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BY THE SMALL GTPASE RAC1

by

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A

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by

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Dedication of this thesis shall go to the three women who maintain residence on my back, my grandmother, Helen, my mother, Veronikia, and my daughter, Angelina.

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REGULATION OF NET1A SUBCELLULAR LOCALIZATION

BY THE SMALL GTPASE RAC1

Publication No._____

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Activation of Rho family small G proteins is thought to be a critical event in breast cancer development and metastatic progression. Rho protein activation is stimulated by a family of enzymes known as guanine nucleotide exchange factors (Rho GEFs). The neuroepithelioma transforming gene 1 (Net1) is a Rho GEF specific for the RhoA subfamily that is overexpressed in primary breast tumors and breast cancer cell lines. Net1 isoform expression is also required for migration and invasion of breast cancer cells *in vitro*. These data indicate that Net1 may be a critical regulator of metastatic progression in breast cancer. Net1 activity is negatively regulated by sequestration in the nucleus, and relocalization of Net1 outside the nucleus is required to stimulate RhoA activation, actin cytoskeletal reorganization, and oncogenic transformation. However, regulatory mechanisms controlling the extranuclear localization of Net1 have not been identified. In this study, we have addressed the regulation of Net1A isoform localization by Rac1. Specifically, co-expression of constitutively active Rac1 with Net1A stimulates the relocalization of Net1A from the nucleus to the plasma membrane in breast cancer cells, and results in Net1A activation. Importantly, Net1A localization is also driven by endogenous Rac1 activity. Net1A relocalizes outside the nucleus in cells spreading on collagen, and when endogenous Rac1 expression was silenced by siRNA, Net1A remained nuclear in

spreading cells. These data indicate that Rac1 controls the localization of the Net1A isoform and suggests a physiological role for Net1A in breast cancer cell adhesion and motility.

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CHAPTER 1: INTRODUCTION

Genes commonly associated with Breast Cancer Initiation and Progression

Breast cancer is the most widespread malignancy specific to women in the developed world and is the second leading cause of death, with approximately half a million fatalities worldwide per year (1, 2). This high death rate continues despite improvements in diagnostics and treatments. Breast cancer is a heterogeneous collection of diseases with diverse pathological factors, including aberrant hormone and growth factor regulation, genetic variations in key oncogenes and tumor suppressors, and altered cell-cell and cell-matrix adhesion. Metastatic progression is the cause of death in breast cancer, yet our understanding of the mechanisms controlling metastasis is not fully understood.

Heightened activity or expression of particular extracellular ligand receptors, including the hormone receptors (HR) estrogen receptor (ER) and progesterone receptor (PR), and the epidermal growth factor receptor (EGFR) 2 (HER2/Neu), is recognized as a critical factor contributing to disease progression in human breast cancer. In fact, HR over-expression occurs in 70% of breast tumors, while HER2 positive (+) breast tumors can be detected in approximately 30% of patients (3-5). HR+ tumors are often more treatable and less aggressive compared to HR negative (-) tumors, which tend to exhibit greater metastatic spread and are not receptive to endocrine therapy (5).

Estrogen is important in the development of reproductive tissues in women and for multiple physiological functions in adults. For example, binding of ligand induces the ER to interact with estrogen response elements (ERE) in the promoters of estrogen responsive genes, and also recruits transcriptional co-activators to promote the transcription of these genes. ER activation also elicits non-genomic activation of

multiple signaling cascades, including the PKC, PKA, PI3K-Akt, and MAPK pathways (6, 7). In cancerous ER+ tissues, estrogen promotes vascular endothelial growth factor (VEGF) and Transforming Growth Factor-β (TGF-β) secretion to stimulate carcinoma cell growth (6). Endocrine therapy approaches for ER+ tumors include ER antagonists or selective estrogen receptor modulators (SERM) (2). Tamoxifen is an ER antagonist that prevents the binding of estrogen to the ER, while aromatase inhibitors, such as Anastrozole, are SERMs that inhibit estrogen biosynthesis (2, 8).

HER2 is a proto-oncogene with relatively low expression in normal breast tissue that is inversely proportional to the expression levels of ER, and is down-regulated by estrogen (5). Binding of ligand to the HER2 causes it to dimerize with other EGFR family members, stimulating the MAPK, PI3K and PLC pathways (5, 9). Over-expression of HER2 in breast cancer correlates with an up-regulation of cyclin D1 expression, which is an essential regulator of G1/S phase progression in the cell cycle, promoting tumor cell growth (10). HER2+ tumors are generally sensitive to therapy using HER2-specific monoclonal antibodies, such as trastuzumab (5, 9, 10). However, recurrence of breast cancer in patients receiving anti-HER2 therapy indicates that additional treatment approaches are needed.

Tumor suppressor genes that have been shown to be mutated in breast cancer and to contribute to breast cancer risk include the *BRCA1/2* and *TP53* tumor suppressor genes (11). For example, inheritance of mutations in the *BRCA1* or *BRCA2* genes, which are responsible for DNA repair, significantly increases the risk of breast cancer (3, 12). *BRCA1* mutation-containing cancers typically lack ER, PR, and HER2 expression, and occur in only 5% to 10% of breast cancer cases. However, about 30% of sporadic breast cancers exhibit reduced BRCA1 mRNA and protein expression (3, 13). On the other hand, *TP53* gene mutations are the most common

genetic alteration in human cancer and have been detected in human breast tumors (14). The *TP53* gene regulates essential cellular processes such as DNA repair and apoptosis. Thus, p53 mutations typically allow a cancer cells to evade death in the presence of genetic imbalances or apoptotic stimuli (11).

Another hallmark of breast cancer is the process of epithelial to mesenchymal transition (EMT), which involves the loss of cell polarity, cell-cell and cell-matrix adhesion, and an increase in cell motility (15, 16). Precursor tumor cells undergo EMT through aberrant regulation of adherens junctions (AJ), tight junctions (TJ), and integrin-extracellular matrix (ECM) adhesion. Studies have shown that dysregulation of AJs, such as the loss of E-cadherin expression, and TJs, such as dysregulation within the Partitioning defective (Par) complex, lead to breast cancer tumorigenesis in cells and in animal models which incorporate much of the diversity of human breast cancer (17-20).

Abnormal regulation of cell-matrix adhesions also promotes tumor invasive potential. Integrins constitute the major transmembrane receptor that binds to the ECM and have been implicated in tumor cell survival, invasion, migration, and anchorage at metastasis sites (21). For example, the alpha6-beta4 (α6β4) and α6β1 integrins promote tumor cell survival and motility, and the α1 and α2 integrins have been implicated in controlling the expression of the matrix metalloproteinase (MMP) MMP-3/stromelysin-1, promoting tumor cell invasion (21-24). The α3β1 and $\alpha_v\beta1$ integrin dimers are also associated with tumor cell migration and invasion due to MMP-9 production (25, 26). However, the mechanisms underlying the dysregulation of cell adhesion in breast cancer are not fully understood.

Rho GTPases and Cancer

The Rho family of small GTPases contains 23 members and is part of the Ras superfamily of monomeric GTPases. Amongst the Rho GTPases, RhoA, Rac1, and Cdc42 are the most extensively characterized (27). Rho family small G proteins regulate many critical processes of cell physiology, including actin cytoskeletal organization, cell cycle progression, cell motility, gene expression, vesicle trafficking, cell polarity, proliferation, cell-cell and cell-matrix adhesion (28-31). Rho proteins act as molecular switches, cycling between inactive, GDP-bound, and active, GTP-bound states, and upon activation, Rho proteins interact with effector proteins to stimulate multiple cell signaling pathways (Figure 1) (27, 32). Rho protein activation is mediated by guanine nucleotide exchange factors (GEFs) that catalyze GDP release to facilitate GTP loading (33). GTPase activating proteins (GAPs) stimulate the intrinsic GTPase activity of Rho proteins to inactivate their function (34). Guanine nucleotide dissociation inhibitors (GDIs) sequester GDP-bound Rho proteins into the cytoplasm (35).

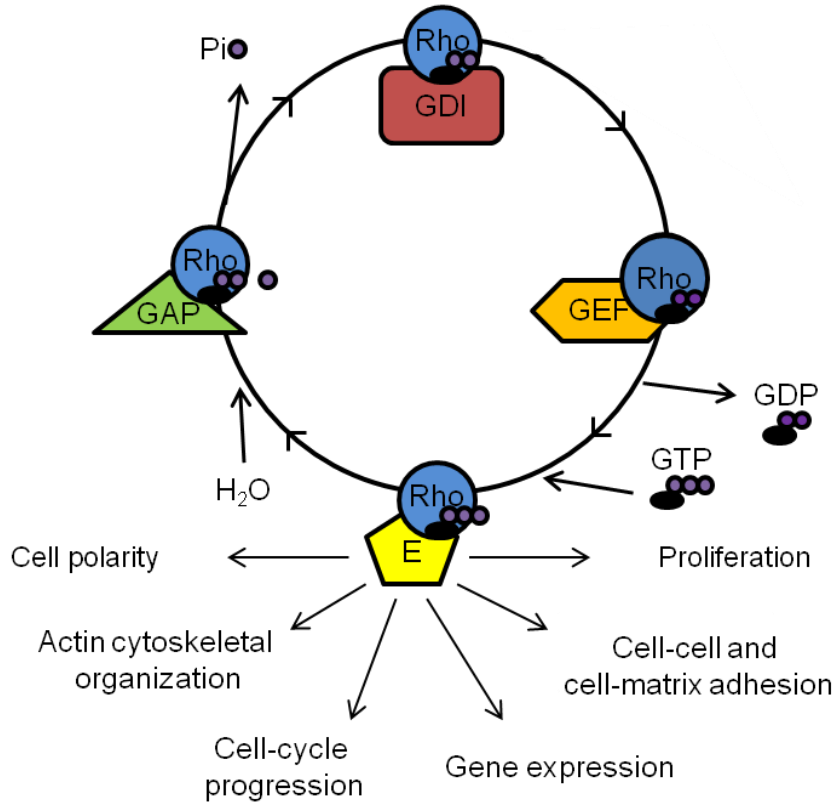


Figure 1. Rho GTPase activation cycle. Rho GTPases cycle between inactive (GDP-bound) and active (GTP-bound) states. Intrinsic Rho GTPase activity is stimulated by Rho GAPs, down-regulating Rho GTPases. GDP-bound Rho GTPases are sequestered in the cytoplasm by GDIs. Rho GEFs catalyze GTP binding by Rho GTPases, stimulating their activation. Active Rho proteins interact with downstream effector (E) proteins to control many cellular processes, including cytoskeletal organization, cell cycle progression, gene expression, vesicle trafficking, cell polarity, proliferation, cell-cell, and cell-matrix adhesion.

