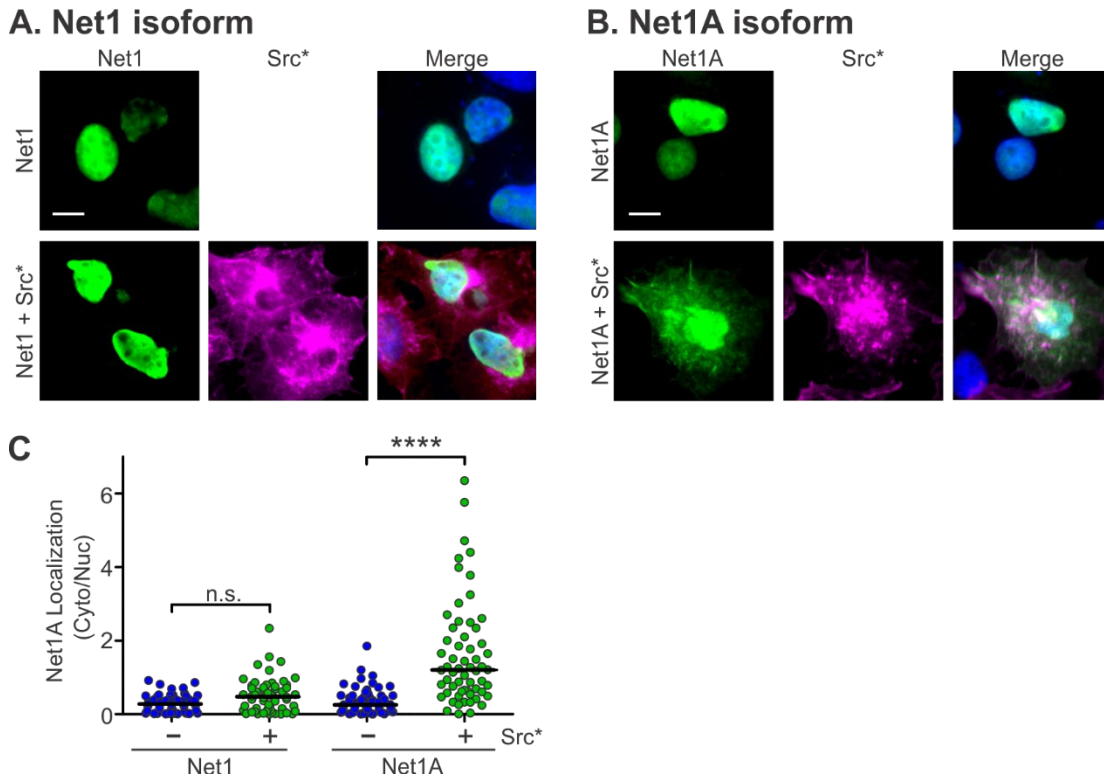


Figure 3. Constitutively active Src stimulates cytosolic localization of the Net1A isoform. MCF7 cells were transfected with expression plasmids for Flag-Myc-Net1 or Flag-Myc-Net1A with or without constitutively active Src (Src*). Cells were serum-starved overnight, fixed, and stained for Myc-epitope (green), Src (magenta), and DNA (blue). Representative images of Net1 (A) or Net1A (B) are shown. (C) Quantification of Net1 and Net1A localization. Data were obtained from three independent experiments. In each experiment, at least 20 cells per condition were quantified. Lines indicate the median values. **** = $p < 0.0001$; n.s. = not significant. Scale bars = 10 μm .

3.2.2. Five tyrosine residues in Net1A are phosphorylated in a Src dependent manner

To identify the mechanism by which Src controls Net1A subcellular localization, we assessed whether Src stimulates tyrosine phosphorylation of Net1A. Thus, constitutively active Src was co-expressed with either HA-Net1 or HA-Net1A in MCF7 cells. The cells were then serum-starved overnight and Net1 isoforms were immunoprecipitated using an anti-HA antibody. Immunoprecipitates were resolved by SDS-PAGE and tested for tyrosine phosphorylation by western blotting with a phospho-tyrosine-specific antibody. Surprisingly, Dr. Carr observed that both Net1 and Net1A were tyrosine phosphorylated in response to active Src expression (**Fig. 4A**). Although Net1 isoforms appeared equally well tyrosine phosphorylated, we decided to identify the Src-stimulated tyrosine phosphorylation sites within Net1A in order to assess whether they were involved in the mechanism by which Src stimulates Net1A cytosolic localization. HeLa cells were transfected with active Src and HA-Net1A, serum-starved, and the HA-Net1A was immunoprecipitated and resolved by SDS-PAGE. The HA-Net1A band was then visualized by Coomassie staining, excised, and subjected to phospho-peptide analysis using LC-MS/MS (Taplin Mass Spectrometry Facility). This analysis showed that expression of active Src stimulated the phosphorylation of 4 residues within Net1A, namely Y126, Y310, Y370, and Y374 (**Fig. 4B**). To determine the necessity of these sites for Net1A relocalization, we used site-directed mutagenesis to generate a 5YF mutant, where each tyrosine phosphorylated residue, plus Y311, was mutated to phenylalanine. Y311 was included in this analysis to preclude its phosphorylation when Y310 was mutated. Constitutively active Src was then co-expressed with wild type Net1A or Net1A-5YF in MCF7 cells, and the cells were serum-starved as before. We observed that active Src was unable to stimulate cytosolic localization of Net1A-5YF, suggesting one or more of the tyrosine phosphorylation sites played an important role in mediating Src-stimulated Net1A relocalization (**Fig. 4C-E**).

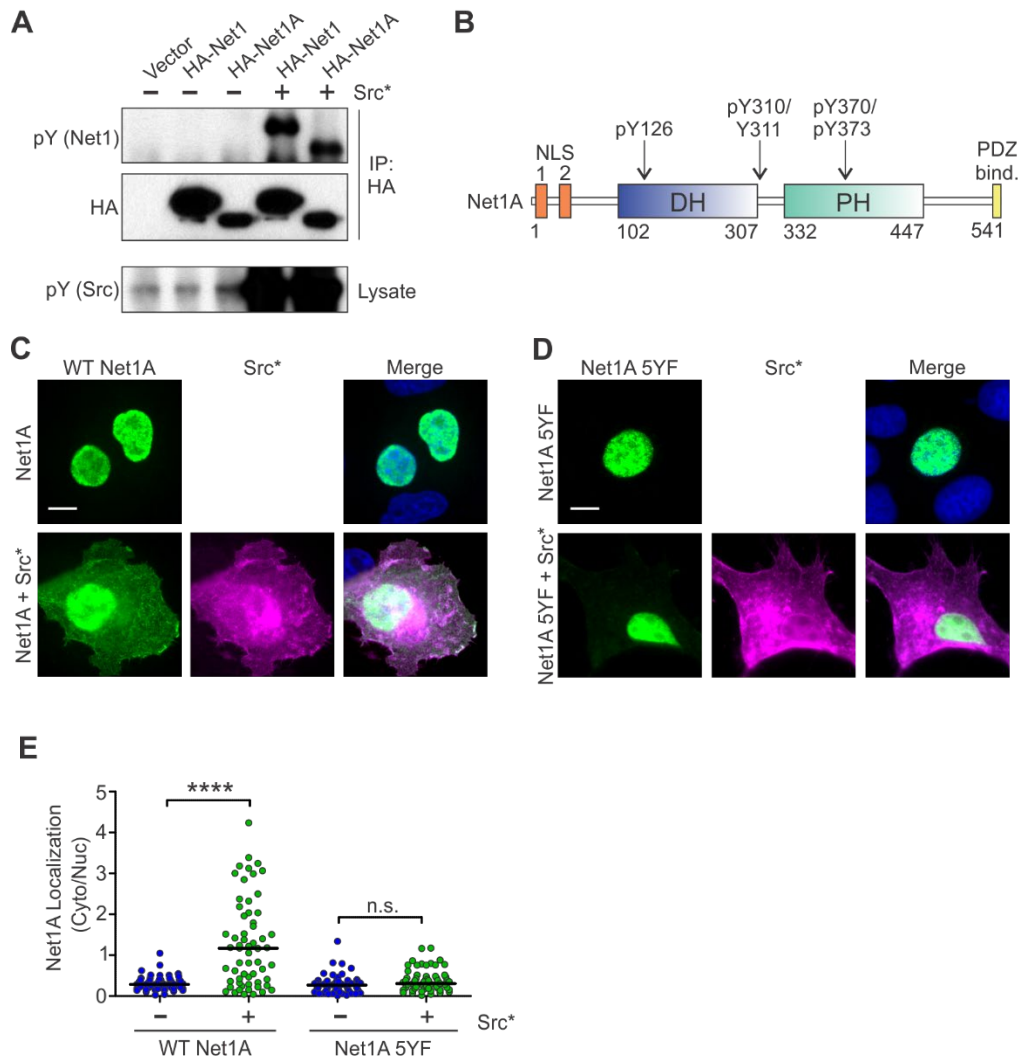


Figure 4. Phosphorylation of Net1A on Y373 is necessary for Src-stimulated cytosolic

localization. (A) MCF7 cells were transfected with HA-Net1 or HA-Net1A with or without constitutively active Src. Cells were serum-starved and Net1 isoforms were immunoprecipitated using an anti-HA antibody. The presence of phospho-tyrosine and HA were detected by western blotting. Shown is a representative experiment of three independent experiments. Experiments performed by Dr. Heather S Carr, used with permission (B) Schematic of Net1A showing Src stimulated phosphorylation sites (arrows) and locations of nuclear localization signal sequences (NLS), Dbl homology and pleckstrin homology domains (DH, PH, respectively), and PDZ binding domain. Numbers refer to the amino acid residues of wild type mouse Net1A. (C, D) MCF7 cells transfected with expression plasmids for wild type Net1A (WT) or Net1A 5YF (5YF) with or without constitutively active Src. Cells were serum starved, fixed, and stained for Myc-epitope (green), Src (magenta), and DNA (blue). Representative images of Net1A (C) or Net1A 5YF (D) are shown. (E) Quantification of Net1A or Net1A 5YF localization. Data were obtained from four independent experiments. At least 20 cells per condition per experiment were quantified. Lines indicate median values. **** = $p < 0.0001$; n.s. = not significant. Scale bars = 10 μm

3.2.3 Mutation of tyrosine 373 is the single mutation with most significant decrease in Src stimulated accumulation

To determine which phosphorylation sites were most important for Net1A relocalization, we created Net1A constructs that contained single or double mutations of the identified phosphorylation sites and co-expressed these mutants with constitutively active Src. Dr. Ulu observed that mutation of Y126 was without effect. Mutation of Y310/Y311 caused a trend towards decreased in Src-stimulated cytosolic localization relative wild type Net1A, but this was not statistically significant. On the other hand, mutation of Y370/Y373 together, or Y373 alone essentially nullified the effect of active Src on Net1A localization (**Fig. 5A**). These data indicate that phosphorylation of Net1A on Y373 is necessary for Src-stimulated cytosolic re-localization.

To confirm that Src stimulated Net1A phosphorylation on Y373, we created an antibody specific for phosphorylated Y373. Using this antibody, we were able to demonstrate that Net1A was phosphorylated on Y373 in response to active Src co-expression. The antibody was specific for pY373, as mutation of this site to phenylalanine eliminated detection of phosphorylated Net1A by western blotting (**Fig. 5B**). Thus, these data confirm that Src can stimulate Net1A phosphorylation on Y373 and validate the use of this antibody for further analysis.

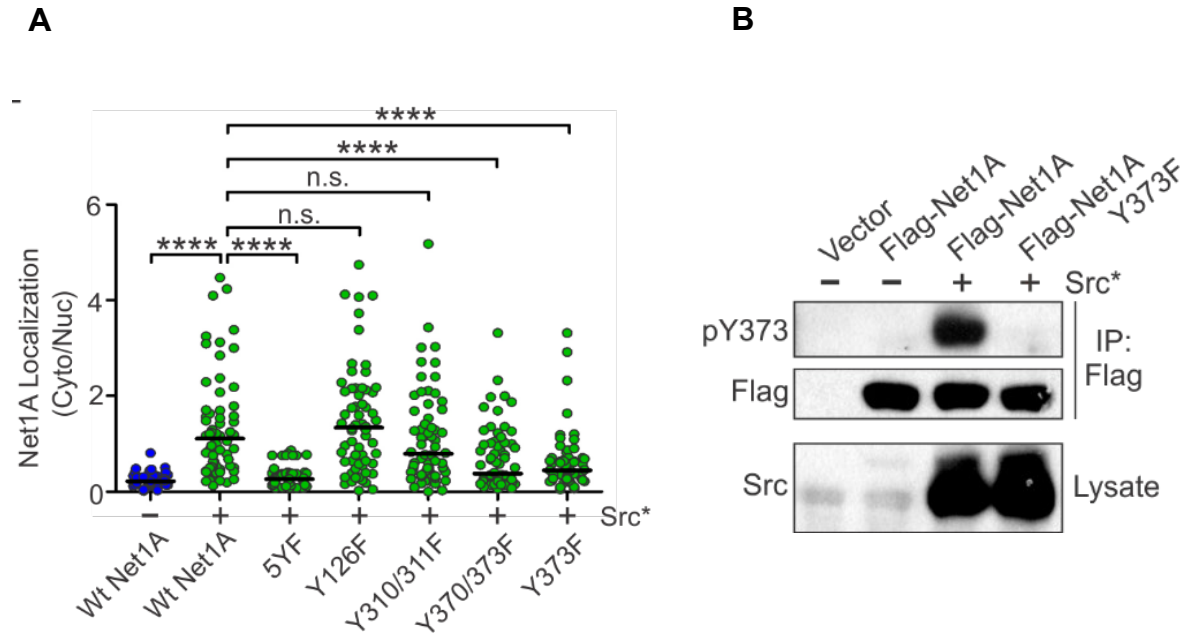


Figure 5. Phosphorylation of Net1A on Y373 is necessary for Src-stimulated cytosolic localization (A) Quantification of the localization of wild type Net1A and the Net1A mutants shown in the presence of active Src. At least 20 cells per condition per experiment were quantified. Lines indicate median values. **** = $p < .0001$; n.s. = not significant. (B) MCF7 cells were transfected with Flag-Myc-Net1A or Flag-Myc-Net1A Y373F plus or minus active Src. Cells were serum-starved and Net1A proteins were immunoprecipitated using an anti-Flag antibody. pY373, Flag-epitope, and Src were detected by western blotting. Data were obtained from three independent experiments. Experiments performed by Dr. Arzu Ulu, used with permission.

3.2.4 ATP-competitive inhibition of Src, FAK, or Abl prevent EGF stimulated accumulation of Net1A

EGFR stimulation activates Src, which can phosphorylate and activate three downstream non-receptor tyrosine kinases (NRTKs) in epithelial cells: Focal Adhesion Kinase (FAK), Abelson non-receptor kinase 1 (Abl1) and Abl2 (Plattner et al. 1999; Mitra and Schlaepfer 2006; Colicelli 2011; Mader et al. 2011) (**Fig. 6A**). To determine which kinase(s) mediated Net1A cytosolic localization downstream of EGF, we inhibited their activities using small molecule inhibitors. MCF7 cells were transfected with HA-Net1A and serum-starved overnight. During this starvation period the cells were also incubated with small molecule inhibitors of Src, FAK, or Abl1/2. The following morning the cells were stimulated with EGF for 15 minutes, which we have shown previously to be the length of time required to maximally stimulate Net1A cytosolic localization in MCF7 cells (Song et al. 2015; Ulu et al. 2018). Interestingly, inhibitors of Src (bosutinib), FAK (PF-562271), and Abl1/2 (imatinib) were all effective at blocking EGF-stimulated Net1A cytosolic localization (**Fig. 6B**). These data suggested that these small molecule inhibitors were not specific for their intended kinases (Rossari et al. 2018), or that Net1A relocalization was dependent on Abl1/2 activity, which function downstream of Src and FAK (Plattner et al. 1999; Mitra and Schlaepfer 2006; Colicelli 2011; Mader et al. 2011).

