Insertional Mutagenesis Screen Identifies HACE1 as a HER2 Cooperating Tumor Suppressor Whose Loss Results In Hyperactive Rac Signaling

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INSERTIONAL MUTAGENESIS SCREEN IDENTIFIES HACE1 AS A HER2 COOPERATING TUMOR SUPPRESSOR WHOSE LOSS RESULTS IN HYPERACTIVE RAC SIGNALING

By

Erik Tadashi Goka

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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INSERTIONAL MUTAGENESIS SCREEN IDENTIFIES HACE1 AS A HER2 COOPERATING TUMOR SUPPRESSOR WHOSE LOSS RESULTS IN HYPERACTIVE RAC SIGNALING

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The transformation from ductal carcinoma in situ (DCIS) to invasive breast cancer (IBC) is a crucial step in breast cancer progression. The specific alterations that govern this transition have not been elucidated. HER2/neu is frequently overexpressed in DCIS but is less common in IBC suggesting additional requirements for transformation. To identify genes capable of cooperating with HER2/neu to fully transform mammary epithelial cells, we used an insertional mutagenesis screen on cells isolated from wild-type neu expressing mice and identified the E3 ligase HACE1 as a HER2 cooperative tumor suppressor gene. Loss of HACE1 expression is commonly seen in clinical multiple cancer datasets including breast cancer. HACE1 down-regulation in normal human mammary epithelial cells (HMECs) results in the accumulation of the activated GTP-bound Rac1 partially transforming these cells. Overexpression of HER2 activates Rac1, which further accumulates upon HACE1 loss resulting in Rac1 hyperactivation. While the knockdown of HACE1 or overexpression of HER2 alone in HMECs is not sufficient for tumorigenesis, HER2 overexpression combined with HACE1 down-regulation fully transforms HMECs resulting in robust tumor formation. The pharmaceutical interference of Rac function
abrogates the effects of HACE1 loss both *in vitro* and *in vivo* resulting in dramatic reduction in tumor burden. Our work supports a critical role for HACE1 in breast cancer progression and identifies patients that may benefit from Rac targeted therapies.
Dedication

I would like to dedicate this dissertation to my wonderful parents, David and Jan Goka
Acknowledgements

There are so many people who have helped me throughout the course of this Ph.D. I would first like to thank my friends and family for their never-ending encouragement and support. I would also like that thank my mentor Marc for all of his support and guidance along the way. Thank you to my committee members for providing crucial feedback and suggestions along the way. Above all, I would like to thank my best friend Kristen Yee for giving me the courage to pursue my dreams and providing me the strength to carry on through the hard times.

Thank you all.
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<td>Ab</td>
<td>Antibody</td>
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<tr>
<td>ADH</td>
<td>Atypical ductal hyperplasia</td>
</tr>
<tr>
<td>AKT/PKB</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic acid</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>DCIS</td>
<td>Ductal carcinoma in situ</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>EGFR/HER1</td>
<td>Human Epidermal Growth Factor Receptor 1</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen Receptor</td>
</tr>
<tr>
<td>ERBB2/HER2</td>
<td>Human Epidermal Growth Factor Receptor 2</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular-signal regulated kinase</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine exchange factor</td>
</tr>
<tr>
<td>GEM</td>
<td>Genetically engineered mice</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-Protein Coupled Receptor</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine-5'-triphosphate</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
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<tr>
<td>HACE1</td>
<td>HECT domain and ankyrin repeat containing E3 ligase 1</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse Radish Peroxidase</td>
</tr>
<tr>
<td>IBC</td>
<td>Invasive breast cancer</td>
</tr>
<tr>
<td>IDC</td>
<td>Invasive ductal carcinoma</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IMEM</td>
<td>Improved modified essential media</td>
</tr>
<tr>
<td>LTR</td>
<td>Long terminal repeat</td>
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<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
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<tr>
<td>MAPKK</td>
<td>Mitogen Activated Protein Kinase Kinase</td>
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<tr>
<td>MAPKKKK</td>
<td>Mitogen Activated Protein Kinase Kinase Kinase</td>
</tr>
<tr>
<td>MMTV</td>
<td>Mouse murine tumor virus</td>
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<tr>
<td>NSC</td>
<td>Non Silencing Control</td>
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<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PH</td>
<td>Plekston homology</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinases</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKI</td>
<td>Protein Kinase Inhibitor</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
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<tr>
<td>RACE</td>
<td>Rapid amplification of cDNA ends</td>
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<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay buffer</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>Ribonucleic Acid interference</td>
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RT  Room Temperature
RT-PCR  Real time polymerase chain reaction
RTK  Receptor Tyrosine Kinase
SD  Standard Deviation
SE  Standard Error
shRNA  short hairpin ribonucleic acid
siRNA  small interfering ribonucleic acid
TAE  Tris-acetate buffer
TBS  Tris-Buffered Saline
TBST  Tris-buffered saline with Tween20
TDLU  Terminal duct lobular unit
TKI  Tyrosine Kinase Inhibitor
UCSC  University of California Santa Cruz
VBIM  Validation based insertional mutagenesis
CHAPTER 1: BACKGROUND