Aberrant signaling, activation, or expression of Rho proteins can lead to increased proliferation, EMT, and transformation, and are critical for cancer cell motility and invasion (36-39). Overexpression of Rho proteins in human cancer seems to be a frequent occurrence. For example, over-expression of RhoA and RhoC has been observed in multiple human cancers, including breast cancer (40-43). Furthermore, because both RhoA and RhoC stimulate cell motility, enhanced RhoA or RhoC expression in breast tumors suggests that they may promote metastatic progression

(44). However, the overexpressed Rho proteins are invariably wild type, meaning Rho GEFs and Rho GAPs still control their activation state.

There are more than 70 Rho GAPs and 70 Rho GEFs encoded by the human genome, which allows for pathway specific regulation of Rho activity (45, 46). Two distinct families of Rho GEFs up-regulate Rho GTPase activity, the canonical Dbl (diffuse B-cell lymphoma) family and the non-conventional DOCK (Dedicator of cytokinesis) or CZH (CDM [Ced-5, Dock180 and Myoblast city]-Zizimin homology) family of Rho GEFs. DOCK family Rho GEFs activate Rac and Cdc42 GTPases to control cell migration, morphogenesis, and phagocytosis, and contain conserved DHR2 (DOCK homology region 2) catalytic domains and conserved DHR1 domains (47). Dbl family Rho GEFs contain conserved, tandem Dbl homology (DH) and Pleckstrin homology (PH) domains (33, 47-49). The catalytic activity of Rho GEFs resides in the DH domain, while the PH domain stabilizes the DH domain-Rho protein interaction and has been observed to control binding of Rho GEFs such as Lbc (Lymphoid blast crisis), Lfc (Lbc's first cousin), and Dbs (Dbl's big sister) to phosphatidyl-inositol lipids (48, 50-53). Many canonical Rho GEFs, such as Dbl, Vav (sixth letter of the Hebrew alphabet), Neuroepithelioma Transforming Gene 1 (Net1), and Lfc, were first identified as oncogenes in NIH3T3 cell transformation assays. In all cases their transforming activity was dependent on their ability to stimulate Rho protein activation (49, 54-56). In addition, over-expression of Rho GEFs such as T-cell lymphoma invasion and metastasis-inducing protein 1 (Tiam1), leukemia-associated Rho GEF (LARG), and Net1 has been observed in human tumors (57-59). However, the requirement for particular Rho GEF activity in tumor development and progression has not been evaluated at length.

Regulation of Rho GEF activity

Regulatory mechanisms controlling Rho GEF activity have only been extensively studied for a few proteins. Almost all Rho GEFs display specificities for different Rho family small G proteins and can be regulated by post-translational modifications, such as phosphorylation, ubiquitylation, PDZ (PSD-95/Disc-large/ZO-1)-domain protein interaction, and subcellular localization (33, 60-62).

For example, Tiam1 catalyzes nucleotide exchange specifically for Rac1, while the Rho GEF XPLN is specific for RhoA and RhoB, but not RhoC (63, 64). Moreover, Rho GEFs such as Vav, Dbl, FRG (FGD1 [faciogenital dysplasia gene product]-related Cdc42 GEF), Tiam1, and Epithelial cell transforming sequence 2 (Ect2) are positively regulated by phosphorylation (65-69). Dbl activity is enhanced by tyrosine phosphorylation by the tyrosine kinase, activated Cdc42 kinase 1 ACK1 (67). FRG is directly phosphorylated and activated by Src kinase, which underlies cell motility inhibition downstream of the endothelin A receptor (68). GEF phosphorylation can also coincide with particular phases of the cell cycle, as indicated by Ect2 up-regulation by phosphorylation during the G2/M phase of the cell cycle (70). Furthermore, phosphorylation can be accompanied by lipid binding, as observed when Vav is activated by binding to phosphoinositide 3, 4, 5-phosphate (PIP_3) and by lymphocyte-specific protein tyrosine kinase (Lck)-mediated phosphorylation (65, 66, 71). In addition, phosphorylation can be induced by ligand stimulation. Exposure to the agonist lysophosphatidic acid (LPA), or increases in intracellular Ca^{2+}, induce protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent protein kinase II phosphorylation of Tiam1, respectively, to increase its activity (69).

Rho GTPase activity can also be down-regulated by ubiquitin-stimulated degradation of Rho GEFs by the proteasome (72). For example, after mitosis, RhoA

activity is reduced by ubiquitin-mediated degradation of the Rho GEF Ect2 (73). Dbl is another Rho GEF that is ubiquitylated by the E3 ligase, CHIP (carboxyl terminus of HSC70-interacting protein) to target it for proteasome degradation (74). Additionally, the Cdc42 specific GEF, hPEM-2 (human homologue of the Ascidian protein Posterior End Mark-2), is ubiquitylated by the E3 ligase, Smurf1 in a Ca^{2+} independent manner, while Net1 has been found to be ubiquitylated following disengagement of E-cadherin (75, 76).

In addition, about 40% of Rho GEFs have a PDZ binding motif (PBM) that is predicted to mediate interaction with PDZ domain containing proteins. However, the PBM has only been shown to be functional for a few Rho GEFs (62). Proteins containing PDZ domains often act as scaffolds to promote specific cellular localization of signaling molecules and increased protein-protein interactions. PDZ-domain proteins also provide a mechanism for regulating Rho GEF subcellular targeting and activation. For example, the neuronal Rho GEF Kalirin-7 interacts with postsynaptic density protein 95 (PSD-95), targeting Kalirin-7 to the PSD to regulate dendritic morphogenesis through Rac1 signaling (77). Two other PDZ-domain proteins, Scribble and Shank, form complexes with the Rho GEF Pak-interacting exchange factor-beta (β-PIX) and localize β-PIX to pre-synaptic sites in neurons to promote exocytosis and dendritic protrusions, respectively (78, 79). Furthermore, the PDZ-domain protein, synectin, binds to and recruits the RhoA-specific GEF, Syntaxin 1A (Syx1), to the plasma membrane, promoting local RhoA activation (80). Finally, Net1 (Net1A isoform) interaction with the PDZ-domain containing protein, Dlg1, prevents proteasome mediated degradation of Net1A to allow for maximal stimulation of RhoA activity (76, 81).

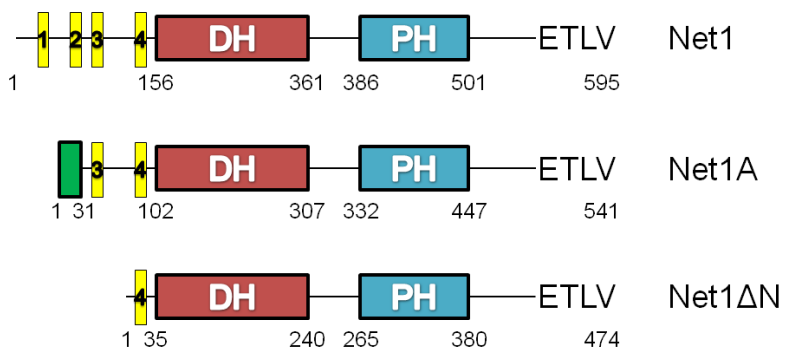
Lastly, subcellular localization is an important mechanism for controlling Rho GEF activity. For example, inactive Rho GTPases and most Rho GEFs are primarily localized in the cytosol. Upon activation, both the Rho proteins and their respective GEFs re-localize to the plasma membrane. However, two Rho GEFs, Ect2 and Net1, diverge from this regulatory paradigm and are localized to the nucleus at a steady state (70, 82). This represents a mechanism to downregulate their activity, as truncation of their respective N-termini relocalizes Ect2 and Net1 outside the nucleus to potentiate RhoA and RhoB activation (70, 82).

The Rho GEF Net1

Net1 is a Rho GEF specific for the RhoA subfamily (33, 60). Net1 was originally cloned from a human neuroepithelioma cDNA library in a screen for novel transforming genes using a focus formation assay in NIH3T3 fibroblasts (83). In this screen, a non-naturally occurring 5' truncated form of Net1 (lacking the first 145 amino acids) was found to have transforming properties. NIH3T3 cells expressing this truncated form of Net1 exhibited increased proliferation, loss of contact inhibition, and anchorage independent growth *in vitro*, and were tumorigenic when injected into nude mice (83). The *NET1* gene encodes a protein of 595 amino acids with tandem DH domain and PH domains, with 155 amino acid amino-terminal and 93 amino acid carboxyl-terminal regulatory domains (Figure 2) (60, 61, 83, 84). The presence of multiple nuclear localization signal (NLS) sequences in the N-terminal regulatory domain of Net1 targets it to the nucleus at steady state (61). The C-terminus of Net1 also contains a Type I PBM (X-S/T-X-V-COOH [X being any amino acid]) allowing for interaction with the PDZ domain containing proteins Dlg1 (Discs large gene 1) and Magi-1 (Membrane-associated guanylate kinase, WW and PDZ domain-containing protein 1)

(81, 85). Interaction with Dlg1 protects the Net1A isoform from proteasome mediated degradation (81, 86).

A.



B.