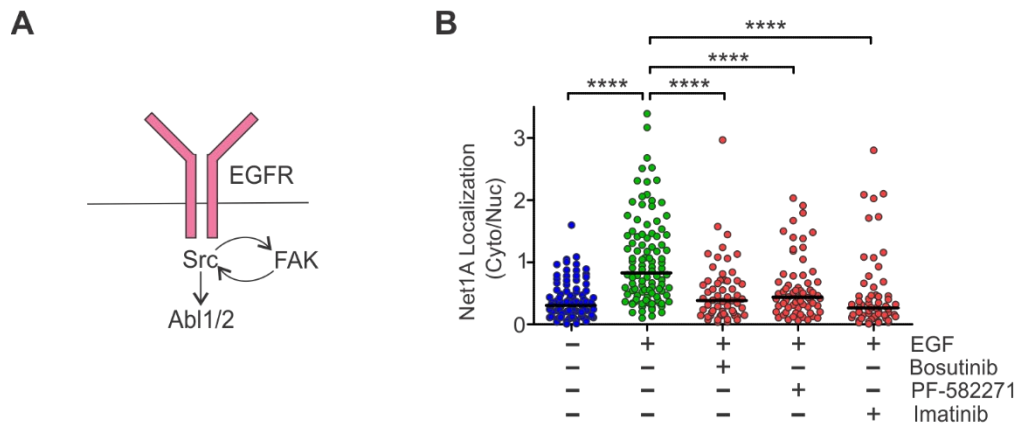


Figure 6. ATP-competitive inhibition of Src, FAK, or Abl prevent EGF stimulated accumulation of Net1A. (A) Schematic of non-receptor tyrosine kinase cascade activated by ligand stimulation of Epidermal Growth Factor Receptor (EGFR). Direct activation of Src by EGFR leads to Focal Adhesion Kinase (FAK) activation. Increased FAK activity drives increased Src activity and, subsequently, activation of Abelson non-receptor tyrosine kinases (Abl1/2). (B) MCF7 cells were transfected with HA-Net1A, serum-starved overnight, stimulated with EGF (100 ng/mL) for 15 min, fixed, and stained for HA-Net1A localization. Prior to EGF stimulation cells were pretreated with DMSO, the Src inhibitor Bosutinib (10 μ M), the FAK inhibitor PF-582271 (10 μ M), or the Abl inhibitor Imatinib (10 μ M) overnight. Data were obtained from three independent experiments with at least 20 cells quantified per condition per experiment. Lines indicate median values. **** = $p < 0.0001$; n.s. = not significant.

3.2.5 Allosteric inhibition of Abl1/2 prevents EGF stimulated accumulation

In support of Abl1/2 mediating Net1A cytosolic localization, the region surrounding Y373 (LYQ-Y373-RQPIP) correlates most closely with a consensus sequence for Abl substrates (AEVIV-Y-**A**APF) (Songyang et al. 1995). Thus, we tested whether an allosteric inhibitor of Abl1/2, GNF5 (Zhang et al. 2010; Karunakaran et al. 2015), was capable of blocking EGF-stimulated cytosolic localization of Net1A. We observed that GNF5 pretreatment significantly decreased EGF-stimulated Net1A cytosolic relocation, supporting the notion that Abl1/2 mediate Net1A relocation downstream of EGFR and Src (Fig. 7 A, B).

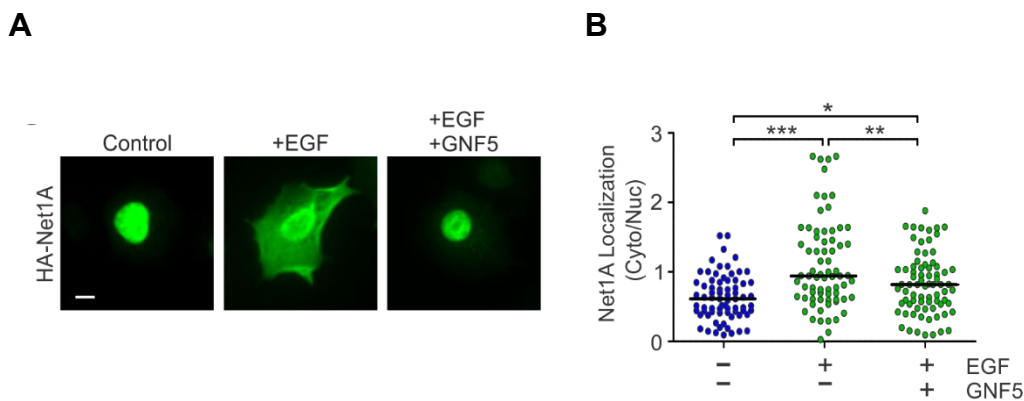


Figure 7. Allosteric Abl inhibition prevents EGF Stimulated Accumulation. (A) Representative images of MCF7 cells transfected with HA-Net1A, serum-starved and treated with or without EGF (100 ng/mL) for 15 minutes. Some cells were incubated with GNF5 (10 μ M) overnight prior to EGF treatment. (B) Quantification of Net1A localization. Data were obtained from three independent experiments. At least 20 cells per condition per experiment were quantified. Lines indicate median values. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. Scale bars = 10 μ m.

3.2.6 Abl1 phosphorylates Net1A on Y373 *in vitro*

To assess whether Abl1 was capable of directly phosphorylating Net1A on Y373, we performed *in vitro* kinase assays with purified, recombinant Src, Abl1, FAK, and Net1A. Net1A phosphorylation was assessed by western blotting using the pY373 antibody that we created, and the activity of each kinase was confirmed by western blotting for phospho-tyrosine of the autophosphorylated kinases as well as cortactin, which is phosphorylated to some extent by each of these kinases (Tomar et al. 2009; Mader et al. 2011; Boateng and Huttenlocher 2012; Cleary et al. 2014). Although all three kinases exhibited significant autophosphorylation and cortactin phosphorylation activities, only Abl1 was capable of phosphorylating Net1A on Y373 *in vitro* (**Fig. 8**).

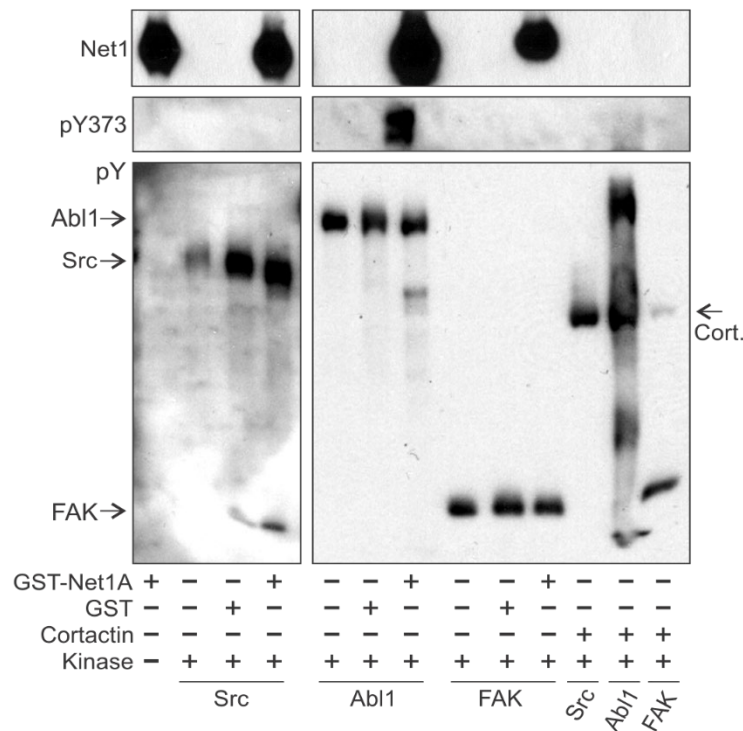


Figure 8. Abl1 directly phosphorylates Net1A on Y373 *in vitro*. Representative western blots from *in vitro* kinase assays to detect Net1A pY373 in the presence of recombinant, active FAK, Src, or Abl1. Data were obtained from five independent experiments.

3.2.7 Abl1 kinase activity is required for phosphorylation of Net1A on Y373 and cytosolic accumulation

To determine whether Abl1 overexpression could drive Net1A cytosolic accumulation in the absence of EGF stimulation, we co-expressed wild type Abl1 with Net1A in MCF7 cells. We also tested whether the kinase activity of Abl1 was important for Net1A relocation by co-expressing either kinase-inactive Abl1 K290R, or Abl1 with an inactive SH2 domain, Abl1 I164E. The mutation of Abl1 I164 to glutamate disrupts the SH2-kinase domain interaction that Abl1 requires for autophosphorylation, as well as substrate phosphorylation (Grebien et al., 2011; Lamontanara et al., 2014; Songyang et al., 1995). We observed that co-expression of wild type Abl1 drove Net1A accumulation in the cytosol to a similar extent as EGF stimulation (**Fig. 9B, C**). On the other hand, neither kinase-dead Abl1 K290R, nor SH2 domain-inactive Abl1 I164E were capable of stimulating Net1A cytosolic localization (**Fig. 9B, D**), consistent with the idea that Abl1 phosphorylates Net1A to drive its cytosolic localization. We confirmed by western blotting that the Abl1 mutants were impaired in their autophosphorylation activity and were expressed at similar levels to wild type Abl1 (**Fig. 9E, F**). We also found that constitutively active Abl1 PP/EE was slightly better than wild type Abl1 at stimulating Net1A cytosolic localization (**Fig. 9D**). Western blotting showed that wild type Abl1 and Abl1 PP/EE were expressed at similar levels, but that Abl1 PP/EE exhibited a greatly elevated autophosphorylation activity (**Fig. 9G**).

We then tested whether Abl1 was able to stimulate Net1A phosphorylation on Y373 in cells. MCF7 cells were co-transfected with HA-Net1A and the various Abl1 mutants, and then serum-starved overnight. HA-Net1A was then immunoprecipitated and tested for pY373 by western blotting. These experiments showed that wild type Abl1 stimulated a modest degree of Net1A pY373 phosphorylation, while constitutively active Abl1 PP/EE was much more efficient at doing so. Neither kinase-inactive Abl1 K290R nor SH2 domain-inactive Abl1 I164E were capable of stimulating Net1A Y373 phosphorylation in cells (**Fig. 9H**).

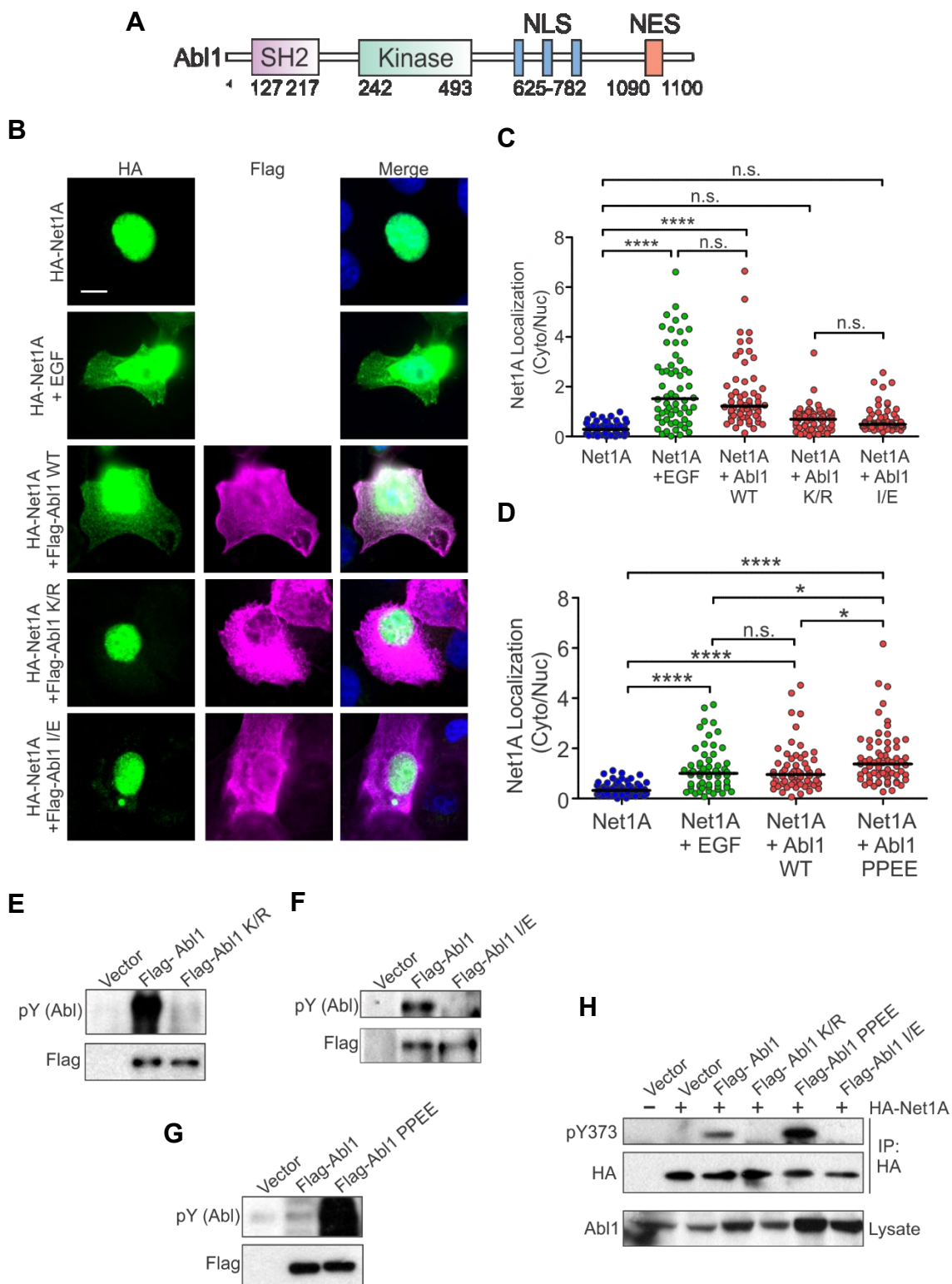


Figure 9. Abl1 directly phosphorylates Net1A on Y373 and stimulates Net1A cytosolic accumulation. (A) Schematic of Abl1 showing locations of SH2 domain, kinase domain, nuclear localization signal sequences (NLS), and nuclear export signal sequences (NES). Numbers refer to the amino acid residues of wild type mouse Abl1. (B) MCF7 cells were transfected with expression plasmids for HA-Net1A, alone or with wild type Flag-Abl1, Flag-Abl1 K/R (kinase-dead), Flag Abl1 I/E (SH2 domain mutant). Cells were serum-starved overnight. Some cells were stimulated with EGF (100 ng/mL) for 15 min. All cells were fixed and stained for HA-Net1A (green), Abl1 (magenta), and DNA (blue). Representative images from 3 independent experiments are shown. (C) Quantification of Net1A localization in the presence of wild type Abl1 or Abl1 mutants (K/R, I/E). Data were obtained from at least three independent experiments. At least 20 cells per condition per experiment were quantified. (E, F) Representative western blots of phosphotyrosine and Flag-epitopes for the Abl1 proteins are shown. (D) Quantification of Net1A localization in the presence of wild type or constitutively active Abl1 (PPEE). Data were obtained from at least three independent experiments. At least 20 cells per condition per experiment were quantified. (G) Representative western blots of phosphotyrosine and Flag-epitopes for the Abl1 proteins are shown. (H) MCF7 cells were transfected with HA-Net1A alone or with wild type Flag-Abl1, Flag-Abl1 K/R, Flag-Abl1 I/E, or Flag-Abl1 PPEE. Cells were serum-starved overnight and HA-Net1A was immunoprecipitated using an anti-HA antibody. The presence of pY373, HA, or Abl1 were detected by western blotting. Representative western blots from at three independent experiments are shown. For graphs, lines indicate median values. * = $p < .05$; **** = $p < .0001$; n.s. = not significant. Scale bars = 10 μm .