1.1 Breast cancer

1.1.1 Breast cancer incidence and mortality

Excluding skin cancer, breast cancer is the most common form of cancer among women in the United States with an estimated 232,340 new cases in 2013 [1]. Breast cancer incidence has been on the rise over the past few decades due to increased awareness combined with better screening techniques. Increased understanding of the biology of breast cancer has led to better therapies dramatically improving patient outcome.

However, breast cancer is still the second leading cause of death with an estimated 39,620 deaths in 2013 [1]. The likelihood of developing breast cancer increases with age and an estimated 1 in 8 women in the United States will develop breast cancer in their lifetime [2].

1.1.2 Breast cancer early detection and screening

Breast cancer is a progressive disease that arises from the normal breast epithelium. Smaller tumors are likely to be earlier stage disease and are less likely to have metastasized than larger (later stage) tumors [3]. In most cases, the earlier stage a breast cancer is detected, the better the survival outcome. The 5-year survival rate for patients diagnosed with DCIS or non-invasive breast cancer is greater than 98% but decrease to less 25% when the cancer has spread to distant organ sites [2]. Therefore, the detection of tumors in individuals with occult disease results in earlier treatment and better patient outcome.
Currently, the American Cancer Society recommends a clinical breast exam (CBE) every three years starting at age 20 for women at average risk of breast cancer and mammograms every year for women starting at 40 [4]. Mammography, the utilization of low dose X-rays to create an image of the breast, is the standard imaging method for breast cancer screening. Images can be detected using film or converted into a digital image that allows electronic manipulation of the image [5]. The use of additional imaging tests such as breast ultrasound that uses sound waves and magnetic resonance imaging (MRI) that uses magnetic fields instead of X-rays to create an image of the breast may be capable of producing a more detailed image of the breast and are used to screen women at higher risk of breast cancer [6]. While the sensitivity of these screening techniques is good [7], studies have shown the chances of having a false positive result after 10 yearly mammograms may be as high as 60% [8]. However, despite the false positives, the goal of screening for breast cancer is to identify as many occult cancers and possible while having an acceptable false positive rate.

1.1.3 Breast cancer progression and Ductal Carcinoma In Situ

The vast majority of human breast cancers are proposed to arise from the normal terminal duct lobular unit (TDLU) and culminate in invasive ductal breast carcinoma. Wellings and Jensen initially proposed the natural progression of breast cancer [9, 10] based on histological observations of a gradual continuity of disease progression (Figure 1-1). While breast cancer progression is a continual process, Wellings and Jensen identified key stages that could be differentiated at
Figure 1-1: Breast cancer progression model. Modified Wellings and Jensen model of breast cancer progression.
the pathologically equivalent levels that are generally referred to as atypical hyperplasias, in situ carcinomas, and invasive carcinomas. The progression from each stage to the next however is a non-obligate process.

Atypical ductal hyperplasia (ADH) is characterized by epithelial cells that are mildly atypical in appearance that pile up on within the ductal lumen. Once these cells fill the ductal lumen but remain within the confines of the basement membrane they are considered ductal carcinoma in situ (DCIS). DCIS is the immediate precursor to invasive ductal carcinoma (IDC) where the cells within the ductal lumen breach the basement membrane and invade into the surrounding stroma. The transition from DCIS to IDC has been implicated as the key transition in this progression model because it is the transition where precancerous cells that are confined within the ductal system invade into the surrounding breast tissues and have the potential to become disseminated disease.