	Net1	Net1A
NLS1	$R^{12}PRRRSRR^{19}$	-----
NLS2	$K^{66}RKRREK^{72}$	-----
NLS3	$P^{87}SNKRVRP^{94}$	$P^{33}SNKRVRP^{40}$
NLS4	$P^{147}TKRRSS^{153}$	$P^{93}TKRRSS^{99}$

Figure 2. Net1 proteins. (A) Net1 is 595 amino acids in length. Net1 contains NLS sequences within the N-terminus, indicated by yellow boxes, followed by tandem DH and PH domains, illustrated by the red and blue boxes, respectively. The PBM sequence, ETLV, lies at the carboxyl terminal end of Net1. The splice variant, Net1A, is identical to Net1 except in the N-terminus. Specifically, Net1A lacks the first 85 amino acids of Net1 and contains a distinct 31 amino acid sequence, as denoted by the green box. Net1ΔN lacks the first 121 amino acids of Net1. (B) Amino acid sequence of the NLS sequences in Net1 and Net1A.

There are two Net1 isoforms that exist in most cells, Net1 and the splice variant Net1A. Expression of Net1 isoforms is controlled by alternative promoters within the *NET1* gene, which are inversely regulated by estradiol (59). The difference between the primary amino acid sequences of Net1 and Net1A lies within their unique amino-termini. The amino-terminus of Net1A lacks the first 85 amino acids of full length Net1 and has its own unique 31 amino acids (81). Net1 contains 4 putative NLS sequences, while Net1A only contains the NLS sequences corresponding to NLS3 and

NLS4 in Net1 (Figure 2). Net1 also contains a putative nuclear export signal (NES) within the PH domain, however, mutation of the NES in Net1 did not affect chromosomal region maintenance protein 1 (CRM1)-dependent export (61). Net1 NLS sequences allow for nuclear import of Net1 isoforms, and truncation of the N-terminal regulatory domain results in the oncogenic form known as Net1ΔN. Net1ΔN mainly localizes outside the nucleus to constitutively stimulate RhoA activity and actin stress fiber formation, indicating that localization of Net1 in the nucleus is a form of negative regulation (60, 61, 87). However, signaling mechanisms controlling the nuclear import or export of Net1 isoforms have yet to be identified.

Both Net1 isoforms are overexpressed in various cancers, including gastric, breast, ovarian, cervical, and pancreatic cancers (59, 88, 89). Moreover, Net1 isoform expression is necessary for migration and invasion of MDA-MB-231 breast cancer cells and gastric adenocarcinoma cells (AGS) *in vitro*, suggesting that Net1 may be a critical regulator of metastatic progression in cancer (88). Furthermore, Net1 and integrin alpha6beta4 (α6β4) co-expression in primary tumors of lymph node-positive breast cancer patients and Net1 overexpression in ER positive (ER^+) breast tumors selects for high risk distant metastasis (59, 89). Thus, understanding regulatory mechanisms controlling Net1 activity may be important for discerning breast cancer metastasis.

Net1 and DNA damage

Net1 localizes to the nucleus, sequestered away from RhoA at the plasma membrane. This is thought to leave Net1 biologically inert. However, Net1-dependent RhoA localization and activation in the nucleus has been observed in response to DNA

damage (90, 91). This indicates that Net1 may have additional, non-classical nuclear functions that are not well appreciated. However, the exact role of Net1 in DNA damage responses is still unclear. For example, it has been shown that Net1 expression is required for activation of the p38 Mitogen Activated Protein Kinase (MAPK)-MAPKAP2 signaling pathway after exposure to genotoxic agents such as ionizing radiation (IR), which promotes survival in HeLa cells (91, 92). On the other hand, in MCF7 breast cancer cells IR-stimulated DNA damage causes Net1- and Ect2-dependent RhoB activation, which inhibits cell survival (93). Thus, the role of Net1 in DNA damage signaling may be cell type specific.

Net1 mediated signaling through RhoA

RhoA activation induces various downstream signal transduction pathways that control actin cytoskeletal rearrangement, transformation, adhesion, and gene expression. For example, TGF-β stimulates Smad activity, which enhances Net1 expression, leading to increased Rho activity and actin stress fiber formation (84, 94). Net1 dependent activation of downstream stress fiber formation is down-regulated by co-expression with the scaffold protein Connector Enhancer of Kinase Suppressor of Ras 1 (CNK1) (87, 95). Importantly, CNK1 co-expression causes Net1 to stimulate RhoA-dependent SAPK/JNK pathway activation. These findings indicate that signaling downstream of Net1 is regulated by associated proteins (60, 95). Net1 also has the ability to induce SAPK/JNK activation independently of the generation of titratable GTP-bound RhoA (60). Moreover, the PBM of Net1 is not required for RhoA dependent cytoskeletal effects, but is necessary for cellular transformation (87). On the other hand, Net1ΔN interaction with Dlg1 reduces the transformation potential of

oncogenic Net1 (81). Altogether, this indicates that Net1 activity is differentially regulated towards RhoA and transformation.

Regulation of Net1 activity

Net1 activity is regulated by phosphorylation, ubiquitylation, and subcellular localization (61, 76, 81, 87, 96). Additionally, TGF-β inhibits Net1 expression by the microRNA miR-24 (94). The exchange activity of Net1 and its ability to stimulate stress fiber formation is reduced by phosphorylation of serines 152 and 153 (S152, S153) by p21 Activated Kinase 1 (PAK1) in response to Rac1 activation (96). Disruption of E-cadherin engagement leads to decreased Net1A and Dlg1 interaction and subsequent Net1A ubiquitylation, thus indicating enhancement of Net1A stability in response to cell-cell contact (76). Increased Net1A stability correlates with increased interaction with Dlg1 and relocalization of Net1A/Dlg1 complexes to the subnuclear structures, the promyelocytic leukemia protein (PML) bodies (76, 81). This indicates Net1A association with Dlg1 which protects Net1A from ubiquitin-mediated degradation and this interaction is regulated by cell-cell contact (76). Lastly, down-regulation of Net1 activity also occurs through nuclear sequestration (61, 87). Deletion mutants of Net1, containing various NLS aberrations or N-terminus abnormalities, exhibit cytoplasmic localization, indicative that the N-terminus of Net1 regulates subcellular localization through these NLS sequences. Extranuclear localization of Net1 isoforms is required for RhoA activation and stress fiber formation. However, stimuli that cause increased nuclear export or decreased nuclear import of Net1 isoforms, thereby relocalizing Net1 outside the nucleus, have not been described.

Ran dependent Nuclear Import and Export

Nuclear import and export are typically regulated by families of binding proteins known as importins and exportins, which function as chaperones to mediate transit through the nuclear pore. Importins are classified as karyopherins (97). Karyopherins have two subunits, importin α and importin β, of which there are six importin α subunits in humans, namely KPNA1, 2, 3, 4, 5, and 6. Importin α binds to the NLS of a protein to be imported into the nucleus, while importin β binds to importin α and mediates docking to the nuclear pore complex (NPC) and translocation through the pore. The importin β subunit can also bind to a NLS protein without the aid of an importin α subunit. The importin/NLS complex binds the GDP-bound Ras family GTPase, Ran (Ras-related nuclear protein). Together, this complex moves through the nuclear pore by forming contacts with nucleoporins. Once inside the nucleus, GDP-Ran interacts with chromatin bound RCC1 (a Ran GEF), which causes GTP binding. GTP-bound Ran triggers a conformational change of importin β to catalyze release of the NLS containing protein from importin α (98, 99). Cargo that needs to be exported outside the nucleus utilizes exportins. There are seven exportins in humans, respectively named CRM1 (Chromosome region maintenance protein 1) (XPO1), CAS (Cellular Apoptosis Susceptibility Protein) (XPO2), XPO T (XPO3), XPO4, XPO5, XPO6, and XPO7. Of these, CAS controls the nuclear export of importins after they shuttle cargo into the nucleus and XPOT mediates the nuclear export of tRNAs (98, 99). A consensus NES has only been defined for CRM1. Exportins bind to the NES of a protein to form a ternary complex with RanGTP. Upon translocation through the nuclear pore, cytoplasmic RanGAP1 stimulates GTP hydrolysis by Ran, causing complex dissociation and the release of the exported cargo into the cytoplasm (98, 99).

Cellular Adherence to Extracellular Matrices and Cell Spreading

Adhesion of cells to the ECM provides structural support for cells and is critical for tissue development and homeostasis. ECM binding also mediates activation of multiple signaling pathways to regulate cellular behavior. The ECM is comprised of an interlocking mesh of assorted fibrous proteins and proteoglycans, such as heparan sulfate, elastins, fibronectins, laminins, and collagens, of which collagen is the most abundant (100-102). Cells bind to the ECM through different cell surface receptors, including syndecans and integrins. The major receptors for cell adhesion to the ECM are integrins (103). Integrins are heterodimeric transmembrane receptors composed of single α and β chains. There are 18 α and 8 β subunits in mammals, which assemble into 24 distinct integrin complexes, allowing for specificity in ECM component binding and intracellular signaling (103). For example, while integrins α1β1 and α2β1 both bind collagen I and IV, α2β1 preferentially interacts with collagen I while α1β1 has a higher affinity for collagen IV (104-106). Integrins behave as bidirectional signal transducers, transmitting signals from the inside of the cell to the outside through cytoplasmic binding to cytoskeletal adaptor proteins, or from the ECM to the inside of the cell through extracellular ligand binding (103).

Following initial adherence to an ECM, cells begin to flatten and spread out. During this process, signaling events are triggered by integrin engagement. Prior to ECM binding, the protein Talin binds to the β subunit of the integrin dimer, inducing a conformational change in an integrin receptor that allows for binding to the ECM. Following binding of the integrin heterodimer to the ECM the focal adhesion kinase (FAK) is recruited. Clustering of multiple ECM-bound integrins allows for the trans-phosphorylation of FAK molecules on tyrosine 397, which creates a binding site for the

tyrosine kinase Src (107, 108). Src binding leads to further tyrosine phosphorylation of FAK as well as increased Src phosphorylation, maximizing the kinase activity of the FAK-Src complex (109). This FAK-Src complex recruits and activates scaffolding and adaptor proteins, such as p130Cas and paxillin. These proteins recruit additional signaling molecules to initiate cell signaling. Among these events, activation of the Rho family proteins Rac1 and Cdc42 is crucial. Their activation is mediated by binding to the Rac1 and Cdc42 exchange factors, the unconventional GEF Dock180-engulfment and motility 1 (ELMO1) complex and β-PIX, which stimulate Rac1 and Cdc42 activation to promote membrane protrusion (110-112). Once activated, Cdc42 and Rac1 promote the extension of filopodia and lamellipodia that allow for early cell spreading. Rac1 stimulation also induces reactive oxygen species production, which inhibits tyrosine phosphatase activity toward p190RhoGAP, thereby activating p190RhoGAP and inhibiting RhoA activity. This diminishes actomyosin contractility in the spreading edge of the cell (113-116).