3.2.8 Abl1 phosphorylates Net1A on Y373 in the cytosol

Abl1 localizes to both the nucleus and cytoplasm (Wen et al. 1996; Taagepera et al. 1998; Wang 2014). To assess whether Abl1 requires nuclear localization to phosphorylate Net1A on Y373, we generated a constitutively active Abl1 construct in which the three nuclear localization signal sequences were mutated to alanines to block nuclear import (Abl1 NLS3^{mut}). When co-expressed with Net1A, we found that this mutant was at least as good as constitutively active Abl1 with intact NLS sequences at phosphorylating Y373 (**Fig. 10A**). This suggests that Abl1 may phosphorylate Net1A in the cytoplasm. To determine whether cytosolic, constitutively active Abl1 drove Net1A cytosolic localization, we co-expressed each construct with HA-Net1A and measured Net1A subcellular localization. We observed that both forms of Abl1 stimulated Net1A cytosolic localization equally well (**Fig. 10B, C**). These data suggest that Abl1 does not need to access the nucleus to drive Net1A cytosolic localization.

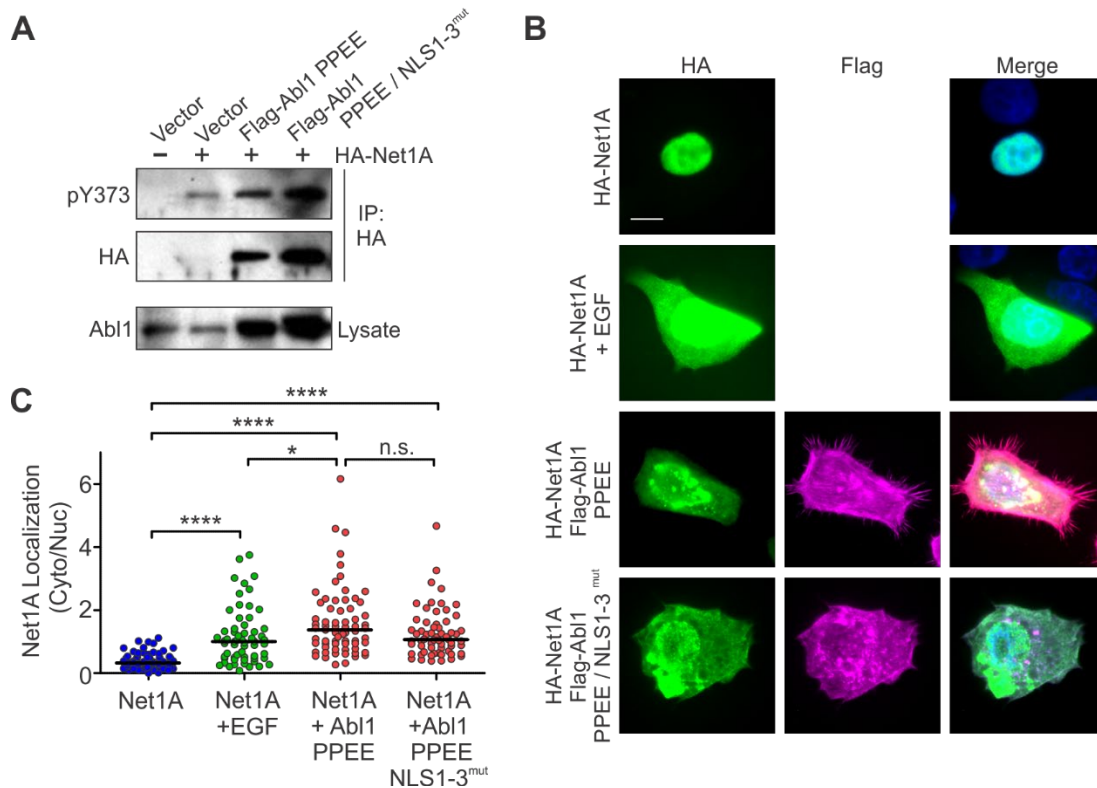


Figure 10. Abl1 lacking nuclear localization signal sequences phosphorylates Net1A on Y373. (A) MCF7 cells were transfected with HA-Net1A alone or with Flag-Abl1 PPEE or Flag-Abl1 PPEE with all 3 NLS sequences mutated (NLS3^{mut}). The cells were serum-starved overnight and HA-Net1A was immunoprecipitated using an anti-HA antibody. pY373, HA, and Abl1 were detected by western blotting. Representative western blots from at least three independent experiments are shown. (B) MCF7 cells were transfected with expression plasmids for HA-Net1A alone or with Flag-Abl1 PPEE or Flag-Abl1 PPEE NLS3^{mut}. Cells were serum-starved overnight. Some cells were stimulated with EGF (100 ng/mL) for 15 min. All cells were fixed and stained for HA-Net1A (green), Abl1 (magenta), and DNA (blue). Representative images from 3 independent experiments are shown. (C) Quantification of Net1A localization in the presence of Abl1 PPEE or Abl1 PPEE NLS3^{mut}. Data were obtained from at least three independent experiments. At least 20 cells per condition per experiment were quantified. Lines indicate median values. * = $p < 0.05$; **** = $p < 0.0001$; n.s. = not significant. Scale bars = 10 μ m.

3.2.9 Inhibition of endogenous Abl does significantly reduces cytosolic relocation of endogenous Net1

To confirm that endogenous Net1A localization is also regulated by Abl1/2, we tested whether Abl1/2 inhibition in the invasive breast cancer cell line MDA-MB-231 altered the steady state localization of Net1 proteins. We have shown previously that MDA-MB-231 cells express a significant amount of endogenous Net1A, and that its expression is required for cell motility and invasion (Carr et al., 2013). We used subcellular fractionation for these experiments, as isoform-specific antibodies suitable for immunofluorescence do not exist. We observed that overnight treatment with GNF5 significantly reduced the localization of Net1A to the cytosol (Fig. 11A, B). There was also a trend towards reduced expression of Net1 isoforms in the nucleus, but this was not significant. These data indicate that endogenous Net1 localization to the cytosol is also controlled by Abl kinases.

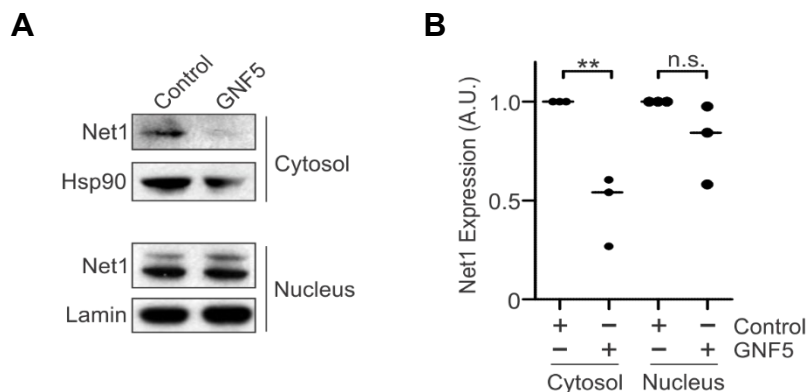


Figure 11. Inhibition of endogenous Abl has significant effects on endogenous Net1 localization. (A, B) Subcellular fractionation of MDA-MB-231 cells. Proteins were analyzed by Western blotting with the indicated antibodies. Hsp90 and Lamin A/C were monitored as controls for cytosolic and nuclear fractions, respectively. Endogenous Net1 expression in each fraction was quantified. Shown are representative western blots (E) and quantification from three replicates (F). Lines indicate median values. ** = $p < 0.01$; n.s. = not significant.

3.3 Discussion

In this chapter, we have shown that active Src stimulates Net1A tyrosine phosphorylation and accumulation in the cytosol, and that phosphorylation of one of these sites, Y373, is primarily responsible for this effect. In addition, we found that Src-dependent Y373 phosphorylation occurs via Abl kinases, and that Abl1 phosphorylates this site *in vitro* and in cells. Importantly, the ability of EGF to stimulate Net1A cytosolic accumulation requires Abl-dependent phosphorylation of Y373. These data indicate that Src and downstream Abl kinases are important regulators of Net1A cytosolic localization.

Our data reinforce the notion that Src is an important regulator of Rho GTPase signaling that either directly phosphorylates or stimulates the phosphorylation of many Rho GEFs, Rho GAPs, and Rho proteins themselves. For example, the Vav1 guanine nucleotide exchange factor is phosphorylated by Src family kinases on multiple sites within its regulatory acidic, zinc finger, and C-terminal SH3 domains, and this directly controls its activation and signaling potential (Bustelo et al. 1992; Margolis et al. 1992; Rodríguez-Fdez and Bustelo 2019; Farago et al. 2020). Similarly, the Rho GEFs FRG, ASEF, β PIX, ARHGEF5, Trio, and GEF-H1 are regulated by Src-dependent phosphorylation (Miyamoto et al. 2003; Itoh et al. 2008; Kuroiwa et al. 2011; DeGeer et al. 2013; Azoitei et al. 2019). Abl proteins, on the other hand, have far fewer substrates in the Rho pathway. Notable exceptions are BCR1 (Rac GAP), p190RhoGAP (RhoA GAP), and SOS2 (Rac GEF) (Colicelli 2011). Instead, Abl proteins tend to regulate actin cytoskeletal organization more directly through phosphorylation of a myriad of linker proteins and actin regulatory proteins (Bradley and Koleske 2009; Colicelli 2011; Wang 2014). Thus, Net1A is somewhat unusual in being directly regulated by Abl proteins instead of Src. In this regard, we should note that active Src promoted the phosphorylation of multiple tyrosines within Net1A (**Fig. 4B**). Although we showed that Y373 phosphorylation was dependent on Abl function, it is an open question as to whether

phosphorylation of the other tyrosines within Net1A is directly catalyzed by Abl kinases. In addition, although we showed that Abl1 directly phosphorylated Y373 *in vitro*, it is possible that Abl2 may also phosphorylate this site in cells.

Our data clearly show that phosphorylation of Y373 was necessary for EGF-stimulated cytosolic accumulation of Net1A, but they do not demonstrate how phosphorylation of this site controls Net1A relocalization. Our previous studies indicate that Net1A continuously cycles between the nucleus and cytoplasm, and that cytosolic accumulation of Net1A can result from mechanisms that prevent its cytosolic degradation or that slow its rate of nuclear import (Carr et al., 2013; Song et al., 2015; Ulu et al., 2018). In addition, we have found that Net1A nuclear export is CRM1-dependent (Ulu et al. 2018) and it is theoretically possible that the rate of Net1A nuclear export can also be regulated. However, our observation that Abl1 lacking its nuclear localization signal sequences was still capable of phosphorylating Y373 and promoting Net1A cytosolic localization (**Fig. 10**) suggests that Y373 phosphorylation may sustain Net1A in the cytosol rather than enhance Net1A nuclear export.

Chapter 4. Net1A Y373D is a gain of function mutant

4.1 Introduction

Elucidating mechanisms controlling the subcellular localization of Net1A are critical to understanding how Net1A regulates RhoA dependent signaling. Cytosolic Net1A drives RhoA-mediated actin cytoskeletal rearrangement as well as increased cell motility and invasion (Song et al., 2015; Ulu et al., 2018). Previous work from the Frost group has identified Net1A S52E and Net1A K83/95Q as gain of function mutants due to their increased cytosolic localization, rather than increased catalytic activity towards RhoA (2013; Song et al., 2015; Ulu et al., 2018). As shown in the previous chapter, our work has established a novel axis of regulation whereby Src activated Abl1 directly phosphorylates Net1A on Y373 and drives cytosolic accumulation of Net1A. This chapter demonstrates that substitution of this site with aspartate causes Net1A to accumulate in the cytosol without ligand stimulation, acting as a gain of function mutant that promotes RhoA-mediated signaling, breast cancer cell motility, and invasion.

4.1.1 Substitution of an aspartate residue for tyrosine 373 results in cytosolic localization of Net1A to drive RhoA-mediated signaling

To determine whether Net1A phosphorylation on Y373 was sufficient for cytosolic localization and downstream signaling, we created Net1A with an aspartate substitution at Y373, which can mimic the negative charge of phosphorylation. When expressed in serum starved MCF7 cells, Net1A Y373D exhibited cytosolic localization. Stimulation with EGF slightly increased cytosolic localization of this mutant, but this was not statistically significant (**Fig. 12A, B**). These data suggest that phosphorylation of Y373 may be sufficient for cytosolic localization of Net1A and indicate that the Y373D mutant may represent a gain of function mutant.

When in the cytosol, Net1A functions as a guanine nucleotide exchange factor for RhoA and stimulates RhoA-dependent phosphorylation of Myosin light chain 2 (pMLC2) and stabilizes F-actin filaments (Amano et al., 1996; Haga & Ridley, 2016; Kimura et al., 1996). Thus, we assessed whether expression of Net1A Y373D stimulated either of these cellular RhoA activity readouts. Interestingly, when expressed in serum-starved cells, Net1A Y373D did not stimulate MLC2 phosphorylation or F-actin accumulation, suggesting that cytosolic localization of this mutant was not sufficient to promote RhoA activation (**Fig 13A-C**). However, when cells were treated with EGF for 15 minutes, Net1A Y373D did significantly enhance pMLC2 and F-actin staining as compared to wild type Net1A (**Fig. 13A-C**). This indicates that expression of Net1A Y373D increases RhoA-dependent signaling. To confirm that this mutant enhances RhoA activation directly, we measured RhoA activity in cells transfected with Net1A constructs. Cells were treated with EGF for 15 minutes prior to harvesting and RhoA activation was normalized to Net1A expression. We observed that cells expressing Net1A Y373D had significantly more RhoA activation than cells expressing either wild type Net1A or Net1A Y373F (**Fig. 12C, D**). These data suggest phosphorylation of Net1A on Y373 is required for efficient RhoA activation in MCF7 cells and that Net1A Y373D acts as a gain of function mutant to increase RhoA activation and RhoA-mediated actin cytoskeletal rearrangement.