Advanced screening techniques, better awareness, and an overall aging of the population has resulted in an increase in the incidence of DCIS which accounts for 20-25% of all newly diagnosed breast cancers [11, 12]. Rates of DCIS between 1975 and 2004 rose 560% while rates for IDC increased by only 18% over the same time period [13]. The optimum treatment regimen of DCIS is lumpectomy with the addition of radiation therapy and hormone therapies for estrogen receptor positive breast cancer [14].

Studies that have looked at histological differentiation, standard biomarkers, as well as gene expression analysis in DCIS have found them to be
nearly identical with that of IDC suggesting that the heterogeneity of breast cancer occurs at the DCIS level [15, 16]. The vast majority of molecular alterations found in IDC are also found in DCIS indicating that although DCIS and IDC have an enormous difference in clinical behavior there are only a handful of key alterations that separate the two. However, it is difficult to distinguish them from each other at the molecular level. The similarities between DCIS and IDC also make it difficult to predict which DCIS will progress to IDC and which DCIS lesions are truly benign making the management of DCIS a difficult task.

Insight on the rates of DCIS that will progress to IDC can be inferred from studies that follow patients with DCIS who have been untreated. Page et al. [17, 18] identified 28 patients whom at the time of biopsy were considered to be free of disease and thus were not provided with any treatment. However, upon subsequent review many years later these patients were changed to having DCIS. Of the 28 patients, 7 (25%) developed IDC within 10 years of their original biopsy and another 2 developed invasive disease 20-30 years after the initial biopsy. This makes the absolute 30-year risk of progressing from DCIS to IDC 32% for the Page et al. patients, suggesting that two-thirds of untreated DCIS may never progress to invasive disease. While this study was a retrospective analysis of patients that most likely represented lower grade DCIS, the study suggests that a significant number of newly diagnosed women who present with DCIS will be over-treated because their disease will not progress to IDC.
1.2 Erbb2 positive breast cancer

1.2.1 ErbB2 positive DCIS and invasive breast cancer

The receptor tyrosine kinase ErbB2, also known as HER2/neu, is a proto-oncogene located on chromosome 17q12 that is frequently amplified in breast cancer resulting in the overexpression of ErbB2 mRNA and ErbB2 protein [19]. Breast cancer patients whose invasive cancers overexpress HER2/neu have some of the poorest patient outcomes. Thus the overexpression of the HER2/neu proto-oncogene results in a different type of breast cancer. While all mammary epithelial cells express low basal levels of HER2/neu, HER2 positivity in the clinical setting is determined by the HerceptTest™ or by HER2 fluorescent in situ hybridization (FISH) which measures HER2 protein or DNA copy number, respectively. HerceptTest™ uses immunohistochemical (IHC) staining of HER2 and staining intensity is measured on a 0 to 3+ scale with cancers scoring 3+ being considered positive [20]. Alternatively, cancers can be considered HER2 positive by FISH if the results of the ratio of HER2 to Chromosome 17 Centrosome (CEP17) is greater than 2.2 since the vast majority of HER2 overexpression is due to gene amplification [21]. Moreover, breast cancers that are driven by HER2 signaling have a distinct molecular gene expression signature, that segregates them into a distinct molecular subtype of breast cancer ([22]). Concordant with the histological outcome, the breast cancers that belong to the ERBB2 molecular subtype have some of the worst patient outcomes [23].
Interest in HER2 arose with correlations of ErbB2 positivity and poor patient outcomes suggesting ErbB2 may be utilized as a prognostic marker in breast cancer in addition to the expression the estrogen receptor and progesterone receptor [19, 24, 25]. Using immunohistochemical techniques, ErbB2 has been documented to be overexpressed in up to 46% of all DCIS lesions [26, 27] and is associated with higher grade DCIS [28]. One study has suggested that HER2/neu positive DCIS is predictive of progression to invasive breast cancer [29]. However, HER2 overexpression accounts for only about 20% of all invasive breast cancer [1, 13, 26]. The high incidence of HER2 expression in DCIS suggests that HER2 expression may contribute to progression of pre-invasive disease; however, the reduction in HER2 positive invasive breast cancer suggests that HER2 expression is not sufficient for progression to invasive transformation and that additional events are required for the progression to invasive ductal carcinoma [28, 30]. Tsuda et al. found no difference in HER2 levels between the in situ and invasive components of breast cancers suggesting that HER2 plays a negligible role in the transformation to an invasive phenotype [31]. Moreover, transgenic mice that express wild-type neu under the control of the mammary specific mouse mammary tumor (MMTV) promoter form spontaneous focal mammary tumors but only after a prolonged latency period [32]. The prolonged latency period further suggest that additional alterations are required for malignant transformation to occur.