In the later stages of spreading, 60 to 90 minutes following adhesion, Rac1 and Cdc42 activities decrease and RhoA activity steadily increases, driving maturation of focal complexes (FC) to focal adhesions (FA), promoting the formation of actin stress fibers. This provides cellular anchorage to the ECM and enhances actomyosin contraction (113, 114, 117, 118). RhoA activation also stimulates Rho-associated protein kinase (ROCK)-mediated phosphorylation of the Rac1 GAP FilGAP, thereby down-regulating Rac1 activity (117, 118). In fibroblasts the increase in RhoA activity at later stages of cell spreading has been shown to require the actions of the Rho GEFs LARG, p115RhoGEF (Lsc), and p190RhoGEF (119, 120). However it is not clear how the activity of these Rho GEFs are coordinated during cell spreading, nor is it known why multiple RhoA directed GEFs are required for efficient spreading. Also,

most of these studies have been conducted in mouse fibroblasts. It is possible that different Rho GEFs may be operative in human breast cancer cells.

Constitutive activation of Rac1 stimulates Net1A extranuclear localization

Previous work in the Frost lab was directed at screening upstream regulators and downstream effectors of Rho proteins to identify factors that would cause relocalization of Net1 isoforms from the nucleus. From these efforts, the lab found that co-expression of constitutively active Rac1 (V^{12}Rac1) caused a dramatic re-localization of Net1A outside the nucleus in transfected MCF7 breast cancer cells. As shown in Figures 3A and 3B, when transfected alone, Net1 and Net1A localized outside of the nucleus in approximately 15% and 25% of transfected cells, respectively (61, 87, 96). However, co-expression of V^{12}Rac1 robustly increased the percent of transfected cells with extranuclear Net1A to more than 80% of cells. This considerable effect, however, was not observed when V^{12}Rac1 was co-expressed with Net1, suggesting that the localization of Net1 and Net1A are regulated differently. Furthermore, using subcellular fractionation followed by Western blotting, the localization of HA-tagged Net1A in the membrane fraction was significantly increased by co-expression of Myc-tagged V^{12}Rac1, indicating that Net1A was re-localized to the membrane (Figure 3C).

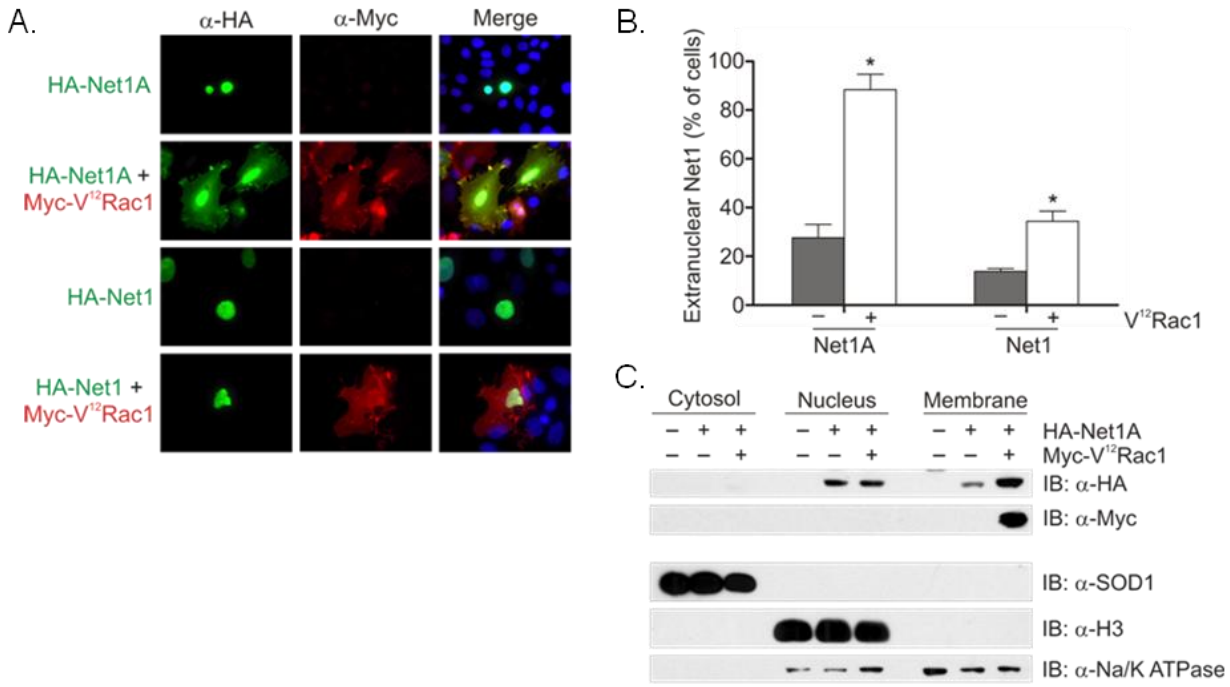


Figure 3. Co-expression of V^{12}Rac1 relocalizes Net1A outside the nucleus. (A) MCF7 cells were transfected with HA-Net1 or HA-Net1A, plus control vector or Myc-V^{12}Rac1, and processed for immunofluorescence by using anti-HA antibodies (green), anti-Myc antibodies (red), and DAPI (stains DNA, blue). A representative panel for each transfection is shown. The constructs used for each condition are indicated to the left of each panel. (B) Quantification of extranuclear Net1 isoform localization. At least 100 cells were counted for each condition. Significance was estimated by Student's *t* test for non-paired values. Error bars represent standard error of the mean. * = P <0.002. (C) Cells were transfected with HA-Net1A alone or with Myc-V^{12}Rac1 and processed for subcellular fractionation. Proteins within each fraction were analyzed by Western blotting using the indicated antibodies. Results of a representative experiment are shown (Frost Lab, data not published).

Since V^{12}Rac1 co-expression relocalized Net1A outside the nucleus, the Frost Lab also tested whether the related Rho GTPase Cdc42 also caused Net1A relocalization in transfected MCF7 cells. In these experiments, Net1A localization was tested only one day after transfection, as expression of constitutively active Cdc42 (V^{12}Cdc42) was toxic to MCF7 cells after longer periods of time (data not shown). In these assays, it was found that co-expression of V^{12}Rac1 caused extranuclear

localization of Net1A in approximately 60% of the cells while V^{12}Cdc42 only caused 30% of the cells to exhibit extranuclear Net1A (data not shown), suggesting that V^{12}Rac1 is more effective than V^{12}Cdc42 for stimulating Net1A relocalization outside the nucleus.

The Frost Lab also determined what aspects of Net1A function were required for Rac1-stimulated relocalization. For these assays the following Net1A mutants were tested: catalytically inactive Net1A (L267E, L/E); the PH domain mutant (W438L, W/L) (60), and the deletion mutant Net1A 1-307, which lacks the PH domain and the C-terminal regulatory domain (Figure 2). Immunofluorescence analysis showed that each Net1A mutant efficiently relocalized outside the nucleus when co-expressed with V^{12}Rac1. Co-expression of wild type Net1A, Net1A L/E, and Net1A W/L with V^{12}Rac1 caused approximately 70% of the cells to contain extranuclear Net1A. Rac1 was slightly less efficient at causing re-localization of Net1A 1-307, with only 60% of the cells exhibiting extranuclear Net1A localization (data not shown). Taken together, these results demonstrate that the catalytic activity, potential phosphatidyl-inositol binding ability, and C-terminal sequences within Net1A are not necessary for stimulation of its relocalization outside the nucleus by V^{12}Rac1.

Extranuclear localization of Net1 is required for RhoA activation, actin cytoskeletal reorganization, and oncogenic transformation. However, regulatory mechanisms controlling the import or export of Net1 have not been identified. It has been determined that the relocalization of Net1A from the nucleus to the plasma membrane in breast cancer cells is regulated downstream of constitutively activation of Rac1. Through the current study, we established that Rac1 expression and activation are necessary for Net1A relocalization during cell spreading. These results suggest that Net1 isoforms are regulated differently and that Rac1-mediated relocalization of

Net1A may be a significant determinant of the ability of cells to adhere to an ECM and for cell motility.

CHAPTER 2: MATERIALS AND METHODS

Antibodies and cDNA constructs

The following antibodies were used: anti-Rac1, anti-Cdc42, and anti-paxillin (BD Biosciences); anti-GAPDH, anti-glutathione S-transferase (GST), anti-Net1, anti-HA, anti-SOD1, and anti-Na^+/K^+ ATPase (Santa Cruz); anti-RhoA (Cytoskeleton); anti-H3 and anti-phosphoFAK (Y397) (Cell Signaling); anti-Myc epitope (AbCam); and anti-α-tubulin (Sigma). Primary antibodies used for Western blotting were detected with horseradish peroxidase-conjugated secondary antibodies (Kirkegaard & Perry Laboratories) via enhanced chemiluminescence (ECL). For immunofluorescence microscopy, Cy2- and Cy3- conjugated anti-mouse and anti-rabbit secondary antibodies were from Jackson ImmunoResearch. TRITC-phalloidin and 4', 6-diamidino-2-phenylindole (DAPI) were from Sigma. Alexa 647-phalloidin was from Invitrogen. Wild type and constitutively active V^{12}Rac1 and V^{12}Cdc42 were in pCMV5M, as described (96). Mouse Net1, Net1A and their respective mutants were in pEF_{HA} (87). DNA sequencing was used to verify all constructs.

Cell culture and transfection

MCF7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (FBS) (Sigma) and antibiotics (100 units/mL penicillin-streptomycin) (HyClone). MCF7 cells were transfected with cDNA expression vectors using Lipofectamine/Plus (Invitrogen) following the manufacturer's instructions. Cells were assayed 48 h after transfection.