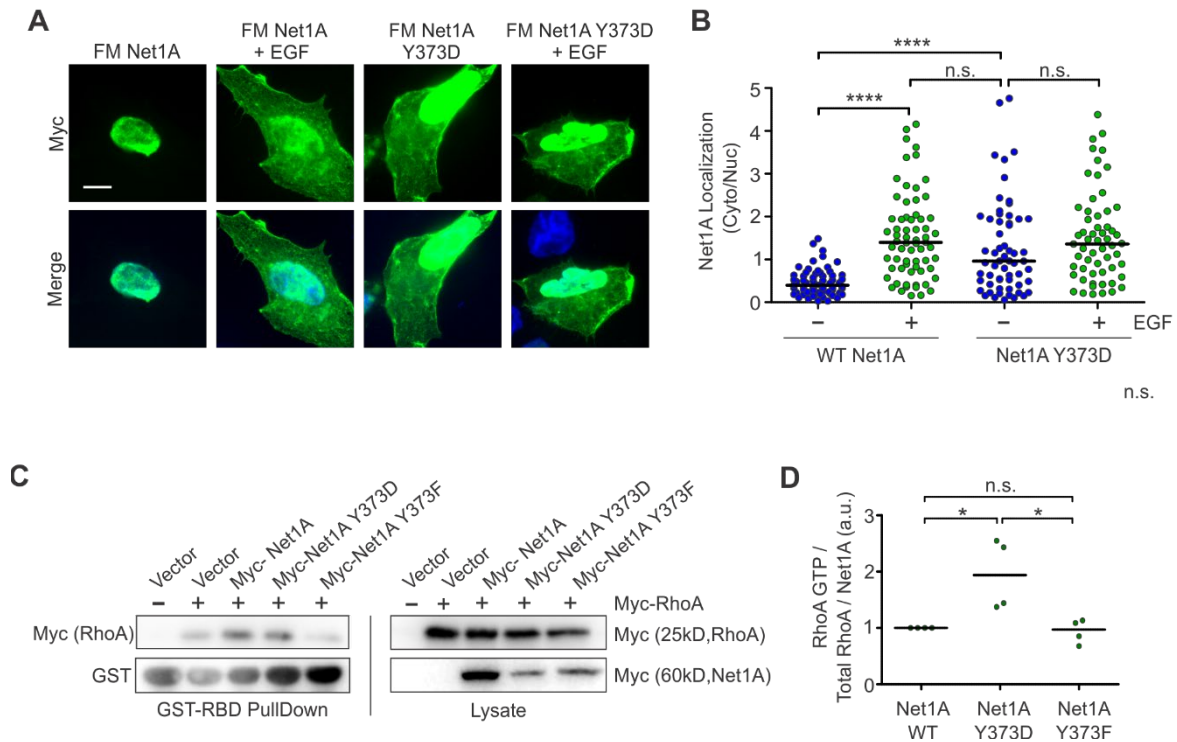


Figure 12. Substitution of aspartate for tyrosine 373 results in cytosolic localization of Net1A and drives RhoA Activation. (A) MCF7 cells were transfected with expression plasmids for wild type Flag-Myc-Net1A or Flag-Myc-Net1A Y373D. Cells were serum-starved overnight. Some cells were stimulated with EGF (100 ng/mL) for 15 min. All cells were fixed and stained for Myc-Net1A (green) and DNA (blue). Shown are representative images from three independent experiments. (B) Quantification of Net1A localization with and without EGF stimulation. Data were obtained from three independent experiments. At least 20 cells per condition per experiment were quantified. (C) MCF7 cells were transfected with pCMV5M-RhoA and wild-type Flag-Myc-Net1A, Flag-Myc-Net1A Y373D, or Flag-Myc-Net1A Y373F. Cells were starved in serum-free media for 4 hours and stimulated with EGF (100 ng/mL) for 15 min prior to harvesting cells. Lysates were incubated with GST-Rhotekin RBD beads for

30 minutes and RhoA activation (Myc-RhoA), Net1A expression (Myc-Net1A), and GST were evaluated by western blotting. Representative western blots from four independent experiments are shown. (D) Quantification of RhoA activation normalized to Net1A expression. Lines indicate median values. * = $p < 0.05$; *** = $p < 0.001$; **** = $p < 0.0001$; n.s. = not significant. Scale bars = 10 μm .

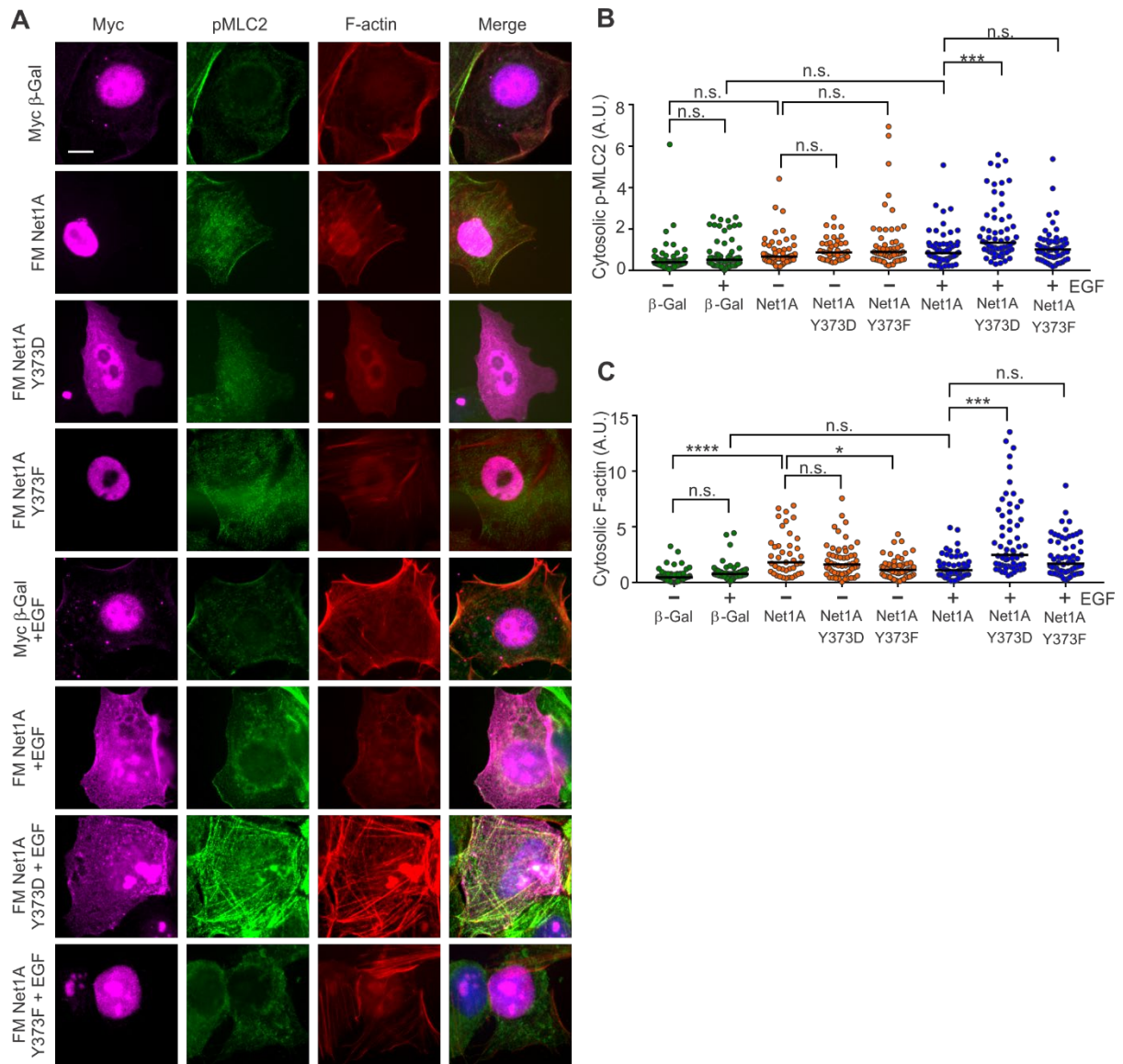


Figure 13. Net1A Y373D drives increased ligand stimulated RhoA-mediated signaling

(A) MCF7 cells were transfected with wild-type Flag-Myc-Net1A, Flag-Myc-Net1A Y373D, Flag-Myc-Net1A Y373F, or Myc-tagged-NLS- β -galactosidase (β -GAL). Cells were then serum-starved overnight. Some cells were stimulated with EGF (100 ng/mL) for 15 min. All cells were fixed and stained for phosphorylated MLC2 (green), F-actin (red), DNA (blue), and the transfected proteins (magenta). Representative images are shown from three independent experiments are shown. (B, C) Quantification of pMLC2 (B) and F-actin (C). Data were

obtained from three independent experiments. For each experiment, at least 20 cells per condition per experiment were quantified.

4.2.2. Net1A Y373D drives increased motility and invasion in breast cancer cells

RhoA activation is crucial for efficient cell motility and previous work from our lab has shown that cytosolic localization of Net1A is required to drive RhoA-mediated cell migration and invasion (Carr, Morris, et al., 2013; Song et al., 2015; Ulu et al., 2018). Thus, we examined whether expression of Net1A Y373D promoted migration and invasion in MCF7 cells, which are a minimally invasive breast cancer cell line (Holliday & Speirs, 2011). MCF7 cells were transfected with a β -galactosidase control vector, wild type Net1A, Net1A Y373D, or Net1A Y373F. To enrich for Net1A-transfected cells, we also co-transfected an empty vector containing a puromycin selection cassette. After 48 hours, transiently transfected cells were selected by puromycin treatment overnight, which consistently resulted in a transfection efficiency of 50%. After puromycin selection, the cells were allowed to recover in full serum media for 2 hours, and then trypsinized and replated in a transwell apparatus without serum. The bottom well contained serum-free media supplemented with EGF. After 6 hours the membranes were fixed and stained with DAPI to evaluate cell motility. We observed that overexpression of wild type Net1A significantly elevated cell migration, and that expression of Net1A Y373D was even more effective. On the other hand, Net1A Y373F was completely unable to promote MCF7 cell motility (**Fig. 14A**). We also assessed the ability of Net1A to stimulate extracellular matrix invasion by replating cells in transwells coated with Matrigel. In these experiments, only Net1A Y373D was able to drive extracellular matrix invasion (**Fig. 14B**). In all experiments, we confirmed that Net1A proteins were expressed at similar levels by western blotting (**Fig. 14C**). Taken together, these data suggest that phosphorylation of

Net1A on Y373 is sufficient to stimulate cell motility and extracellular matrix invasion in otherwise weakly invasive breast cancer cells.

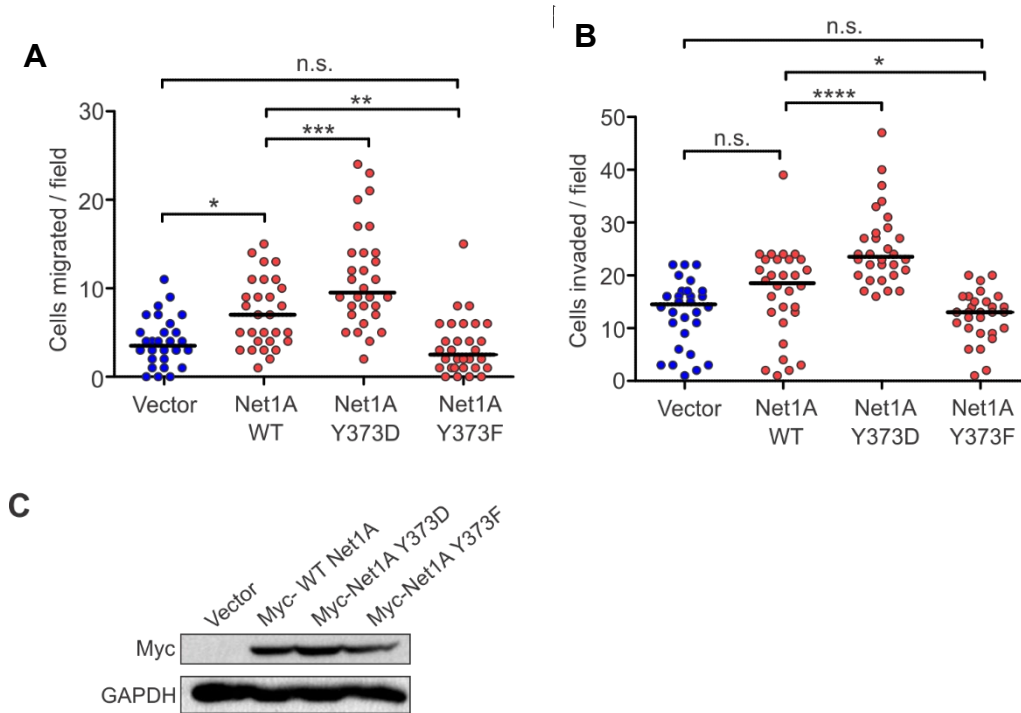


Figure 14. Cytosolic Net1A is required for breast cancer cell motility and invasion.

(A, B) MCF7 cells were transfected with wild type Flag-Myc-Net1A, Flag-Myc-Net1A Y373D, or Flag-Myc-Net1A 928 Y373F. All cells were also transfected with an empty vector containing a puromycin selection cassette. Cells were serum-starved and selected with 1 μ g/mL puromycin overnight and allowed to recover in full-serum media for two hours. Cells were then placed in the upper chamber of a transwell apparatus and allowed to migrate towards serum-free media containing EGF (100 ng/mL) for 6 hours (migration, panel A) or 20 hours (invasion, panel B). Data were obtained from three independent experiments. (C) Western blots showing expression of Myc-tagged Net1A proteins and GAPDH as a loading control. Shown are

representative blots. In all graphs, lines indicate median values. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$; 942 n.s. = not significant.

4.1.3. Net1A is required for Abl1- stimulated cell motility and invasion

We then examined whether Net1A is needed for Abl1-stimulated cell motility, and if this requires Net1A phosphorylation on Y373. MCF7 cells were transfected with control or Net1-specific siRNAs. For this experiment we chose to knockdown all Net1 isoforms, as we had done previously (Carr et al., 2013). One day later the cells were transfected with expression vectors for wild type Abl1, plus Net1A Y373D or Net1A Y373F. The cells were then assessed for EGF-stimulated cell motility, as described above. We observed that wild type Abl1 overexpression stimulated MCF7 cell motility, and that this was abrogated by Net1 knockdown (**Fig. 15A**). Importantly, expression of Net1A Y373D completely rescued Abl1-stimulated cell motility, while Net1A Y373F was unable to do so (**Fig. 15A**). We confirmed Net1 knockdown and overexpression of Abl1 and Net1A mutants by Western blotting (**Fig. 15B**). These data show that endogenous Net1 expression is required for Abl1-driven cell migration and indicate that Net1A phosphorylation on Y373 is essential for rescue of Net1 function downstream of Abl1.

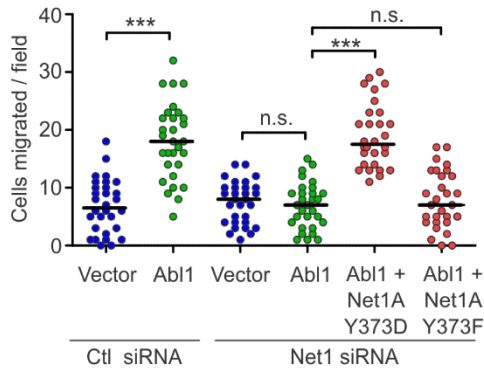
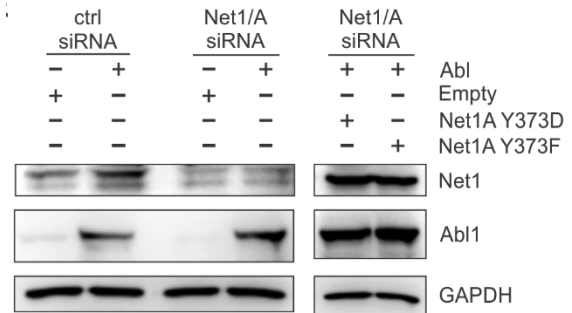
A**B**

Figure 15. Cytosolic Net1A is required for Abl driven breast cancer cell motility.