While HER2 alone is not sufficient to fully transform mammary epithelial cells into mammary carcinoma, HER2 positivity by immunohistochemistry has
been established as a poor prognostic marker for patients once they have
invasive breast cancer [25]. In the past decade, breast cancer has also been
characterized into at least 5 molecular subtypes based on distinct gene
expression signatures defined as Luminal A, Luminal B, HER2 enriched, basal,
and normal like [22]. HER2 overexpression is represented in the HER2 enriched
subtype and a portion of the Luminal B subtype subtypes whose gene expression
is driven by the ErbB2 amplicon. Concordant with previous immunohistochemical
findings, the HER2 enriched molecular subtype has one of the poorest patient
outcomes [33]. Taken together, it appears that the overexpression of HER2 may
play a significant role in developing in situ cancer and while HER2 alone may not
be sufficient for invasive transformation, if invasive ductal carcinoma does
develop it results in a more aggressive form of breast cancer resulting in poor
patient outcome.

1.2.2 ErbB2 mediated signaling pathways

HER2/neu is a receptor tyrosine kinase that is a member of the
HER/EGFR family of receptors. This family of receptors contains an extracellular
domain, a transmembrane domain, and an intracellular kinase domain. HER2 is
a unique member of this family because unlike the other HER family receptors
that bind ligands such as EGF, TNF-α, HRG, and NRG for activation, there are
no known ligands for HER2 [34] (Figure 1-2). HER2 contains a unique
extracellular domain that sits in an open configuration poised for dimerization
with other family members. Activation of HER2 occurs via formation of
heterodimers with other family members once they bind their respective ligands
resulting in a conformational change allowing dimerization to occur. Once
dimerization occurs, the intracellular kinase domains trans-phosphorylate each
other resulting in activation.

Figure 1-2: Members of the ERBB receptor family. EGFR, HER2, HER3, and
HER4 are members of the ERBB transmembrane receptor tyrosine kinase family. Adapted from Baselga and Swain. Nature reviews cancer 2009
HER2 mediated signaling depends on its dimerization partner. HER2/EGFR heterodimerization and subsequent trans-phosphorylation of tyrosine residues in the intracellular domain results in activation of the MAPK signaling pathway (Figure 1-3). Phosphorylated HER2 and EGFR results in the recruitment of cytosolic adaptor proteins such as SHC and GRB2 that contain SH2 and SH3 domains that recognize phosphorylated tyrosine and proline-rich regions, respectively. SHC and GRB2 recruit the Ras guanine exchange factor (GEF) SOS that activates Ras, a GTPase, by facilitating the exchange of GDP for GTP resulting in the activated GTP-bound Ras. Activated Ras then activates the serine/threonine kinase Raf (MAP3K). Raf kinase then activates MEK (MAP2K) which then activates ERK (MAPK). Activation of ERKs results in the activation of transcription factors such as AP1 and MYC that result in expression of genes involved in advancement through the cell cycle resulting in cellular proliferation.

The dimerization of HER2 with HER3 has different signaling effects than that of HER2/EGFR dimerization. The binding of ligands to HER3 such as HRG/NRG results in a conformational change into an open position allowing dimerization. HER3 however, is unique because it lacks a functional kinase domain. Once dimerization occurs with HER2, HER2 phosphorylates HER3 resulting in activation of the AKT pathway (Figure 1-4). Phosphorylation of HER3
results in the recruitment of the regulatory subunit of PI3K (p85) to bind the phosphorylated tyrosine residues of HER3 through its SH2 domain. The catalytic

![Diagram of ERBB2 mediated MAPK signaling in breast cancer.](image)