Small interfering RNAs (siRNA) against human Rac1 were from Sigma. Rac1 siRNA sequences were as follows: Rac1-1 sense, 5'-

AAGGAGAUUGGUGCUGUAAAAUU-3', and antisense, 5'-UUUUACAGCACCAAUCUCCUU-3'; and Rac1-2 sense, 5'-AACCUUUGUACGCUUUGCUCAUU-3', and antisense, 5'-UGAGCAAAGCGUACAAAGGUUUU-3'. The RNA sequences, sense, 5'-GAUCAUACGUGCGAUCAGAUU-3', and antisense, 5'-UCUGAUCGCACGUAUGAUCUU-3', was used as a non-targeting control (Sigma). MCF7 cells were transfected with siRNAs using the INTERFERin transfection reagent (Polyplus) according to manufacturer's instructions. Cells were assayed 96 h after transfection with Rac1 siRNA.

Recombinant protein expression

V17A-RhoA (RhoA(17A)) in pGEX-KG was created by PCR-mediated mutagenesis and verified by DNA sequencing. GST- or GST-RhoA17A proteins were purified as previously described (121). Briefly, BL21(DE3) *E. coli* transformed with pGEX-KG or pGEX-KG/RhoA(17A) were cultured to $O.D._{600}$ = 0.8 and GST protein expression was induced for 12 to 16 h at room temperature following the addition of 50 μM IPTG (isopropyl-β-D-thiogalactopyranoside). Bacterial cells were collected by centrifugation (6,000 x g), lysed in buffer containing 20 mM HEPES pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μg/ml each of aprotinin, leupeptin, and pepstatin A, and insoluble material was pelleted by centrifugation (20,000 x *g*, 30 minutes, 4°C). GST or GST-RhoA17A in the soluble fraction was purified by incubation with glutathione-agarose beads (Sigma) for 1 h at 4°C, followed by two washes with lysis buffer and two washes with lysis buffer lacking Triton X-100 and protease inhibitors. Protein purity was

assessed by Coomassie staining and concentrations were tested by BCA assay (Pierce). Proteins were left attached to the beads and frozen in aliquots.

Rac1 activity was measured by pulldown assay using the p21-binding domain (PBD) of Pak1 cloned into pGEX-2T, a kind gift from Catherine Denicourt (UT-Houston). Prokaryotic expression of the pGEX-2T/PBD construct was performed as described (122). Briefly, BL21(DE3) *E. coli* transformed with pGEX-2T/PBD were grown to $O.D._{600}$ = 0.8 and expression of the GST or GST-PBD fusion proteins in was induced with 400 μM IPTG for 3 h at 30°C. Bacterial cells were pelleted by centrifugation (6,000 x g) and re-suspended in buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 mg/ml lysozyme (Fisher), and 10 μg/ml each of aprotinin, leupeptin, and pepstatin A. After sonication, insoluble material was pelleted by centrifugation (12,000 x *g*, 10 min, 4°C), and soluble proteins were incubated with glutathione-agarose beads for 1 h at 4°C followed by three washes using buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin A. Protein purity was assessed by Coomassie staining and concentrations were tested by BCA assay. Proteins were left attached to the beads and frozen in aliquots.

Net1 activity pulldowns

Active Net1 pulldown experiments were performed as described (121). Briefly, cells were lysed in lysis buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 1 mM DTT, 1 mM PMSF, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin A), sonicated for 30 s, and clarified by centrifugation (13,000 x *g*, 5 min,

4°C). Lysates concentrations were determined by BCA assay and equal amounts of lysate were mixed for 1 h at 4°C with 20 µg of GST or GST-RhoA17A beads. Beads were pelleted by centrifugation and washed 3 x in lysis buffer, resuspended in 25 µl sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris-HCl pH 6.8, 100 mM DTT, 2% SDS, 0.02% bromophenol blue, and 10% glycerol), boiled for 5 min, and separated by SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to polyvinylidene fluoride (PVDF) membrane (GE, Millipore) and immunoblotted with the indicated antibodies.

Rac1 activity assays

Active Rac1 pulldown experiments were performed as described (122). Briefly, suspended and adherent cells were lysed in buffer containing 50 mM Tris-HCl pH 7.5, 200 mM NaCl, 10 mM $MgCl_2$, 1% NP-40, 5% glycerol, 1 mM PMSF, and 10 µg/ml each of aprotinin, leupeptin, and pepstatin A and incubated on ice for 5 min. Insoluble material was pelleted by centrifugation (13,000 × *g*, 10 min, 4°C). Lysate concentrations were determined by BCA assay and equal amounts were incubated with GST or GST-PBD beads for 1 h at 4°C. The beads were pelleted and washed 3 times in wash buffer (25 mM Tris-HCl pH 7.5, 40 mM NaCl, 30 mM $MgCl_2$, 1% NP-40, 1 mM DTT, 1 mM PMSF, and 10 µg/ml each of aprotinin, leupeptin, and pepstatin A), followed by two washes with wash buffer lacking NP-40. Lysates were subsequently prepared for SDS-PAGE, transferred to PVDF membrane and immunoblotted for Rac1 and GST proteins.

Cell spreading assays

Cells were trypsinized (HyClone), washed three times with phosphate-buffered saline (PBS), and resuspended in 0.5% de-lipidated bovine serum albumin (BSA) (Sigma) in DMEM for 1 h at 37°C. Cells were then replated on tissue culture dishes or glass coverslips previously coated with collagen IV (10 μg/ml) (BD Biosciences). Coverslips or tissue cultures dishes were coated with Collagen IV diluted in PBS for 1 hr at room temperature then washed with PBS and stored at 4°C until ready for use. Cells were harvested at the appropriate times for biochemical or microscopic analysis.

Immunofluorescence microscopy

Cells grown on collagen IV coated coverslips were washed in PBS and fixed in 4% paraformaldehyde for 10 min at 37°C. Cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min at room temperature. Coverslips were then washed in PBS plus 0.1% Tween 20 (PBST), followed by blocking with 1% BSA in PBST for 30 min. Cells were incubated with primary antibodies diluted to 1-3 μg/ml in PBST + 1% BSA for 1 h at 37°C. Coverslips were washed 3 x in PBST for 5 min and incubated with secondary antibodies diluted to 0.5-2 μg/ml in PBST + 1% BSA for 1 h at 37°C. Coverslips were washed and mounted on slides with FluorSave Reagent (Calbiochem). Epifluorescence images were captured with a Zeiss Axioskop microscope equipped with a Zeiss AxioCam MRm MC100 SPOT digital camera and AxioVision software. For quantitative analysis, images were serially acquired with the same illumination and exposure parameters, and the average fluorescence intensity in regions of interest for each transfected cell was performed using Image J software.

CHAPTER 3: RESULTS

Constitutive activation of Rac1 stimulates Net1A activity

Net1 activity towards RhoA is inhibited by nuclear sequestration (82, 87). Because co-expression of V^{12}Rac1 with Net1A causes its relocalization outside the nucleus, we examined whether this also resulted in an increase in the catalytic activity of Net1A. MCF7 breast cancer cells were transfected with expression plasmids for HA-Net1, HA-Net1A, or HA-Net1ΔN alone or with Myc-V^{12}Rac1. After two days the cells were harvested and equal amounts of lysate were incubated with recombinant GST-RhoA(17A) bound to glutathione-agarose. GST-RhoA(17A) is a nucleotide-free RhoA mutant that binds tightly to active Rho GEFs (121). After incubation, GST-RhoA(17A) complexes were pelleted by centrifugation, washed, and resolved by SDS-PAGE. The presence of HA-tagged Net1 proteins in these complexes was then assessed by Western blotting. Net1 activity was calculated by dividing the level of Net1 in the GST-RhoA(17A) pulldowns by the amount of Net1 in the lysate, normalized to the amount of GST-RhoA(17A) used in the pulldown (GST-RhoA(17A) bound Net1 / total Net1 in lysate / GST-RhoA(17A) in pulldown).

As shown in Figure 4, both HA-Net1 and HA-Net1ΔN displayed a high level of activity in MCF7 cells irrespective of whether V^{12}Rac1 was co-expressed. This was expected for Net1ΔN, as it has been reported previously to function as a constitutively active form of Net1 in other cell types (60, 82). Similarly, Net1 has been reported to be constitutively active (87, 91). On the other hand, HA-Net1A expressed alone in MCF7 cells displayed very little activity. However, co-expression of V^{12}Rac1 strongly activated Net1A, increasing its ability to bind to GST-RhoA(17A) by nearly three-fold. Interestingly, co-expression of V^{12}Rac1 also increased the expression of each Net1

isoform. Since Net1A has been observed previously to be targeted for proteasome-mediated degradation (76), this may indicate that co-expression of V^{12}Rac1 stabilizes Net1 proteins in general. In this regard, others in the Frost Lab have observed that co-expression of V^{12}Rac1 significantly increases the half-life of transfected HA-Net1A (data not shown). Importantly, our method for quantifying Net1 isoform activity included normalization for Net1 expression, indicating that increases in Net1A activity caused by V^{12}Rac1 co-expression cannot be discounted due to altered Net1A expression. Taken together, these data indicate that co-expression of constitutively active Rac1 strongly stimulates the activity of Net1A, but not Net1, similar to its effects on the extranuclear localization of Net1 isoforms.