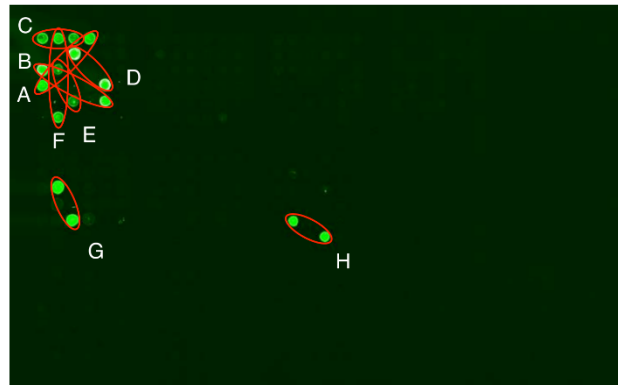
(A) MCF7 cells were transfected with control or siRNAs targeting both Net1 isoforms. Twenty-four hours later the cells were transfected with expression vectors for wild type Abl1 plus Net1A Y373D or Net1A Y373F. After 48 hours, the cells were serum-starved overnight, placed in the upper chamber of a transwell apparatus, and allowed to migrate towards EGF (100 ng/mL) in serum-free media for 6 hours. Data are from three independent experiments. (B) Representative western blots to show efficient knockdown of endogenous Net1 and expression of Abl1, Net1A Y373D, and Net1A Y373F. In all graphs, lines indicate median values. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$; n.s. = not significant.

4.2. Net1A phosphorylation on Y373 creates a predicted SH2 domain binding site

ELM and scan site prediction algorithms show that phosphorylation of Net1A on Y373 creates a potential Src Homology 2 (SH2) domain binding site, suggesting interaction of Net1A with one or more SH2 domain containing proteins may regulate cytosolic accumulation. Tyrosine phosphorylation status can be rapidly changed, thus SH2 domain mediated interactions are often involved in cellular processes that require dynamic spatiotemporal regulation, such as actin cytoskeletal remodeling, GTPase activation, and phospholipid signaling (Jaber Chehayeb & Boggon, 2020; Kaneko et al., 2012; M.-J. Park et al., 2016). Using a protein microarray containing 90 of the 120 SH2 domains in the human genome (Liu et al., 2006), we observed that a pY373 Net1A peptide interacted with the SH2 domains of Abl1, Abl2, Bcr-Abl1, Crk, CrkL, p120RasGAP, CSK, and SOCS7 (**Fig 16**). Abl1 and Abl2 are nonreceptor tyrosine kinases that are activated downstream of DNA damage, adhesion, and oxidative stress (Colicelli, 2011; SHAUL & BEN-YEHOYADA, 2005; Tang et al., 2012; J. Y. J. Wang, 2014). They are integral regulators of actin cytoskeleton rearrangement, and their dysregulation has been implicated in several human cancers, including in breast cancer (Bradley & Koleske, 2009; Dong et al., 2017; Ganguly & Plattner, 2012; Hantschel, 2012; J. Wang & Pendergast, 2015). Bcr-Abl is a fusion protein best known for its driving role in leukemia, but Abl1 fusion proteins are extremely rare in breast cancer (Edwards & Howarth, 2012). Crk family adapter proteins function as signaling integrators that drive breast cancer motility and invasion *in vitro* and contribute to breast tumorigenesis in patients (Fathers et al., 2012; Rodrigues et al., 2005). They do not have catalytic activity but are required for the timely complex formation for signal transduction downstream of extracellular stimuli, such as growth factor stimulation and adhesion (Birge et al., 2009; Feller, 2001; Ng et al., 2015; T. J. Park & Curran, 2014; Rodrigues et al., 2005). The Crk family includes Crk I, Crk II, and CrkL. The SH2 domains of Crk I and Crk II are identical and contain a PxxP motif that is absent in CrkL (Antoku & Mayer, 2009). Thus,

in the microarray the SH2 domain of CRK is representative of both Crk I and Crk II. The SH2 domain of CRKL was plated separately in the microarray. p120RasGAP is best known for its function as a Ras GAP, as it's named, but has also been studied for its role as a scaffold protein. It has been shown to recruit p190RhoGAP to the plasma membrane at sites where suppression of Rho activity is necessary (Chehayeb et al., 2019; Sharma, 1998; Tomar et al., 2009). C-terminal Src Kinase, CSK, is responsible for phosphorylating a variety of proteins, but was originally identified for its role as a negative regulator of Src family kinases. The C-terminal phosphorylation of Src family kinases creates an intramolecular binding site for the Src SH2 domain, thereby occluding the kinase domain. (Okada, 2012; Okada et al., 1991). Finally, Suppressor of cytokine signaling 7 (SOCS7) complexes with E3 ligases to mediate ubiquitylation and degradation of complexed proteins (Babon et al., 2009; Croker, Ben A., Kiu, Hiu., Nicholson, 2012; Kile et al., 2002). To start to determine if any of these predicted interactions affect Net1A regulation, we evaluated how knockdown and overexpression of p120RasGAP and Crk II would affect Net1A cytosolic accumulation. Of note, some experiments shown in this section were not repeated three times, so the figure legends will indicate how many replicates are included.

A



A. ABL2/ARG SH2
B. CRK SH2
C. BCR-ABL1 SH2
D. CRKL SH2
E. CSK SH2
F. ABL1 SH2
G. RASA1_N SH2
H. SOCS7 SH2

Net1A pY373 - 936737 - 100 μ g
Biotin-NERHLYQV[pY]RQPIPVQE

B



α GST

Figure 16. SH2 domain containing proteins that interact with Net1A pY373 peptide. A biotinylated pY373 peptide was screened against an SH2 domain containing protein microarray (MD Anderson Protein Array and Analysis Core). (A) SH2 domains binding the pY373 peptide were visualized using streptavidin conjugated fluorophore. Shown are binding of Abl1, Abl2, Bcr-Abl1, CrkL, Crk, p120RasGAP (RASA1-N), CSK, and SOCS7. (B) The array was re-probed with an anti-GST antibody to confirm the presence of all GST-tagged SH2 domain peptides fixed on the array.

4.2.1. Knockdown of potential interactors prevents Net1A cytosolic accumulation

We have shown that phosphorylation of Net1A on Y373 is required for EGF-stimulated cytosolic accumulation and sought to determine what role the predicted SH2 domain proteins would play in this process. MCF7 cells were transfected control or p120RasGAP specific siRNAs. One day later, the cells were re-transfected with a plasmid encoding HA-Net1A and were subsequently assessed for EGF-stimulated subcellular localization, as described previously. We observed that control siRNA transfection had no effect on EGF-stimulated cytosolic accumulation of Net1A. However, knockdown of p120RasGAP with two different siRNAs significantly reduced cytosolic Net1A (**Fig. 17A, C**). The knockdown was confirmed by western blotting (**Fig. 17B**). These data suggest that p120RasGAP expression is required for EGF-stimulated cytosolic localization of Net1A.

We then tested whether expression of Crk family proteins was also required for Net1A cytosolic localization. Crk II and CrkL, more so than Crk I, are particularly important for focal adhesion maturation (Birge et al., 2009). Since Net1A has also been implicated in focal adhesion maturation (Carr, Zuo, et al., 2013), we first evaluated the necessity of Crk II for Net1A cytosolic accumulation. MCF7 cells were transfected with control or Crk II specific siRNAs and subsequently transfected with an expression vector for HA-Net1A. Cells were serum-starved and then stimulated with EGF for 15 minutes to evaluate Net1A localization. Crk II knockdown significantly reduced the amount of Net1A accumulating in the cytosol after ligand stimulation (**Fig. 18A, C**). Knockdown was confirmed by western blotting (**Fig. 18B**). Thus, Crk II also seems to be important for Net1A to accumulate in the cytosol after EGF stimulation.

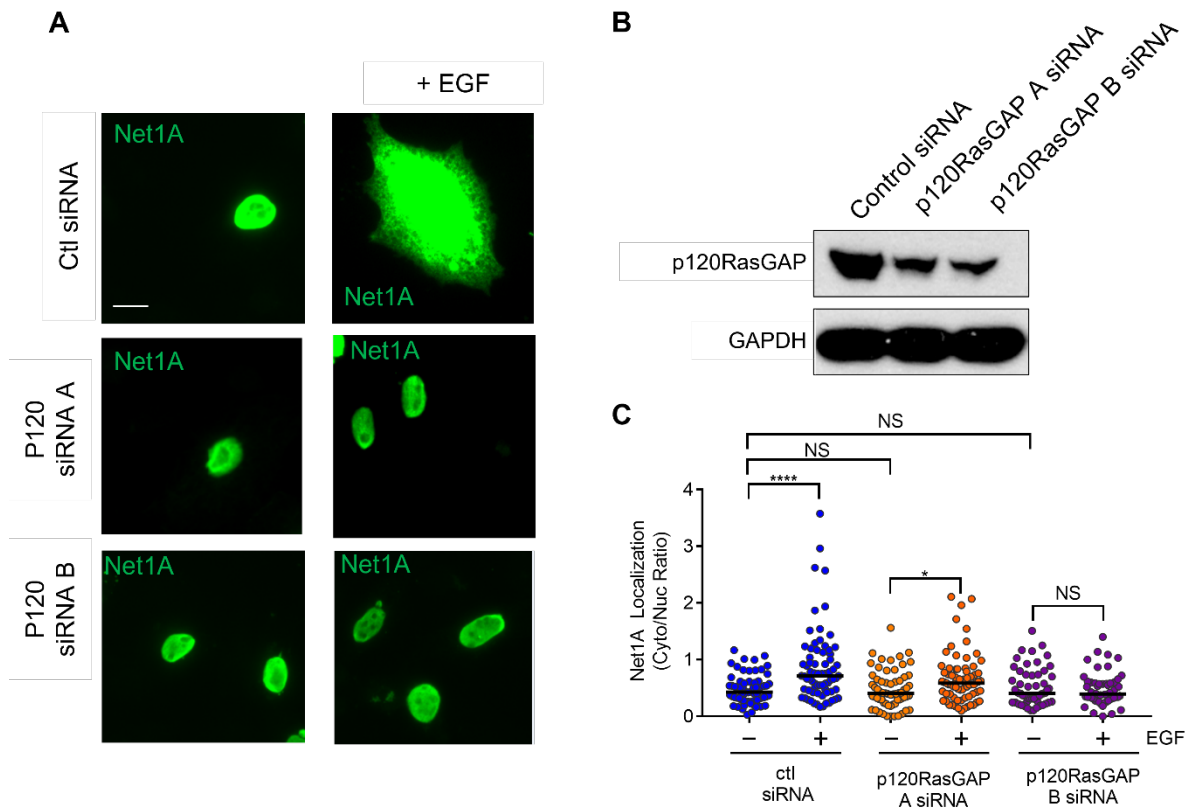


Figure 17. p120RasGAP is required for EGF stimulated Net1A cytosolic accumulation³⁷³. (A) MCF7 cells were transfected with control or siRNAs targeting p120RasGAP. Twenty-four hours later the cells were transfected with expression vectors for wild type HA-Net1A. After 48 hours, the cells were serum-starved overnight, and stimulated with EGF for 15 minutes, fixed, and stained for Net1A localization. (B) Representative western blots to show efficient knockdown of p120RasGAP. (C) Net1A localization data were quantified from three independent experiments. In all graphs, lines indicate median values. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$; n.s. = not significant. Scale bar = 10 μm .

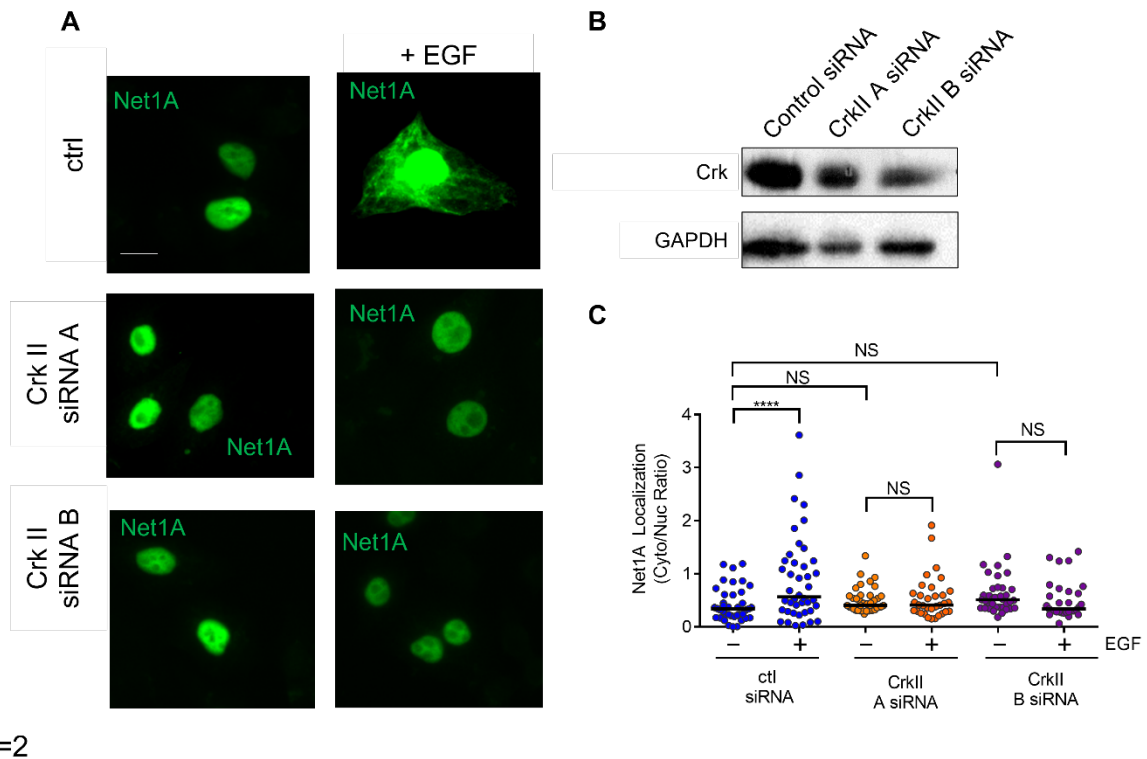


Figure 18. Crk II is required for EGF stimulated Net1A cytosolic accumulation. (A) MCF7 cells were transfected with control or siRNAs targeting Crk II. Twenty-four hours later the cells were transfected with expression vectors for wild type HA-Net1A. After 48 hours, the cells were serum-starved overnight, and stimulated with EGF for 15 minutes, fixed, and stained for Net1A localization. (B) Representative western blots to show efficient knockdown of Crk II. (C) Net1A localization was quantified from two independent experiments. In all graphs, lines indicate median values. **** = $p < 0.0001$; n.s. = not significant. Scale bar = 10 μ m.

4.2.2. Co-expression of Net1A with either p120RasGAP or Crk II drives EGF independent cytosolic relocalization.

Based on the knockdown experiments and the SH2 domain microarray, we predicted that EGF stimulated Abl1 to directly phosphorylate Net1A on Y373, and this would facilitate interactions between Net1A pY373 and the SH2 domains Crk II or p120RasGAP. Although we were unable to co-immunoprecipitate Net1A with either Abl1, Crk II, or p120RasGAP, we observed that coexpressing Net1A with either Crk II or p120RasGAP drove cytosolic accumulation of Net1A in the absence of EGF stimulation. Surprisingly, co-expression of p120RasGAP with either wildtype Net1A or Net1A Y373F seemed to drive cytosolic accumulation of Net1A. However, EGF stimulation remained a stronger driver of cytosolic accumulation than p120RasGAP coexpression (**Fig. 19A**). Interestingly, Crk II seemed more efficient at driving cytosolic accumulation of Net1A Y373F than wildtype Net1A, comparable to our standard relocalization stimulus EGF. It is unclear whether this is due to higher levels of Crk II expression, as this is an immunofluorescence experiment. Further work is needed to determine the exact nature of this regulation.