**Figure 1-3: Simplified representation of ERBB2 mediated MAPK signaling in breast cancer.** A schematic representation of Shc proteins activating the Ras and MAP kinase cascade. Upon stimulation by growth factors, RTK undergoes dimerization and tyrosine phosphorylation. Subsequently, Shc proteins are recruited and phosphorylated on tyrosine residue via forming a complex with RTK through the SH2 and/or PTB domain of Shc. The phosphorylated Shc proteins then associate with Grb2 adaptor protein through its tyrosine phosphorylation site to the SH2 domain of Grb2; the latter is constitutively complexed with SOS through its SH3 domains. These events result in the

Cell Proliferation  |  Cell Migration  |  Cell Differentiation  |  Cell Survival
translocation of SOS to the plasma membrane and subsequently activate membrane-bound Ras in the exchange of GDP for GTP and trigger the activation of MAP kinase cascade, resulting in cell proliferation, differentiation, migration, invasion, and survival. Adapted from Alam et al. Endocr Relat Cancer 2008

**Figure 1-4: Simplified representation of ERBB2 mediated AKT signaling in breast cancer.** A schematic representation of AKT signaling in breast cancer. Upon stimulation by growth factors, RTK undergoes dimerization and tyrosine phosphorylation. Subsequently, PI3K is recruited and activated. Subsequently, PI3K phosphorylates PIP2 generating PIP3. PIP3 recruits PDK1 which then activates AKT. Activation of AKT results in numerous downstream signaling effects such as enhancing metabolic reprograming, increasing angiogenesis, increasing protein synthesis as well as reducing programmed cell death. Adapted from Alam et al. Endocr Relat Cancer 2008
subunit of PI3K (p110) then phosphorylates the D3 hydroxyl group of PI(4,5)P2 (PIP2) generating PI(3,4,5)P3 (PIP3), a potent second messenger. Proteins that contain pleckstrin homology (PH) domains such as PDK1 and AKT bind PIP3. PDK1 phosphorylates AKT, resulting in activation of AKT. Activated AKT binds numerous downstream effectors resulting in a variety of cellular effects involved in protein synthesis, cell cycle progression and cell survival. Activation of mTOR by AKT results in activation of 4EBP and p70S6K, proteins involved in protein synthesis. 4EBP, binds and sequesters eIF4E, the translation initiation factor required for cap-dependent translation. Hyperphosphorylation of 4EBP by mTOR results in dissociation from eIF4E allowing cap-dependent translation. Additionally, mTOR activates p70S6K, a kinase that phosphorylates the 40S ribosomal subunit S6, thereby activating protein translation.

AKT allows for cell cycle progression by repressing GSK3 and the transcription factor FOXO. The inhibition of GSK3 results in stabilization of cyclin D1 and c-myc. Inhibitory phosphorylation of FOXO represses the transcription of the cyclin dependent kinase inhibitor p27 resulting attenuating the repression of cell cycle inhibition. FOXO repression also reduces the expression of FASL and BIM, cellular mediators of apoptosis. BAD is a pro-apoptotic protein that aids in cytochrome-c release from the mitochondria resulting in caspase-mediated apoptosis. Phosphorylation of BAD by AKT results in the suppression of BAD and thus the caspase mediated apoptotic machinery resulting in cell survival. Together, HER2 downstream signaling through EGFR and HER3 results in enhanced proliferation, protein translation, and cell survival.
1.2.3 Models of ERBB2 positive breast cancer

While there are a number of ways to study HER2+ breast cancer, by far the most widely established method is through the use of established breast cancer cell lines in vitro. The ability of breast cancer cells to be propagated under tissue culture conditions provides an unlimited self-replicating source of research material and can thereby grown to almost infinite quantities. However, subpopulations of cell lines that have been around for many years may arise that may have different genotypes and behave differently than other subpopulations of the same cell line [35] indicating that cell lines may change in culture over time [36].