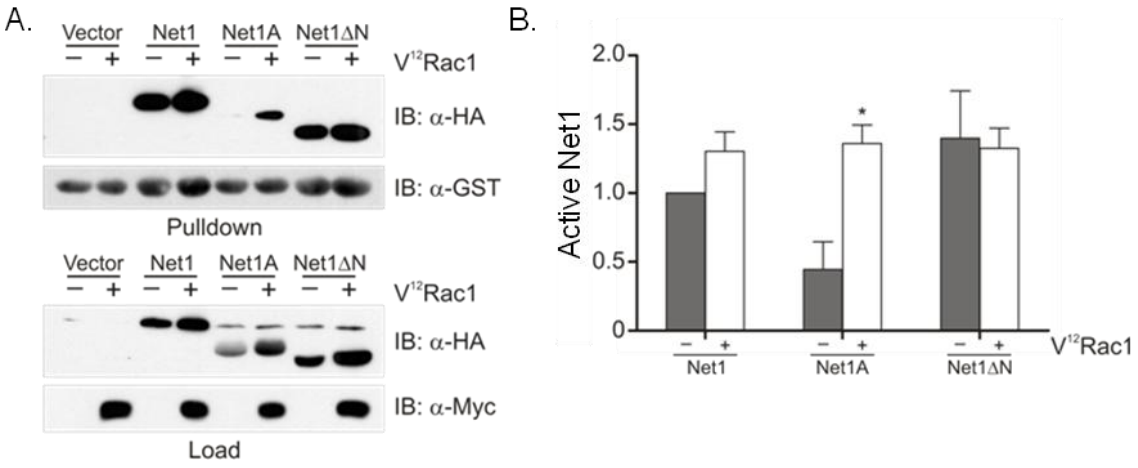


Figure 4. Co-expression of V^{12}Rac1 stimulates Net1A activation. (A) Cells were transfected with HA-Net1, HA-Net1A, or HA-Net1ΔN, alone or with Myc-V^{12}Rac1, as indicated. Equal amounts of lysate from each population of cells were incubated with glutathione-agarose-bound GST-RhoA(17A). Binding of Net1 proteins to GST-RhoA(17A) was analyzed by Western blotting (top panel). Expression of HA-Net1 proteins and Myc-V^{12}Rac1 in the lysates is shown in the bottom panels. Results of a representative experiment are shown. (B) Quantification of Net1 isoform activity. Active Net1 was defined as the amount of HA-Net1 protein in the GST-RhoA(17A) pulldown divided by that in the lysate, and values were adjusted according to the amount of GST-RhoA(17A) in the pulldowns. Data were then normalized to the level of activity exhibited to HA-Net1 transfected alone. The results from 6 independent experiments are shown. Significance was determined by Student's *t* test for non-paired values. * = $p < 0.005$.

Endogenous Rac1 controls Net1A localization

When over-expressed alone, approximately 25% of the HA-Net1A transfected cells exhibited extranuclear localization of Net1A (Figure 1B). This begged the question of whether endogenous Rac1 activation accounts for the extranuclear localization of Net1A in these cells. To test this idea, MCF7 cells were transfected with non-targeting (control) or Rac1-specific siRNAs. Two days later, the cells were transfected with an HA-Net1A expression vector. Two days after that the cells were fixed and stained for HA-Net1A localization. As shown in Figure 5A, inhibition of Rac1 expression drastically reduced the extranuclear localization of HA-Net1A. Quantification of these results showed that inhibition of Rac1 expression decreased the number of cells exhibiting extranuclear localization of Net1A from nearly 30% to 10%. These effects were consistent using two siRNAs targeting distinct sequences within human Rac1, both of which strongly inhibited Rac1 expression (Figure 5C). These data indicate that basal activation of endogenous Rac1 in MCF7 cells drives the extranuclear localization of transfected Net1A, and support the notion that Net1A relocalization stimulated by exogenously expressed V^{12}Rac1 reflects a valid physiological mechanism controlling Net1A localization.

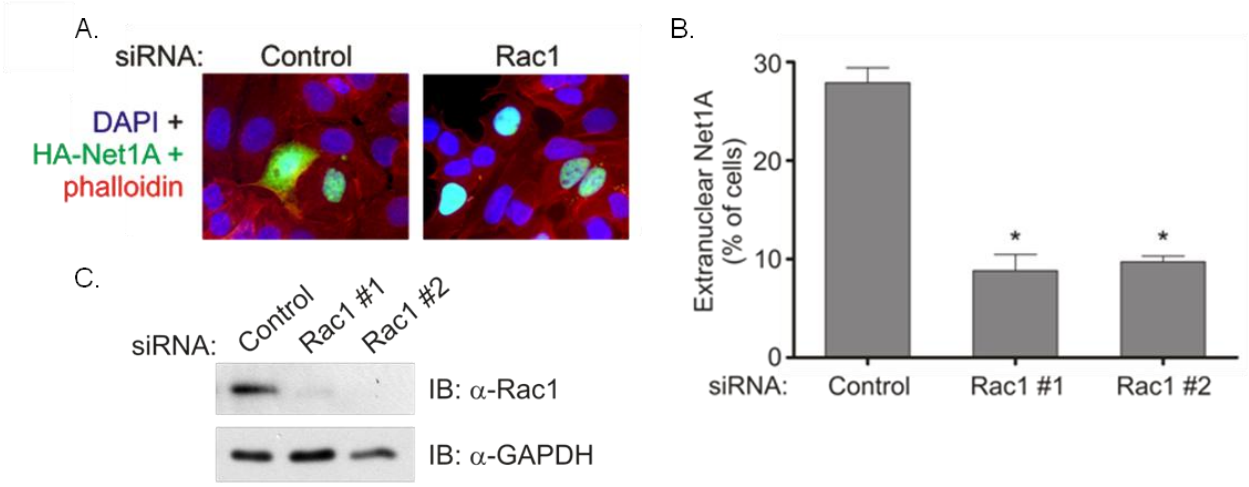


Figure 5. Endogenous Rac1 controls extranuclear localization of Net1A. Cells were transfected with non-targeting control or Rac1-specific siRNAs. Two days later, the cells were re-transfected with an expression vector for HA-Net1A. Two days after that, the cells were fixed and stained for HA-Net1A localization. (A) Representative panels showing HA-Net1A localization (green) in control and Rac1 siRNA transfected cells. DNA is stained blue and F-actin is stained red. (B) Quantification of HA-Net1A localization in control and Rac1 siRNA transfected cells. Shown is the average of three independent experiments. Significance was determined by Student's *t* test for non-paired values. * = $p < 0.0002$. (C) Representative Western blot confirming inhibition of Rac1 expression.

Spreading on collagen IV stimulates Rac1 activity and Net1A relocalization

Our data shows that expression of constitutively active Rac1 causes Net1A relocalization outside the nucleus and results in Net1A activation. Thus, we wanted to test whether stimuli that are known to activate endogenous Rac1 also regulated Net1A localization in MCF7 cells. To test this, we examined whether cell spreading after trypsinization caused Rac1 dependent Net1A relocalization, since this process is known to strongly activate Rac1 in other cells types (110-112). To determine whether replating of MCF7 cells on collagen stimulated endogenous Rac1 activation, actively growing cells at 80% confluency were trypsinized and replated on dishes coated with collagen IV. At different times the cells were lysed and tested for Rac1 activation

using a conventional pulldown assay (122). In this assay, cell lysates are incubated with the recombinant Rac1 binding domain from Pak1 fused to GST (GST-PBD). Since only active Rac1 binds to this domain, the amount of Rac1 bound is directly proportional to the degree of Rac1 activation. Rac1 activation can be detected by Western blot, and is quantified by dividing the amount of Rac1 in the pulldowns by the amount of Rac1 in the lysate, normalized to the amount of GST-PBD used in the pulldown. In these assays, we observed that endogenous Rac1 was strongly activated within 5 min after replating, with peak Rac1 activation occurring within 30 minutes of replating. By 90 minutes Rac1 activation had returned to baseline (Figures 6A and 6B). Thus, these experiments demonstrate that replating MCF7 cells on collagen IV strongly induces endogenous Rac1 activity.

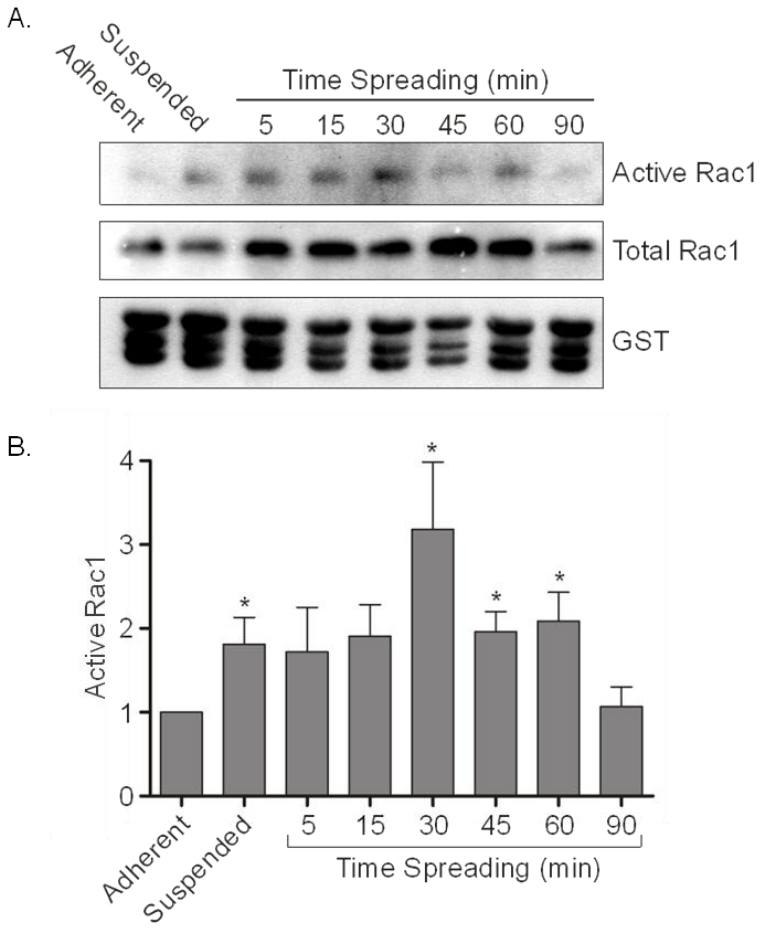


Figure 6. Replating MCF7 cells on collagen strongly activates endogenous Rac1. (A) Endogenous active Rac1 was pulled down from cell lysates with recombinant GST-PBD and analyzed by Western blotting using anti-Rac1 antibodies. The upper panel shows Rac1 bound to GST-PBD, the middle panel shows Rac1 in the lysate. GST-PBD in the pulldowns is shown in the lower panel. Shown is a representative experiment. (B) Quantification of Rac1 activation following replating on collagen IV. Shown is the average of three independent experiments. Significance was determined by Student's *t* test for non-paired values. * = $p < 0.05$.