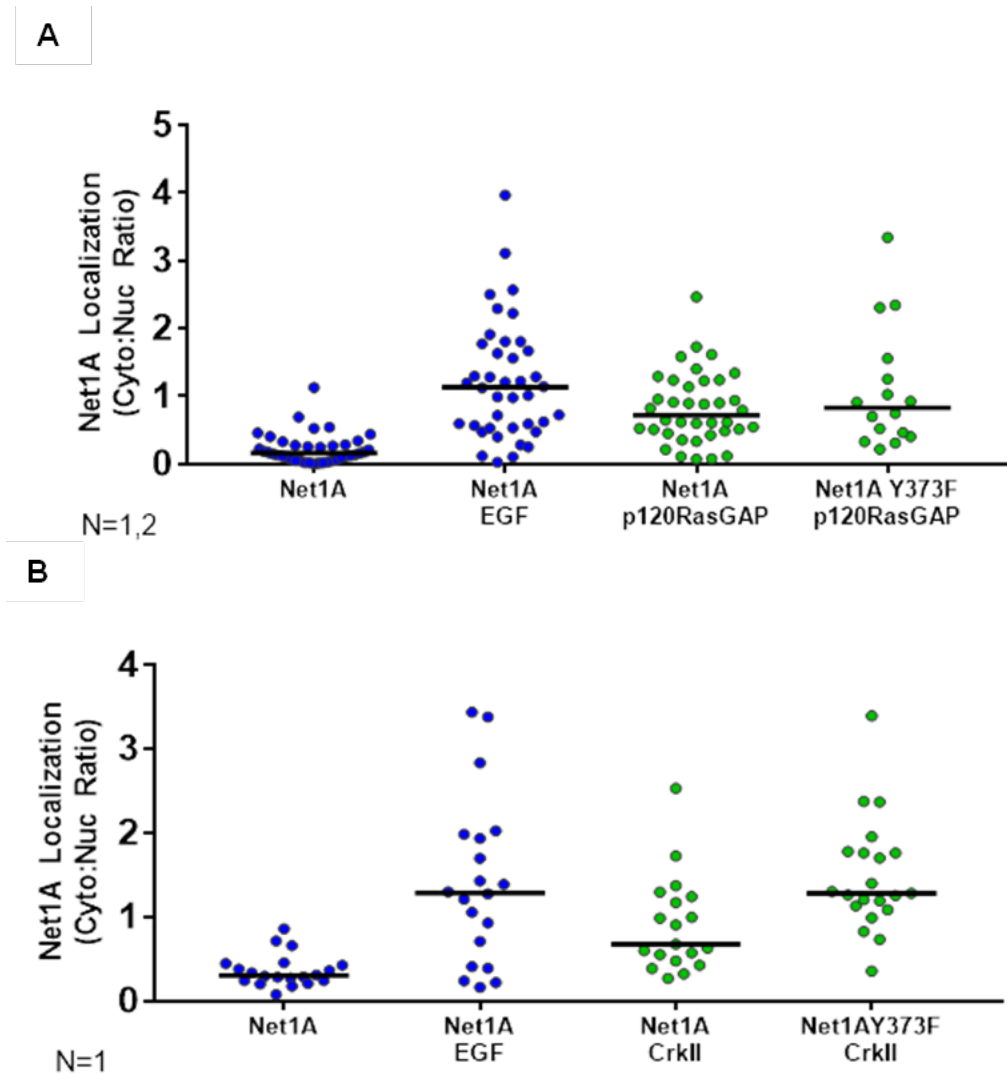


Fig 19. Co-expression of Net1A with either p120RasGAP or Crk II drives EGF independent cytosolic relocalization regardless of Y373 phosphorylation status.

(A) Quantification of Net1A localization with p120RasGAP coexpression. MCF7 cells were transfected with expression plasmids for wild type Flag-p120RasGAP, HA--Net1A or HA-Net1A Y373F. Cells were serum-starved overnight. Some cells were stimulated with EGF (100 ng/mL) for 15 min. All cells were fixed, stained, and evaluated for Net1A localization. Data are from two independent experiments, except for Net1A Y373F + p120RasGAP, which was done once. (B) Quantification of Net1A localization with Crk II coexpression

4.2.3. Net1A Y373D evades degradation and accumulates in the cytosol

Previously, we have shown Net1A Y373D accumulates more significantly in the cytosol than wild type Net1A (**Fig.12A, B**), and we wanted to see if phosphorylation of Y373 modulated proteasome-mediated degradation of Net1A. We have shown that Net1A can be cleared from the cytosol by proteasome-mediated degradation (Carr et al., 2009). We expressed wild type Net1A, Net1A Y373F, or Net1A Y373D in MCF7 cells. After 24 hours, the cells were serum-starved and then treated with the proteasome inhibitor MG132 for 2 or 6 hours. Consistent with previous localization experiments, wild type Net1A and Net1A Y373F accumulated in the cytosol significantly less than Net1A Y373D in unstimulated cells. However, treatment with MG132 significantly increased wild type and Y373F Net1A cytosolic localization, suggesting that their presence in the cytosol was limited by the proteasome. On the other hand, Net1A Y373D was present in the cytosol in the absence of MG132, and proteasome inhibition did not increase this cytosolic localization. Although protein levels still need to be evaluated, this initial data suggests that Net1A Y373D exhibits cytosolic localization in MCF7 cells because it is resistant to degradation by the proteasome.

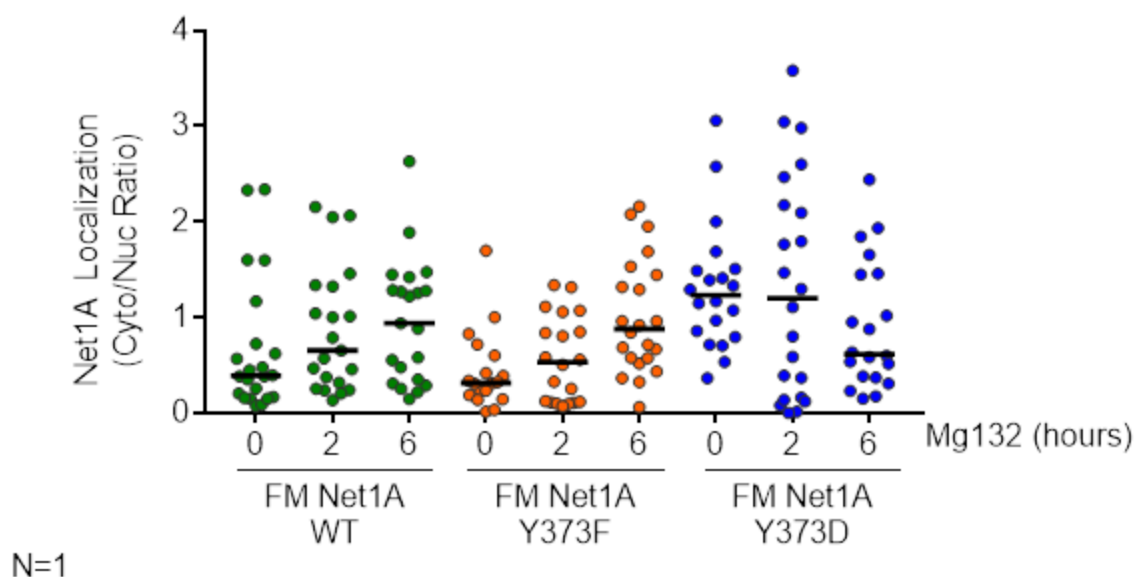


Figure 20. Net1A Y373D accumulates in the cytosol due to reduced proteasome-mediated degradation. MCF7 cells were transfected with expression vectors for wild type FM-Net1A, FM-Net1A Y373F, or FM- Net1A Y373D. After 48 hours, the cells were serum-starved overnight, then treated with 10 μ M Mg132 for zero, two, or six hours. Cells were fixed and stained to assess Net1A localization.

4.3 Discussion

In this chapter, we have shown that substitution of Y373 with the acidic amino acid aspartate promotes Net1A cytosolic accumulation in the absence of EGF stimulation. Overexpression of Net1A Y373D promotes ligand-stimulated RhoA activation and promotes MCF7 cell motility and invasive capacity. Moreover, we found that knockdown of Net1 isoforms inhibits Abl1-stimulated motility in MCF7 cells, and that this can be rescued by expression of Net1A Y373D, but not Net1A Y373F. We also showed data suggesting that p120RasGAP and Crk II are required for ligand-stimulated Net1A cytosolic accumulation. Preliminary experiments also suggest Net1A Y373D is protected from proteasome-mediated

degradation, making it more cytosolic. Taken together, these data indicate Net1A Y373D is a gain of function mutant that is required for Abl1-mediated breast cancer cell motility, due to its cytosolic localization.

Our finding that Net1A Y373D was a relatively poor activator of RhoA in the absence of EGF stimulation was somewhat surprising, as we have observed previously that substitution of the JNK phosphorylation site S52 with glutamate allowed Net1A to promote RhoA activation in the absence of agonist (Ulu et al., 2018). This indicates that Net1A Y373D still requires additional activating events to signal to RhoA. This may include phosphorylation of one of the other Src-stimulated tyrosine phosphorylation sites or additional mechanisms, such as S52 phosphorylation. Nevertheless, once activated, Net1A Y373D was a potent activator of RhoA and downstream signaling (**Fig. 12,13**). In addition, Net1A Y373D also increased MCF7 cell motility and even more strongly enhanced Matrigel invasion (**Fig. 14**). This ECM invasion-promoting effect may be due in part to the ability of Net1A to drive invadopodia formation (Ulu et al., 2018) and further suggests that Net1A may have an important role in promoting metastatic progression. Further work is necessary to improve our understanding of Net1A driven motility and invasion, potentially utilizing 3D culture models. The tumor microenvironment is comprised of several cell types, including fibroblasts and adipocytes, immune cells, and extracellular matrix structures. It has been shown that culturing cells on rigid, 2D substrates has altered cell signaling when compared to cells culture in a more biomimetic 3D culture (Rijal & Li, 2016; Simian & Bissell, 2017). Although the transwell assays are one of the better assays when evaluating motility and invasion of 2D cultured cells, certainly more so than the less quantitative scratch assay, they are still limited. Transwells allows us to mimic the tumor microenvironment in some ways, such as the use of chemoattractants to drive motility and Matrigel to mimic the ECM. However, the cells are only placed in these transwells for a few hours, unlike cells grown for several days in 3D culture.

Blanche *et al.* showed that MCF7 and MDA-MB-231 cells which were cultured in a 3D environment, using PEG hydrogels embedded with mesenchymal stromal cells from human donors, had different secretomes to MCF7 and MDA-MB-231 cells grown on plastic. This study was unique in that it cultured common, stable breast cancer cells in a much more biomimetic setting and found several differences in cell signaling (Blanche *et al.*, 2019). Although *in vitro* assays cannot fully recapitulate the metastatic process, they provide important mechanistic insights into understanding how specific factors affect cancer cell motility and metastasis. Moving forward, it may be valuable to see how Net1 expression alters paracrine signaling to drive motility and invasiveness in stable breast cancer cells and in patient tumor sample cells grown in 2D versus 3D cultures.

The ability of Abl1 to regulate Net1A Y373 phosphorylation may have important implications for breast cancer progression. We have shown previously that Net1 is required for efficient tumorigenesis and metastasis in the polyoma middle t antigen (PyMT) mouse genetic model of breast cancer (Zuo *et al.*, 2018). PyMT functions as a plasma membrane scaffold to initiate Src and PI3K signaling, both of which are necessary for mammary gland tumorigenesis (Guy *et al.*, 1992, 1994; Hutchinson *et al.*, 2001). Hence, our current data may provide an important indication as to how Net1 contributes to cancer in this model. In addition, we have found that Net1 and $\beta 4$ integrin co-expression correlate with poor distant metastasis-free survival in estrogen receptor positive breast cancer patients (Gilcrease *et al.*, 2009). We have also found that breast cancer patients with a Net1-dependent gene expression signature exhibit worse overall survival (Zuo *et al.*, 2018). These data agree with the findings of others indicating that high Net1 expression correlates with worse outcome in breast cancer patients (Ahmad *et al.*, 2014a; Dutertre *et al.*, 2010; Zuo *et al.*, 2018). How Net1 contributes to breast cancer progression is an open question. Net1 undoubtedly promotes breast cancer cell migration and invasion (Carr, Zuo, *et al.*, 2013; Ulu *et al.*, 2018), and *Net1* deletion significantly

reduced RhoA activation within mammary gland tumors in the PyMT mouse model (Zuo *et al.*, 2018). However, Net1 may make important contributions in addition to controlling cell motility. Even with a strong cytoplasmic localization stimulus, a significant fraction of Net1A remained in the nucleus, and in fact, the Net1 isoform essentially never re-localized to the cytosol (**Fig. 1**). Thus, Net1 may have an important nuclear role in breast cancer. In this regard, others have shown that nuclear, catalytically-inactive Net1 controls Smad2-dependent transcription downstream of the TGF β family member Nodal (Wei *et al.*, 2017). Thus, it may be that transcriptional regulation is an important aspect of Net1 function that contributes to cancer progression.

A role for Net1A in Abl1-regulated events may be relevant to other cancers in addition to breast cancer. For example, BCR-Abl is the transforming oncogene in chronic myeloid leukemia (CML), and Abl1 plays important roles in other solid tumors including lung, breast, and gastric tumors (Wang, 2014). In support of this, Net1 has been shown to play important roles in small cell lung cancer, CML, AML, and gastric cancer (Ahmad *et al.*, 2014b; Murray *et al.*, 2008; Sun *et al.*, 2019; Zong *et al.*, 2020). Our data indicating that Net1 expression is necessary for Abl1-stimulated MCF7 cell motility (**Fig. 15**) supports a role for Net1 in Abl1-regulated events. Future work will be required to assess whether Abl1 regulates Net1 function in other cancers.

Net1A pY373 was predicted to interact with several SH2 domain containing proteins implicated in actin cytoskeleton rearrangement (**Fig. 16**), including Abl1, Crk, and p120RasGAP. We initially evaluated these three proteins to determine what their role was in regulating Net1A localization. We observed knockdown of p120RasGAP or Crk II significantly reduced EGF-stimulated accumulation of Net1A in the cytosol (**Fig. 17, 18**). Interestingly, overexpression of either p120RasGAP or Crk II also drove Net1A cytosolic accumulation (**Fig. 19**). Unfortunately, we were unable to co-immunoprecipitate Net1A with the predicted

interactors, which suggests the affinity is too low for co-IP, as is the case for many SH2 domain-phosphorylated protein interactions (Jaber Chehayeb & Boggon, 2020), or that the mechanism of SH2 domain containing proteins regulating Net1A localization is very dynamic and difficult to capture.

p120RasGAP inhibits RhoA activity through its recruitment of p190RhoGAP to the cytoskeleton upon adhesion stimuli, such as integrin stimulation (Sharma, 1998). Early on in migration, RhoA is activated at the leading edge, but it is rapidly inhibited to allow for Rac1-dependent lamellipodia formation (le Clainche & Carlier, 2008; Pertz et al., 2006). Net1A may be required for this initial RhoA activation, as we observe maximal cytosolic accumulation of Net1A merely 15 minutes after EGF stimulation. It's possible that within these 15 minutes, EGF drives phosphorylation of Net1A on Y373, which allows for relocalization. Once cytosolic, Net1A is recruited to the plasma membrane, where it binds to p120RasGAP and activates RhoA. The N-terminal SH2 domain of p120RasGAP, predicted to interact with Net1A pY373, is also required to recruit p190RhoGAP to the cytoskeleton upon integrin stimulation, and p190RhoGAP associates with the cytoskeletal fraction about 20 minutes after replating cells on an ECM (Sharma, 1998). It is also possible Net1A and the p190RhoGAP compete for binding to p120RasGAP to regulate RhoA activity over the course of early cell spreading. Based on Net1A availability in the cytosol, p120RasGAP may bind to a pool of Net1A in the cytosol to activate RhoA to mediate early events in cell spreading. As RhoA signaling needs to be turned off, cytosolic Net1A is degraded over time, and p120RasGAP binds the more available p190RhoGAP to inactivate RhoA.