Another criticism of established cell lines is that they do not contain the relevant components of the tumor microenvironment. To address this criticism, established HER2+ breast cancer cell lines are xenografted orthotopically into the mammary fat pads of immunocompromised mice. Xenograft models of breast cancer allow the tumor cells to grow in their tissue of origin and have a microenvironment that mimics that of their original host. The cancer cells must recruit blood vessels (neovascularization) in order to grow as well as establish the primary niche for the cancer to grow in by activating surrounding stromal components. Xenograft model systems have allowed helped bridge the gap of cancer therapeutics from cells in tissue culture to utilization in human. HER2+ breast cancer xenografts have been instrumental in developing HER2 targeted therapies such as Trastuzumab [37] and Lapatinib [38]. However, the HER2+ breast cancer cell lines lack the intratumoral heterogeneity that is present in
HER2+ breast cancer patients and do not fully represent the actual patient populations that eventually relapse on HER2 targeted therapies. Additionally, immunocompromised mice must be used which makes studying components of the host immune system difficult in this model system. Another pitfall of the established breast cancer cell lines is that they are often obtained from patients with progressive disease (pleural effusions) making it difficult to study the earlier stages of carcinogenesis.

The gold standard for studying cancer initiation and progression has been the utilization of genetically engineered mouse (GEM) models. Transgenic mice that overexpress wild-type neu, under the control of the mouse mammary tumor virus-long terminal repeat (MMTV-LTR) form focal mammary tumors after a prolonged latency period (Figure 1-5,[32]). Furthermore, the similarity of the knock-in mouse model to HER2-initiated human breast cancers is highlighted by the observation that the spontaneous tumors harbor several chromosomal alterations including deletions and amplifications that mimic primary human breast cancer [39]. Furthermore, early lesions in these mice closely resemble the solid and comedo forms of HER2+ ductal carcinoma in situ (DCIS), progress to large focal mammary tumors and in some cases have the ability to metastasize to distant organs completing the full spectrum of breast cancer progression [39].
Figure 1-5: Spontaneous mammary tumor formation in MMTV-*neu* mice. Transgenic mice expressing wild-type neu under the control of the mammary specific MMTV promoter generate spontaneous focal mammary tumors after a latency period of 205 days. Adapted from Guy et al. PNAS 1992
1.3 Forward screening approaches

1.3.1 cDNA screening approaches

The ability to grow cells using formulated growth media has provided cancer researchers a unique tool to investigate biology at a functional level [40]. Unlimited proliferative ability allows eternal propagation the same cell lines resulting in a model system with stable phenotypic characteristics over time. Most importantly, mammalian cell lines are asexual diploids whose daughter cells can faithfully recapitulate the genome of their parent. Therefore, somatic-cell genetic alterations that occur in a parental cell become ingrained in their progeny. This principle has been utilized in cancer research because cancer derived cell lines harbor the same genetic alterations as the cancer they came from. To investigate the functionality of the genetic makeup of a cancer cell, the genetic material from cancer cell lines have been isolated and cloned [41]. Upon generation of cDNA libraries that contain the genetic makeup of the parental cell, one can transfer the genetic material from one cell type to another using episomally replicating plasmids or DNA sequences that can be integrated into the host genome through the use of viruses that contain integrase.

Upon exchange of genetic material from one cell type to another, an appropriate functional assay must be utilized in order to successfully obtain a gene that contributes to your desired phenotype [42]. While the use of cDNA libraries has been used to identify a number of oncogenes or genes that drive a particular phenotype [43-46], the number of genes in your library is limited by the number
of available cell lines to harvest cDNA from as well as biased on the expression profile of the parental cell line.

1.3.2 RNAi screening approaches

Since its discovery in *C. elegans* [47], RNAi has been a common strategy to study gene functionality. RNAi permit modulation of endogenous mRNAs by reducing levels of endogenous mRNA transcripts resulting in the decrease of the protein product. Generating libraries of siRNAs or shRNAs allows forward genetic screens for genes whose disruption yields a particular phenotype. Large-scale RNAi screens have identified genes involved in numerous aspects of cancer progression as well as susceptibility genes that have the potential for therapeutic purposes [48-51]. The benefits of using an RNAi based screening approach are: first, sequences of all genes are immediately known upon identification of siRNA or shRNA sequence that yielded the desired phenotype and second, the lack of the need for viable cells to identify genes of interest. However, the use of RNAi also comes with problems. Many of the oligonucleotide sequences may yield variable or incomplete knock down of the target gene. Moreover, some sequences may target numerous genes enhancing ambiguity in gene identification due to off-target silencing. Lastly, the use of RNAi screens are limited to searching for genes whose loss-of-function provides a phenotype of interest.