We then examined whether replating cells on collagen would stimulate Net1A relocalization. MCF7 cells were transfected with an HA-Net1A expression vector, trypsinized, and replated on collagen. At different times, the cells were then fixed and stained for Net1A localization, DNA, and F-actin. As a positive control, adherent cells were co-transfected with V^{12}Rac1. To more precisely measure HA-Net1A localization,

we quantified the fluorescent intensity of HA-Net1A in the nucleus and cytoplasm, and displayed the results as a ratio of cytoplasmic to nuclear Net1A (Cyto/Nuc). Typically, nuclear localization of HA-Net1A in MCF7 cells is indicated by a Cyto/Nuc ratio of about 0.8, and an increase in this ratio signifies increased localization of Net1A outside the nucleus. By this analysis we observed that HA-Net1A was mainly localized in the nucleus in adherent cells before trypsinization and replating, and quickly relocalized outside the nucleus upon replating. Peak relocalization of HA-Net1A outside the nucleus occurred 60 min after replating onto collagen, and by 90 min HA-Net1A was largely re-localized inside the nucleus (Figures 7A and 7B). Altogether, these data suggest that integrin engagement onto a collagen matrix stimulates Rac1 activation, which in turn stimulates Net1A relocalization outside the nucleus.

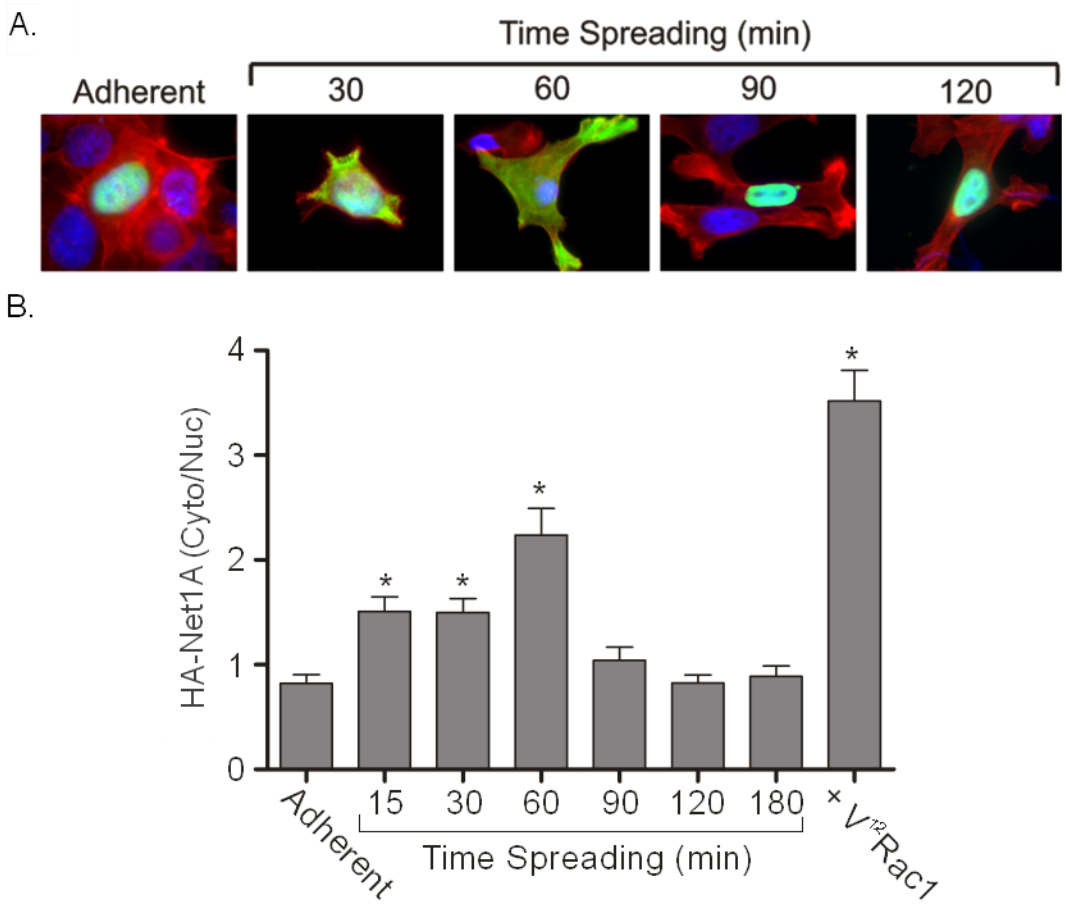


Figure 7. Relocalization of Net1A in cells spreading on collagen IV. (A) Cells were transfected with HA-Net1A and then replated on collagen IV coated coverslips. At different times, the cells were fixed and stained for HA-Net1A (green), DNA (blue), and F-actin (red). A representative experiment is shown. (B) Quantification of HA-Net1A subcellular localization. Fluorescence intensity of HA-Net1A staining was analyzed using Image J software and is depicted as the ratio of intensities in the cytoplasm and nucleus (Cyto/Nuc). Shown is the average of three independent experiments. Significance was determined by Student's *t* test for non-paired values. * = $p < 0.0001$.

Rac1 controls Net1A relocalization during cell spreading

To determine the role of Rac1 on Net1A relocalization during cell spreading, we transfected MCF7 cells with control or Rac1-specific siRNAs. One day later, the cells were re-transfected with an HA-Net1A expression vector. Two days after that, the cells were trypsinized and replated on collagen coated coverslips. At different times the cells were fixed and stained for HA-Net1A localization, DNA, and F-actin. In these

experiments cells depleted of Rac1 exhibited a reduced rate of spreading, consistent with a pivotal role for Rac1 in early spreading events in other cell types (108, 111, 112). Importantly, we also observed that inhibition of Rac1 expression completely inhibited the relocalization of Net1A during cell spreading (Figures 8A and 8B). These data indicate that Rac1 expression is necessary for Net1A relocalization outside the nucleus in spreading cells.

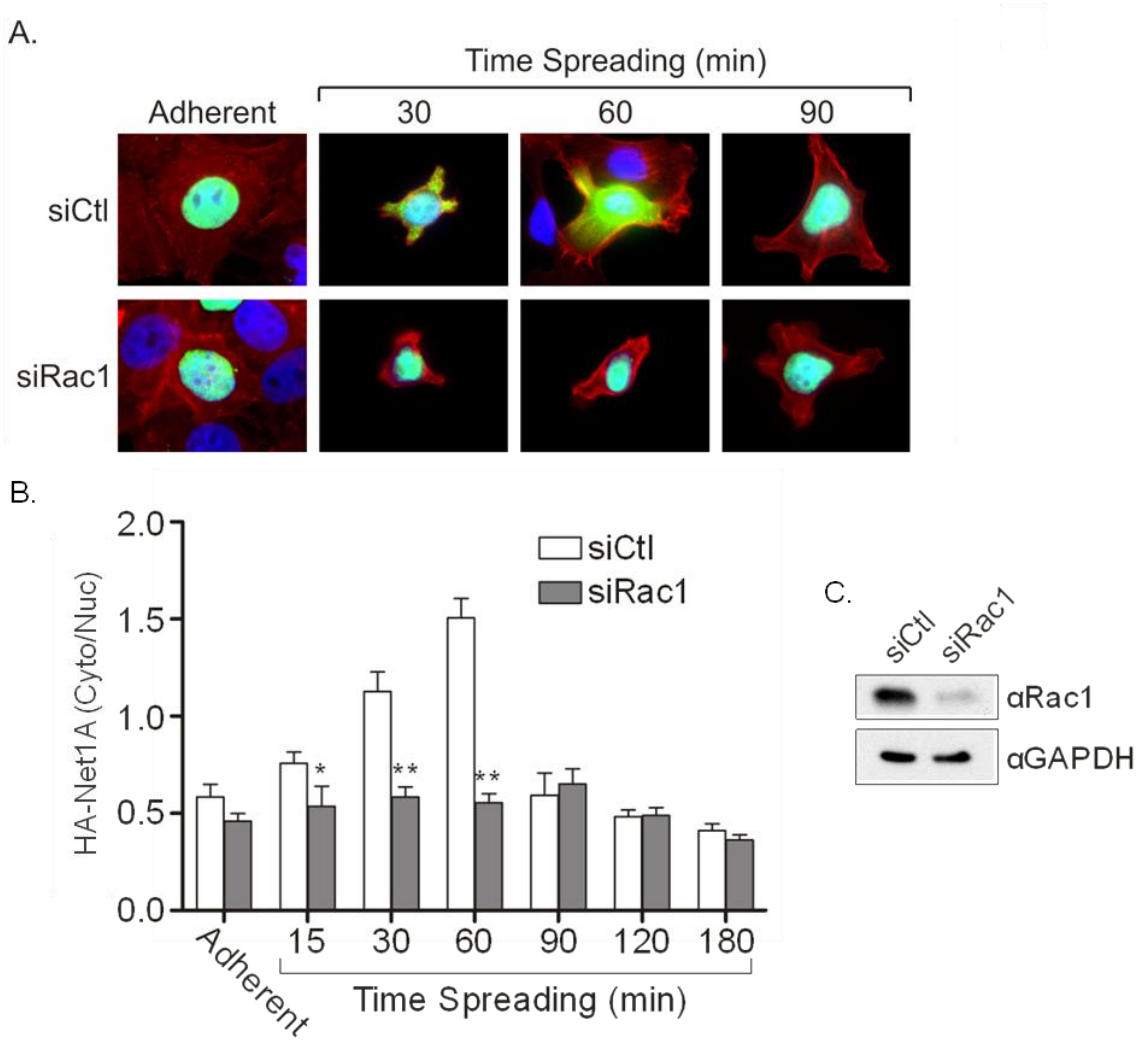


Figure 8. Net1A relocalization during cell spreading requires Rac1 expression. (A) Cells were transfected with control or Rac1-specific siRNAs, and then two days later re-transfected with HA-Net1A expression vector. Two days after that, the cells were trypsinized and replated on collagen coated coverslips. At different times the cells were fixed and stained for HA-Net1A (green), DNA (blue), and F-actin (red). Shown is a representative experiment. (B) Quantification of HA-Net1A subcellular localization. The fluorescence intensity of HA-Net1A in the cytoplasm and nucleus was quantified. The average of three independent experiments is shown. Significance was determined by Student's *t* test for non-paired values. * = $p < 0.05$; ** = $p < 0.0001$. (C) Representative Western blot demonstrating knockdown of Rac1 expression.