Crk II may have several roles in regulating Net1A localization: (1) Crk II is a potent transactivator of Abl1, which drives Net1A Y373 phosphorylation; (2) The predicted interaction between Net1A and Crk II may also enhance cytosolic accumulation of Net1A by facilitating JNK1-mediated phosphorylation of Net1A on S52; or (3) Crk II utilizes its SH2 domain to bind

Net1A pY373 and serves as a cytosolic anchor. The efficient activation of Abl1 relies on the interaction between the N-terminal SH3 domain of Crk II and the proline-rich region of Abl1, which releases Abl1 from its autoinhibited conformation. This facilitates phosphorylation of Crk II on Y221, which then interacts with its own SH2 domain and is released. Abl1 is then in an open, active conformation, available to phosphorylate other substrates (Reichman et al., 2005; Sriram et al., 2011). In Figure 15, we showed that knockdown of Crk II significantly reduced Net1A relocalization, and this is potentially due to a lack of efficient activation of endogenous Abl1. In the absence of Crk II, ligand stimulation may not relocalize Net1A to the cytosol because Abl1 is not as strongly activated and cannot efficiently regulate Net1A phosphorylation or localization. In preliminary experiments, we observed that overexpressing Crk II resulted in increased Net1A cytosolic accumulation, although this was not as potent as EGF stimulation (**Fig. 19B**). It is possible that overexpression of Crk II drove increased activation of endogenous Abl1, resulting in increased phosphorylation of Net1A on Y373 without EGF stimulation. Several groups have shown that the SH3 domain of Crk II is also required for JNK1 activation, particularly downstream of Rac1 or EGFR activation (Birge et al., 2009; Girardin & Yaniv, 2001; Kim et al., 2019), and Rac1 activates JNK1-dependent phosphorylation of Net1A S52 to prevent nuclear re-entry (Ulu et al., 2018). siRNA knockdown of Crk II may also prevent the formation of a protein complex of Crk II, Net1A and JNK, preventing S52 phosphorylation and significantly reducing cytosolic accumulation of Net1A. Finally, although wild type Net1A accumulated in the cytosol with Crk II co-expression, initial experiments show that Net1A Y373F seemed to accumulate in the cytosol to a greater extent (**Fig. 19B**). This suggests the hypothesis that Crk II serves as a cytosolic anchor for Net1A by binding to pY373 is likely incorrect and other factors are at play.

The simplest model would suggest that Net1A pY373 separately interacts with the SH2 domains p120RasGAP and Crk II and that either protein can serve as a cytosolic anchor to

promote accumulation of Net1A. However, the limited data showing co-expression of Net1A and p120RasGAP or Crk II drives relocalization of Net1A Y373F suggests a more complex mechanism likely involving the convergence of multiple post-translational modifications (**Fig. 19**). As previously shown, Abl1 directly phosphorylates Net1A on Y373 (**Fig. 6, 7**). It is possible that the interaction detected by the microarray is a result of this site being directly phosphorylated by Abl1 (**Fig. 16**). Abl1 is a processive kinase and uses its SH2 domain to hold onto its substrates and phosphorylate other sites (Dolker et al., 2014; Lamontanara et al., 2014; Mayer et al., 1995), so this predicted interaction may not form a complex, but rather enable Net1A phosphorylation on other sites, such as Net1A Y311. This project was only focused on understanding how phosphorylation of Y373 affected cytosolic accumulation, but one or more of the remaining tyrosine residues from the mass spectrometry data may be phosphorylated by Abl1 or Abl2 (**Fig. 4B**). In addition to Net1A pY373, Net1A pY311 is predicted to interact with p120RasGAP and CrkII (**Fig. 21**). Since both wild type Net1A and Net1A Y373F relocate to a similar extent with p120RasGAP overexpression, p120RasGAP may bind without preference to either Net1A pY373 or Net1A pY311. Further work is required to evaluate how Net1A Y311F localizes with p120RasGAP and if phosphorylation of Y373 and Y311 work together to regulate Net1A cytosolic accumulation. Interestingly, Net1A Y373F seems to accumulate in the cytosol more with coexpression of Crk II, as compared to wild type Net1A, which suggests that pY373 negatively regulates Crk II interaction. It is possible that Crk II binds to Net1A pY311 but cannot do so if Y373 is phosphorylated due to some steric hindrance. Further experiments evaluating how Crk II overexpression affects Net1A Y311F localization would provide more mechanistic insights. It is highly likely that multiple phosphorylation events work together to regulate Net1A subcellular localization. Currently, this data strongly suggests a role for both p120RasGAP and Crk II in Net1A cytosolic accumulation.

Net1A Y373D is a gain of function mutant due to its increased cytosolic localization and ability to drive ligand stimulated RhoA activation. Whether it is a gain of function mutant in terms of enhanced SH2 domain interactions remains unclear. We observed that Net1A Y373D exhibits cytosolic localization in unstimulated cells (**Fig. 12A, B**), but some biochemists caution against the use of aspartic acid substitution to create a tyrosine phosphomimetic to recreate a gain of function mutation. The charge distribution and structure are quite different between an aspartic acid residue and a phosphorylated tyrosine residue. However, even though the charge distribution is not perfectly recapitulated by a tyrosine to aspartate substitution, it may still recapitulate SH2 domain interactions. Crystallography studies have shown that SH2 domain interactions are mediated by two clefts, one that interacts with the phosphorylated tyrosine residue and a cleft that interacts with a hydrophobic residue at the +3 position, (Marengere & Pawson, 1994). Net1A Y373 has a proline at the +3 position, suggesting that this aspect of any SH2 domain interaction remains intact in the Net1A Y373D mutant. Although neither Net1A Y373D nor Net1A Y311D was predicted to interact with any tested SH2 domain containing proteins by microarray (**Fig. 22**), this needs to be further assessed in cells.

Both wild type Net1A and Net1A Y373F accumulate in the cytosol when the proteasome is inhibited, but Net1A Y373D seems to saturate the cell's tolerance for cytosolic Net1A in unstimulated cells without MG132, potentially leading to nuclear re-import of excessive cytosolic Net1A Y373D after extended proteasome inhibition. Further work is needed to assess if there are any changes in protein levels and to confirm the localization data shown in figure 20. Although interactions associated with Net1A pY373 remain unclear, it appears that Net1A Y373D is protected from proteasome-mediated degradation. Future experiments should assess the stability of Net1A Y373D vs. wild type Net1A or Net1A Y373F to substantiate this finding. In this regard, one must be careful reading too much into the

western blotting of wild type Net1A and Net1A mutants as shown in this work, as the expression of Net1A was assessed following cell lysis in Triton and RIPA buffers, which would not solubilize may actin cytoskeletal associated proteins. Indeed it is possible that Src and Abl1 stimulate Net1A relocalization to the actin cytoskeleton, as they themselves partially localize to this domain. Further work is needed to assess whether (1) phosphorylation of Net1A on Y373 promotes accumulation by protecting from proteasome mediated degradation and (2) the extent to which Net1A Y373D recapitulates this regulation.

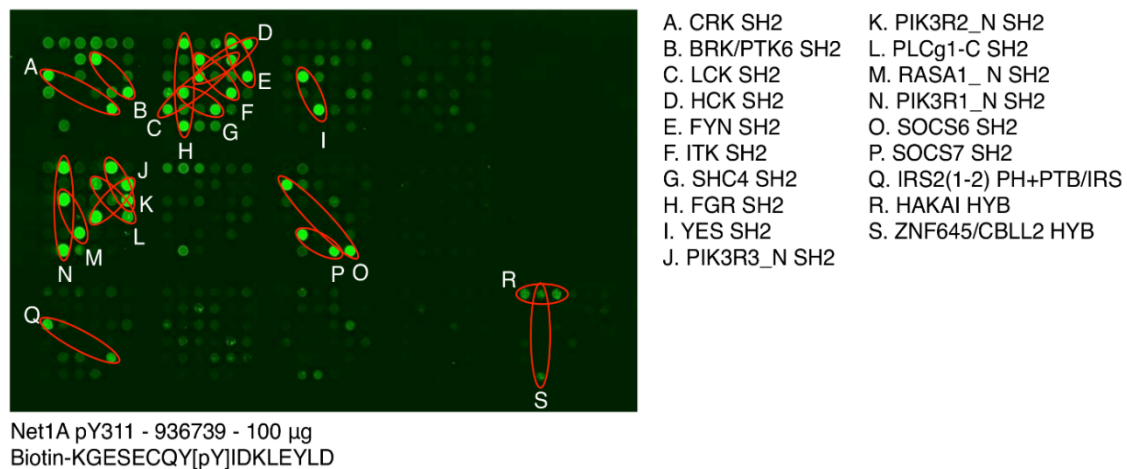


Figure 21. SH2 domain containing proteins predicted to interact with Net1A pY311. A biotinylated peptide pY311 peptide was screened against SH2 domain containing protein microarray (MD Anderson Protein Array and Analysis Core). Predicted interactions were visualized using streptavidin conjugated fluorophores and include Crk, BCR/PTK6, Lck, Hck, Fyn, Yes, ITK, SHC, FGR, PLCγ, SOC6, SOC7, IRS, HAKAI, CBL2, and PI3K regulatory subunits.

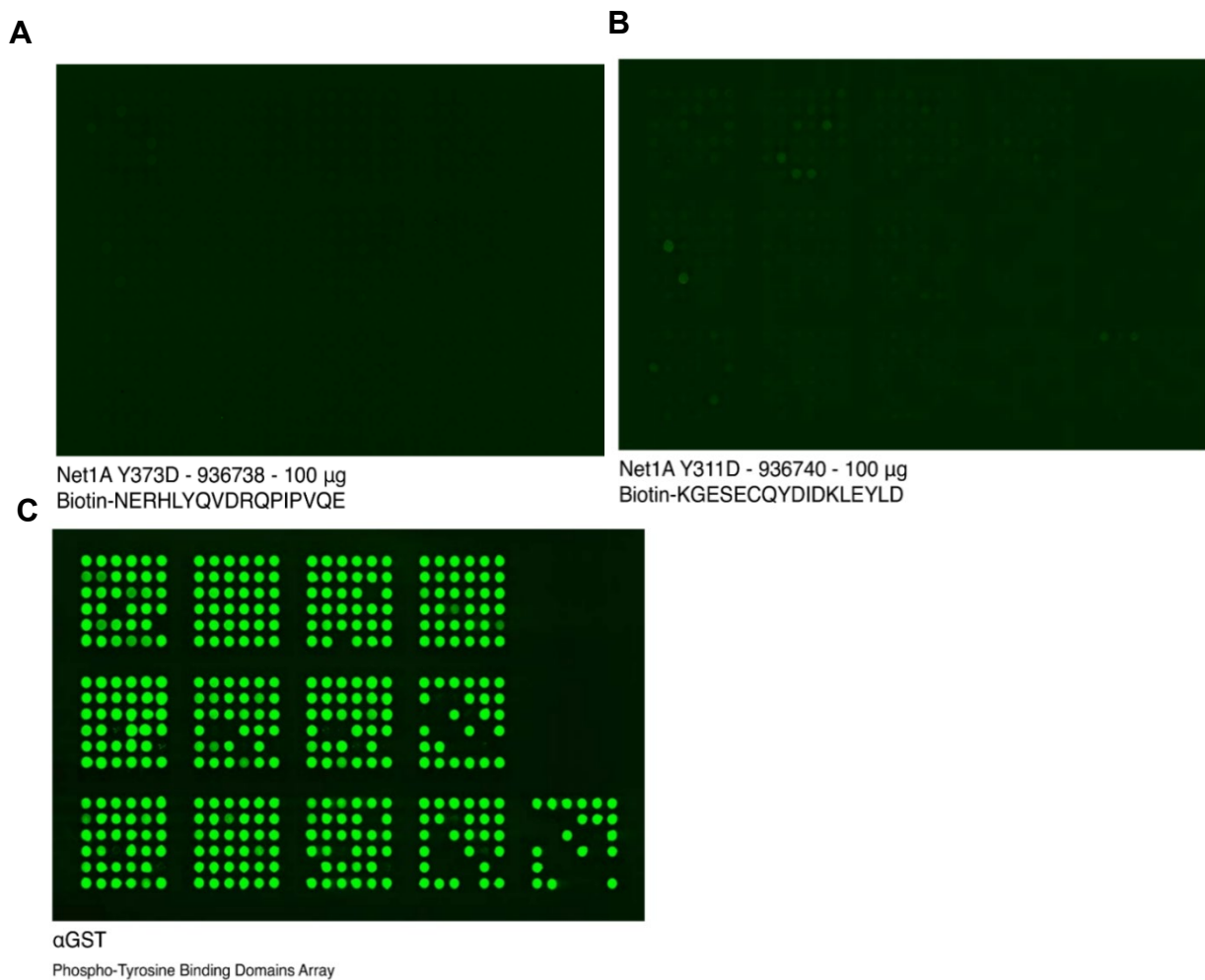


Figure 22. SH2 domain containing proteins predicted to interact with Net1A Y373D and Net1A Y311D (A) Biotinylated Y373D and Y311D peptides were screened against SH2 domain containing protein microarray (MD Anderson Protein Array and Analysis Core). No predicted interactions were visualized using streptavidin conjugated fluorophores. (B) The array was re-probed with an anti-GST antibody to confirm the presence of all GST-tagged SH2 domain peptides fixed on array.

Chapter 5. Discussion and Future Directions

5.1 Summary

The roles of EGFR, Rho GTPases, and nonreceptor tyrosine kinases, like Src and c-Abl, have been well studied for their contributions to breast cancer metastasis (Elsberger et al., 2009; Finn, 2008; Humphries et al., 2020; Nyati et al., 2006) and Net1A is a convergence point of regulation by these key metastatic pathways. Relocalization of Net1A in breast cancer cells can be driven by ligand stimulation, such as EGFR signaling, integrin stimulation, Rac1 signaling, and constitutively active nonreceptor tyrosine kinase activity (Carr, Morris, et al., 2013; Carr, Zuo, et al., 2013; Song et al., 2015; Ulu et al., 2018). The integration of this signaling results in careful regulation of Net1A subcellular localization. This work has shown a novel axis of Net1A regulation via EGFR-Src-Abl1. Abl1 phosphorylation of Net1A on Y373 is necessary for driving cytosolic accumulation and Abl1 requires both an intact kinase domain and SH2 domain to regulate this process. Interestingly, this regulation most likely occurs in the cytosol, as Abl1 lacking nuclear localization signals is capable of phosphorylating Net1A on this site. Net1A Y373D acts as a gain of function mutant and localizes in the cytosol, even in the absence of ligand stimulation; however, stimulation with EGF is required for Net1A Y373D to drive RhoA activation, phosphorylation of myosin light chain activation, and F-actin stabilization. Expression of Net1A Y373D in minimally invasive MCF7 cells also drives increased motility and invasion. Interestingly, we found that Net1 expression is required for Abl1 driven motility. While the exact mechanism underlying the cytosolic accumulation of Net1A Y373D remains unclear, preliminary experiments point to phosphorylation of Net1A being protective from the proteasome. This protection may require phosphorylation of sites other than Y373, but evidence suggests a role for p120RasGAP and CrkII in maintaining Net1A in the cytosol. Taken together, this points to Net1A as an important integrator of pro-

metastatic signaling, downstream of EGFR, Src, and Abl1, and suggests therapeutic targeting of Net1A signaling may improve outcomes for breast cancer patients.

5.2 Mechanistic Mysteries

One question that has remained since identifying Net1 as an oncogene is how nuclear export is regulated. Both Net1 and Net1A have nuclear localization signals, but no nuclear export signals and Net1A seems more sensitive to relocalization stimuli (Garcia-Mata et al., 2007; Song et al., 2015). Downstream of several stimuli, including integrin, EGF, and LPA stimulation, we see cytosolic relocalization and accumulation of Net1A (Carr, Morris, et al., 2013; Song et al., 2015; Ulu et al., 2018). All three of these stimuli have been shown to drive motility in cancer cells and to activate RhoA (Costa et al., 2013; Hu et al., 2021; van Nieuw Amerongen et al., 2000). While canonically, it was thought Rac1 was primarily responsible for regulating membrane protrusion in early stages of cell motility, recent work utilizing RhoA activation biosensors showed RhoA was also active at membrane ruffles and nascent lamellipodia (Kurokawa & Matsuda, 2005; le Clainche & Carlier, 2008; Pertz et al., 2006; Ridley, 2011). We see Net1A accumulate in the cytosol after fifteen minutes of EGF stimulation, suggesting that Net1A relocalization is important for RhoA activation early in migration and cell spreading. Previous work has demonstrated that knockdown of both Net1 isoforms significantly reduces RhoA activation and phosphorylation of myosin light chain in human breast cancer cells; however, Net1A is specifically important for trailing edge retraction, focal adhesion maturation, FAK activation, and amoeboid invasive activity (Carr, Zuo, et al., 2013). Taken together, this suggests that EGF stimulation may rapidly relocalize Net1A to initially activate pools of RhoA at membrane ruffles. While the specific order of Net1A post-translational modifications remains unclear, there are likely specific mechanisms

that target cytosolic Net1A to distinct pools of RhoA, either at the leading edge or the trailing edge, to tightly regulate RhoA activation and breast cancer cell motility.

The Hall group showed that the PH domain was necessary and sufficient to drive export, and recent work from the Frost lab has confirmed this export is CRM1 dependent (Schmidt & Hall, 2002; Ulu et al., 2018). This suggests that Net1A interacts with an NES containing auxiliary protein to mediate its nuclear export. Xu *et. al.* identified over 200 NES-containing proteins reported to utilize CRM1 for their nuclear export. Several proteins identified had roles in signal transduction, stress response, and cell cycle- all processes in which Net1 has been implicated (Xu et al., 2012). Of those proteins, there were several that have established roles in regulating Net1 localization and activity, including c-Abl, FAK, CDK1, ER α , paxillin, and members of the MAPK pathway (Carr, Morris, et al., 2013; Carr, Zuo, et al., 2013; Dutertre et al., 2010; Ulu et al., 2018, 2021). Of these, both paxillin and c-Abl were confirmed to interact with CRM1 via CRM1 binding pull down assays performed by Xu *et. al.*. Our initial hypothesis was that pY373 would mediate NES dependent nuclear export of Net1A, as Y373 is in the PH domain and several SH2 domain containing proteins that may interact with Net1A either have an NES, such as Abl1, or complex with NES containing proteins, such as Crk II. However, our data pointed us in a different direction and showed that phosphorylation of Net1A on Y373 is most likely not a mechanism for nuclear export, but rather a mechanism for cytosolic retention. Of note, we were unable to test how constitutively active, nuclear Abl1 affected Net1A localization, because expression of constitutively active Abl1 trapped in the nucleus caused cell death. It is possible that endogenous Abl1 that is not constitutively active is more nuanced in its regulation of Net1A and can indeed shuttle Net1A out of the nucleus by phosphorylating it on Y373, complexing with Net1A using its SH2 domain, and recruiting CRM1 to mediate the nuclear export of both proteins. Alternatively, Src signaling can drive phosphorylation of Net1A on other sites, besides just Net1A Y373, so

another Src-dependent phosphorylated tyrosine may mediate Net1A nuclear export. Paxillin has been shown to relocate from the nucleus to the cytosol in a CRM1-dependent manner upon EGF stimulation in prostate cancer cells (Sen et al., 2010, 2012). One possible mechanism of Net1A nuclear export could be mediated by paxillin. Downstream of EGFR signaling, both Src and FAK are activated. FAK can translocate to the nucleus, phosphorylate paxillin, and facilitate paxillin nuclear export. Paxillin is phosphorylated by FAK and an intact FAT domain of paxillin is required for its nuclear export (Sathe et al., 2016). If Net1A is phosphorylated in the nucleus upon EGF stimulation, EGF stimulation would drive paxillin phosphorylation and nuclear export. It is possible that paxillin binds Net1A and mediates CRM1-dependent nuclear export of both itself and Net1A. Interestingly, FAK dependent phosphorylation of paxillin also creates a binding site for the SH2 domain of Crk, leaving the SH2 domain of paxillin available to mediate complex formation at the focal adhesion (Schaller & Parsons, 1995). It is possible that paxillin binds Crk and uses its available SH2 domain to bind Net1A and the entire paxillin-Crk-Net1A complex is exported. Additionally, FAK has itself been shown to both bind Net1A and contain two nuclear export signals (Ossovskeya et al., 2008, Carr et al., 2013) suggesting FAK may regulate Net1A nuclear export directly, not necessarily through paxillin. All of these proteins are very important for focal adhesion formation and maturation, so they likely exit the nucleus and are targeted to sites of focal adhesions. This may be yet another role for Crk in Net1A regulation, which would explain why Crk knockdown had such a significant decrease in EGF stimulated Net1A cytosolic accumulation. Further work is certainly needed to uncover the exact mechanism regulating Net1A nuclear export.

Another remaining gap in knowledge is the exact order of post-translational modifications on Net1A and whether this order regulates how efficiently Net1 accumulates in the cytosol. Thus far, it is known that both EGFR activation and integrin stimulation regulate

Rac1 activation, which controls JNK-dependent phosphorylation of Net1A on S52 and recruits Net1A to focal adhesions (Carr, Morris, et al., 2013; Ulu et al., 2018). Additionally, acetylation of Net1A on K83 and K95, near the N-terminal nuclear localization signals, is required for cytosolic accumulation of Net1A (Song et al., 2015). Finally, this work demonstrates a requirement for phosphorylation of Net1A on Y373 for cytosolic accumulation. Whether these modifications synergize to maintain cytosolic Net1A or whether pools of Net1A are modified differently remains unclear.

5.3 Potential Prognostic and Therapeutic Roles for Net1/Net1A

While both Net1 and Net1A are overexpressed in breast cancer, Net1 seems to play a more important role in regulating mitosis. On the other hand, Net1A, which accumulates more readily in the cytosol, drives RhoA activation that promotes focal adhesion formation, motility, and invasion (Carr, Morris, et al., 2013; Dutertre et al., 2010b; Menon et al., 2013; Ulu et al., 2021). The *Net1* gene is highly expressed in estrogen responsive tissues of mice, and whole body *Net1* deletion in mice slowed delayed mammary gland development. Additionally, a surprising finding that *Net1* deletion significantly reduces *Cyclin D1* and *ERα* expression, suggesting broader implications for hormone receptor signaling in these *Net1* knockout animals. While RhoA is certainly important for events in mammary gland development, the loss of efficient estrogen signaling likely contributes more to the inhibited ductal branching, extension, and disorganized epithelial architecture identified in this model (Zuo et al., 2014). In ER+ MCF7 cells, estradiol selectively induces the expression of Net1 over Net1A, due to alternative promoter regulation. This is mediated by the DDX5/DDX17 family of coregulators that facilitate RNA polymerase II recruitment and histone acetylation at the Net1 promoter preferentially. This suggests that Net1, more so than Net1A, and ERα have some feed forward regulation that cooperates to maintain levels of *ERα*, *Cyclin D1* and *Net1* expression to

facilitate normal mammary tissue development and prevent aberrant RhoA activation during mitosis.

In more invasive tumors, the balance of Net1:Net1A expression may be skewed due to increased TGF- β signaling. There is a higher incidence of the Net1 gene expression signature in basal subtype breast cancers (Zuo et al., 2018). Basal subtype breast cancers tend to be more aggressive and are defined as being negative for hormone receptor expression. An absence of high levels of estrogen signaling may reduce the preferential transcription of the Net1 isoform, increasing Net1A expression. Additionally, TGF- β signaling has been shown to drive transcriptional upregulation of the Net1A isoform through the activation of the Smad2/3 and MEK/ERK MAPK pathway (Papadimitriou et al., 2012). An analysis of 5000 human breast tumor samples from the bc-GenExMiner 4.0 database revealed elevated TGF- β 1 and TGF- β 3 expression in the most aggressive basal-like breast tumors (Hachim et al., 2018). Taken together, this suggests that more invasive breast cancers, such as basal subtype breast cancers, rely on mechanisms that upregulate transcription of the Net1A isoform preferentially over Net1. With increased levels of Net1A, the tumor cells have increased expression of the isoform which readily relocalizes to the cytosol. Thus, cytosolic accumulation of Net1A could drive aberrant RhoA activation which can lead to a more migratory and invasive phenotype.

An assessment of 74 ER+ breast tumor samples found high levels of *Net1* expression to correlate with reduced distant metastasis free survival (Dutertre et al., 2010a). Interestingly, the Duterte group showed elevated levels of the Net1A isoform mRNA specifically, not Net1, was a poor prognostic marker. (Dutertre et al., 2010a). Of course, mRNA is not always indicative of protein levels, and Net1A translation and degradation are highly regulated (Ahmad et al., 2014; Carr et al., 2009; Lai et al., 2015; Papadimitriou et al., 2012; Song et al.,

2015). However, there is currently no antibody that allows us to distinguish between Net1 and Net1A, so it remains to be seen whether this occurs at the protein level.

Targeting Net1A specifically has proven difficult, as no antibodies exist that can distinguish between isoforms yet. Additionally, development of small molecule inhibitors that target either Net1 or Net1A has been unsuccessful. In general, the use of small molecules to target Rho GEFs has proven a challenge. Molecules that bind to available surfaces on Rho GEFs tend to have structural properties which make them reactive, and thus highly toxic to cells. For example, a virtual screen of four million compounds identified Y16, which targets the Rho GEF LARG (Shang et al., 2013). Although it is selective, it contains highly reactive pyrazolidine-3,5-diones which make it questionable for use as a therapeutic. A DOCK GEF subfamily inhibitor was also discovered, CPYPP; however, it too contained the highly reactive pyrazolidine-3,5-dione motif and was later discovered to also inhibit the Dbl GEF Trio (Gray et al., 2020). Although this may seem bleak, screening and drug development is an ever-improving endeavor. With improvements in targeting previously “undruggable” molecules, such as Ras, we are hopeful small molecules will be identified that specifically, efficiently, and safely target Rho GEFs, like Net1A (Ostrem et al., 2013).

The proteolysis-targeting chimera (PROTAC) is a recently developed small molecule which can drive degradation of target proteins. Unlike competitive ligands, a PROTAC molecule is catalytic, containing three elements: a ligand which binds the target protein, a ligand which binds the E3 ubiquitin ligase, and a linker region (Gadd et al., 2017). Much of the work understanding molecular mechanisms underlying metastasis have identified protein overexpression as oncogenic. Thus, targeting these for degradation may drastically improve outcomes for cancer patients. This technology already been further developed by several notable pharmaceutical companies, such as AstraZeneca, GlaxoSmithKline, Merck, and more. Among others, PROTAC molecules have been developed to target tyrosine kinases

that are overexpressed in cancers, including FAK and Bcr-Abl. Recently, a PROTAC targeting the estrogen receptor and one targeting the androgen receptor have shown tolerability in Phase I clinical trials (Gao et al., 2020), making this a promising avenue for developing therapeutics. However, some challenges remain with this technology, including difficulty evaluating a PROTAC molecule's pharmacokinetics. Additionally, the size of these molecules is relatively large compared to traditional small molecule inhibitors, so adsorption can be a difficult challenge to overcome (Li & Song, 2020). Further work is needed to better understand the full extent of exploiting PROTAC as a therapeutic agent, but it is promising for targeting overexpressed proteins, such as Net1A, that drive breast cancer cell motility, invasion, and metastasis.

The use of peptide aptamer technology may be the key to developing molecules capable of distinguishing between Net1 and Net1A, for diagnostic or therapeutic use. Originally described by Roger Brent, peptide aptamers consist of a short amino acid sequence typically 5-20 amino acid residues (Reverdatto et al., 2015), conformationally constrained by a protein scaffold, typically a bacterial thioredoxin scaffold. These molecules have nanomolar to picomolar binding affinity, similar to antibodies, but are far less immunogenic and smaller. TRIP α was the first peptide aptamer developed to inhibit the RhoA-specific GEF domain of Trio and was very successful in decreasing Trio-mediated RhoA activation in COS cells. Additionally, it was specific enough to inhibit RhoA activation, but not affect RhoG activation, as Trio has separate GEF domains (S. Schmidt et al., 2002). Peptide aptamers are useful tools because they can both be packaged for therapeutic delivery or used for high-throughput screening, such as AptaScreen, to interrogate small molecule libraries to identify those that bind to similar target site (Baines & Colas, 2006). The development of antibodies that differentiate between Net1 and Net1A has been very difficult because the proteins are nearly identical; however, there are 31 amino acids in the N-terminal portion that are different

between isoforms. If peptide aptamers could be successfully developed against isoforms of Net1 and Net1A, this would allow us to separate the isoforms in *in vitro* studies and improve our understanding of how each isoform's function is regulated. It may also be used for prognostic and therapeutic development. Several studies have shown high *Net1* expression correlates to reduced distant metastasis free survival (Dutertre et al., 2010b; Gilcrease et al., 2009; Zuo et al., 2018), but aptamer based IHC would allow us to assess protein levels in patient samples to determine if one isoform was more dominant in primary tumors or in metastatic tissue. Furthermore, if patient samples showed elevated Net1A specifically, this would suggest a higher risk for metastasis and may alter the course of treatment that could improve the patient's survival rate. The packaging of molecules, such as aptamers, for therapeutics is still in early stages, as toxicity, degradation, and renal filtration remain primary concerns. More work has to be done to demonstrate their feasibility, but there is a lot of potential for nanocarrier and lipid-based systems to deliver RNA aptamers. (Zhou & Rossi, 2014). To date, several aptamer-based therapies have entered clinical trials, including some that have received FDA approval (Catuogno & Esposito, 2017). Pegaptanib is the first and it is an anti-VEGF aptamer approved by the FDA to treat macular degeneration. Other molecules in clinical trials include those targeting TLR4 to reduce inflammatory response (*First in Human Clinical Trial of ApTOLL in Healthy Volunteers - Full Text View - ClinicalTrials.Gov*, n.d.), Regado 1A for its role in anticoagulation for patients with thrombotic disorders, (Dyke et al., 2006), and AS1411 targeting nucleolin for patients with metastatic, clear-cell renal carcinoma relapsing after tyrosine kinase inhibitor treatment. (Rosenberg et al., 2014). Hopefully, as large scale, high-throughput screening makes aptamer libraries more accessible, aptamers against Net1 and Net1A can be exploited to improve diagnostic studies or develop more personalized therapies to treat metastatic breast cancer patients.

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