CHAPTER 4: DISCUSSION

Net1 activity towards RhoA is negatively regulated by nuclear localization (61, 87). However, stimuli causing the redistribution of Net1 isoforms outside the nucleus have not been published. In the present study, we have demonstrated that expression of constitutively active Rac1 stimulates Net1A re-localization to the plasma membrane and results in an up-regulation of Net1A activity. Interestingly, Net1 appears to maintain a high basal activity (Figure 4). Moreover, Rac1 activation is necessary for Net1A relocalization outside the nucleus during cell spreading on a collagen matrix. Thus, these data represent the first mechanism for regulating the subcellular distribution of Net1A, and demonstrate that the localization of Net1 isoforms is differentially regulated.

Little is known about mechanisms controlling the nuclear import and export of Net1A. Nuclear import and export are typically regulated by the small GTPase Ran, which controls import and export by chaperone proteins known as importins and exportins, respectively (98, 99). We hypothesize that Rac1 activation should alter Net1A localization either by negatively regulating Net1A-importin association, up-regulating Net1A-exportin association, or a combination of the two mechanisms. Net1A contains two predicted NLS sequences in its amino-terminus (Figure 2). Others in the Frost Lab have shown that both of these NLS sequences contribute to the nuclear localization of Net1A, although the first NLS (NLS3) seems to be the more important of the two sequences (not shown). In this regard, the Frost lab has also observed that the importin α subunit, KPNA2, co-immunoprecipitates with Net1A (not shown). In addition, KPNA2 mediates the nuclear import of Rac1 (123). So conceivably, the first NLS (NLS3) of Net1A may bind to KPNA2 to mediate nuclear Net1A import. In this regard it would be important to show whether KPNA2 mediates

nuclear import of Net1A, and then to determine whether Rac1 activation alters KPNA2-dependent Net1A nuclear import.

It would also be important to determine whether nuclear export mechanisms contribute to Rac1 mediated relocalization of Net1A. Previous work has shown that the PH domain of Net1A mediates CRM1 dependent nuclear export of the N-terminal truncation mutant, Net1ΔN, from the nucleus (61). However, others in the Frost Lab have observed that treatment of MCF7 cells with the CRM1 inhibitor, leptomycin B, does not block nuclear export stimulated by constitutively active Rac1 (not shown). Thus, if nuclear export of Net1A contributes to its Rac1 dependent re-localization, then one or more exportins other than CRM1 is likely to control this event. Further work is necessary to evaluate how Rac1 activation affects Net1A interaction with the relevant importin or exportin.

The mechanism by which Rac1 signals to cause Net1A relocalization is presently not known. Rac1 initiates intracellular signaling by stimulating the activation of a known set of effector proteins. Thus, identifying the Rac1 effector responsible for Net1A relocalization is important to understanding how Net1A localization and activity are controlled in breast cancer cells. There are approximately 30 effectors for Rho, Rac, and Cdc42 GTPases (32). However, since V^{12}Rac1 is more effective than constitutively active Cdc42 at causing Net1A relocalization, and RhoA activation does not affect Net1A localization (not shown), we would expect that potential effectors should be preferentially be activated by Rac1.

Rac1-dependent effectors that may be responsible for relocalization of Net1A include the kinases MLK2 and 3, MEKK1 and 4, PAKs1-3, the phospholipase PLC-β2, and the scaffolding proteins POSH, POR1, p140Sra-1, and IQGAP1-3 (82, 124-134). Although this list of effectors is long, the respective function of the possible Rac1

effectors can be utilized to identify likely candidates. For instance, the Rac1 effector p70 S6 kinase regulates mRNA translation (32, 135), so it is unlikely to control the ability of Net1A to affect actin cytoskeleton organization. In addition, the Frost lab has shown previously that the Rac1/Cdc42 effector PAK1 phosphorylates Net1 in response to Rac1 activation, which inhibits its ability to stimulate actin stress fiber formation (a RhoA phenotype) (96). As the PAK1 phosphorylation sites are adjacent to the second NLS (NLS4) in Net1A, it is possible that PAK1 mediates Rac1 effects on Net1A localization. Future work will be required to identify the Rac1 effector that controls Net1A localization.

Because we observed that V^{12}Rac1 expression caused extranuclear localization of Net1A, we examined whether stimuli that promote endogenous Rac1 activation also impact Net1A localization. For these experiments, we focused on Rac1 activation stimulated by cellular adhesion, since this is known to require Rac1 at early times, followed by RhoA activation at later times (108). Importantly, we found that cell spreading dramatically stimulated the relocalization of Net1A outside the nucleus (Figure 7). Furthermore, we determined that Rac1 was activated by cell spreading in MCF7 cells, and that its expression was required for Net1A relocalization (Figures 5 and 8), Moreover, we also observed that the catalytically-inactive Net1A mutant, Net1A L267E, also relocalized outside the nucleus during cell spreading (not shown), suggesting that spreading induced Net1A subcellular redistribution independently of Net1A-stimulated RhoA activation. My preliminary data has also suggested that Net1A activity is enhanced during cellular spreading, peaking at 60 min following adhesion, and returning to basal activity by 90 min (not shown). Thus, the subcellular localization of Net1A is regulated by Rac1 during cell spreading, similar to what we

observed in cells transfected with V^{12}Rac1. This indicates that our over-expression experiments are likely to reflect a physiologically relevant regulatory mechanism.

We then asked whether Net1A re-localization was important for cell spreading. Although the results are preliminary, we have observed that MCF7 cells transfected with siRNA targeting both Net1 isoforms exhibited a reduced rate of cell spreading in comparison to cells treated with control siRNA (not shown), suggesting that Net1A is important for efficient cell spreading. In this regard, the Rho GEFs, LARG, Lsc, and p190RhoGEF have been shown to contribute to cell spreading in fibroblasts plated on fibronectin and collagen (119, 120). In the future, it will be important to determine whether they also contribute to cell spreading in MCF7 breast cancer cells, and to investigate the relationship of Net1A to their function.

Not surprisingly, the kinetics of Rac1, Net1A, and RhoA activities during spreading in MCF7 cells fits with cellular spreading dogma; Rac1 is activated and RhoA is down-regulated during early spreading events, and in later spreading stages RhoA and Rac1 activities are reversed. For example, we observed that Rac1 is quickly activated by replating on collagen, reaching a peak by 30 min after replating (Figure 6). Moreover, the kinetics of Rac1 activation coincides with the rapid relocalization of Net1A outside the nucleus (Figure 7). Net1A relocalization and activity kinetics correlates with peak RhoA activity observed in various cell lines at 30 to 90 min of spreading on fibronectin and collagen (119, 120, 136, 137). However, RhoA activation in response to Rac1 stimulated Net1A redistribution in MCF7 cells has not yet been confirmed by our group.

Our data are consistent with the following model for Net1A regulation during cell spreading. We have observed that following adhesion to the ECM, cell spreading activates Rac1. Active Rac1 mediates a rapid increase in Net1A nuclear export, or

decreased nuclear import. By unknown mechanisms, extranuclear Net1A maximally translocates to the plasma membrane and becomes maximally activated. Concurrently, since V^{12}Rac1 co-expression increases the half-life of Net1A (not shown), and interaction with the scaffolding protein Dlg1 has been reported to increase Net1A stability (76), relocalized Net1A may be stabilized by interaction with Dlg1, which typically localizes to the plasma membrane in epithelial cells. During the latter stages of cell spreading, Net1A continues to contribute to RhoA activation, leading to efficient focal adhesion and actin stress fiber formation. Our findings provide a physiological role for the Net1A isoform and suggest an added level of control to cellular spreading mechanisms, where not only does Rac1 activity antagonize RhoA activity following adhesion through Rho GAP activation, but also prepares for RhoA activation mediated by temporal and spatial activation of Rho GEFs such as Net1A.

CHAPTER 5: CONCLUSIONS

Breast cancer metastasis is the critical event leading to death in breast cancer patients. Understanding the mechanisms controlling metastatic progression of tumor cells is thus extremely important, yet this process is not well understood. It is known that activation of Rho family small G proteins, especially RhoA, contributes to breast cancer metastasis. However, there is little data showing how RhoA activation is regulated in breast cancer. Previous work from our lab indicates that Net1 isoforms specifically regulate RhoA activity. It has also been previously established that activity of Net1 isoforms are regulated by subcellular localization. Net1 isoforms are nuclear proteins and must be localized outside of the nucleus in order to activate RhoA. We have shown that Rac1 activating stimuli, specifically cellular anchorage to extracellular substrates, relocalize Net1A outside the nucleus in a Rac1 dependent manner and increase Net1A activity. These findings suggest that Net1A relocalization may be critical for efficient cellular spreading.

In future research efforts, it would be important to elucidate the mechanisms controlling nuclear import and export of Net1A using over-expression, co-immunoprecipitations, and RNAi approaches. How Rac1 stimulation alters the nuclear import and export dynamics of Net1A should also be studied. Ways that Rac1 may alter Net1A localization may be through increased Net1A export and/or decreased importin-Net1A association. This may occur through Rac1 stimulated post-translational modification of Net1A, or through altered protein binding to Net1A. Rac1 stimulates intracellular signaling by activating a known set of effector proteins. The Rac1 effector that mediates Net1A redistribution could also be identified using a combination of RNAi and over-expression methods. Furthermore, continued investigation into the role of Net1A in breast cancer cell spreading is necessary. It will

be interesting to verify if the mechanisms regulating Rac1-induced Net1A relocalization in MCF7 cells also contributes to cell spreading in metastatic breast cancer cells. Since cell spreading dynamics are thought to reflect similar mechanisms used in cell migration and ECM invasion, Rac1 may also regulate Net1A-dependent breast cancer cell migration and invasion. With this understanding of Net1A regulation, novel therapeutic targets can be developed for use in the diagnosis and treatment of breast cancer.

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