1.3.3 Insertional mutagenesis screening approaches

Insertional mutagenesis occurs when an exogenous DNA sequence integrates into the host organisms genome resulting in alterations of an
endogenous gene. Slow growing retroviruses have utilized this system to enhance cellular proto-oncogenes such as Myc and Src [52]. The use of retroviral or lentiviral systems have been utilized to allow the approximate at random integration of exogenous elements into the genome. The insertion of an enhancer element typically results in enhanced gene expression which has led to the discovery of numerous oncogenes [53]. Depending on the location of the insertional element, integrating vectors can manipulate the host genes in a number of different ways. Integration can lead to enhanced transcription or translation of proto-oncogenes, generate chimeric or truncated transcripts by intragenic insertion, or inactivation/disruption of tumor suppressor gene expression [54]. Utilization of retroviruses such as the mouse mammary tumor virus (MMTV), a tissue specific virus, has led to the identification of numerous cancer related genes such as Wnt1 [55] and genes that belong to the Wnt and Fgf pathways [56] in a relatively unbiased manner. Some insertional mutagens may have preferential insertion sites described as insertional hotspots. Additionally, retroviruses require the target cell to be actively replicating in order to for integration into the host genome to occur. However, newer lentiviral-based systems seem to have alleviated a number of these concerns. Overall, insertional mutagenesis allows for unbiased forward genetics screening that have significantly improved our knowledge of the molecular pathogenesis of cancer.

1.3.4 Validation Based Insertional Mutagenesis (VBIM)

An ideal method in performing a forward genetics insertional mutagenesis screen require that mutations/integrations should: one, be able to affect any
gene; two, be able to identify both gains and losses of function; and three the target gene of interest should be easily identifiable [57]. Insertional mutagenesis is based off the principle insertion of a defined exogenous DNA fragment into the genome. If the inserted DNA fragment includes a strong promoter, integration could lead to the over-expression of an mRNA encoding a full-length or truncated protein, an anti-sense RNA, depending on the position and orientation, or disruption of endogenous gene translation/mRNA stability [58].

To achieve the given requirements, Lu et al. designed a reversible promoter insertional technique that was dubbed a lentiviral validation-based insertional mutagenesis (VBIM) system (Figure 1-6, [59]). The utilization of a lentiviral backbone enables the insertional element to integrate approximately at random into any cell type, including non-dividing cells. Moreover, the LoxP sites were built into the LTRs to allow removal of the insertional element through the use of Cre recombinase activity. The ability to remove the inserted promoter allows the ability to distinguish spontaneous mutants from those derived by the targeted mutation. A strong CMV promoter is used to drive the transcription on an internal GFP reporter gene as well as a N-terminus flag epitope. The use of an internal ribosome entry site (IRES) that lies downstream of the GFP reporter allows translation of the encoded protein downstream of the insertion site. A splice donor/splice acceptor site is also utilized to allow proper splicing to occur as well as maintenance of the conserved reading frame. Upon insertion and
Figure 1-6: Validation Based Insertional Mutagenesis (VBIM). (A) Functional components of the VBIM lentiviral backbone, (B) Integration into the host DNA generates the VBIM provirus, (C) The CMV promoter drives downstream transcription of chimeric VBIM and gene mRNA, (D) Translated protein products.
proper splicing, the promoter can potentially allow the synthesis of a GFP reporter and Flag-tagged fusion protein. Together, the VBIM technique fulfills the requirements for an effective forward genetic insertional mutagenesis screening system.

1.4 Hect domain and ankyrin repeat containing E3 ubiquitin ligase 1 (HACE1)

1.4.1 Identification and characterization of HACE1

The analysis of chromosomal arrangements has lead to the discovery of numerous oncogenes and tumor-suppressor genes (1). In Wilms’ tumor, a renal neoplasm that primarily affects children, a common rearrangement of the 6q21 region had been reported including t(5;6)(q21:21) and t(2;6)(q35;q21) translocations (2-5). While allelic loss of 6q21 was uncommon in Wilm’s tumor, deletions of 6q21 were well characterized in numerous human cancers including breast, ovarian, and prostate cancers (12-15) suggesting the presence of a possible tumor suppressor gene within that region.

To investigate the possibility of such a gene, Anglesio et al. mapped the 6q21 chromosomal to a non-coding region upstream of a novel open reading frame (ORF). Protein domain analysis predicted that the ORF encoded a 909 amino acid protein that possessed six N-terminal ankyrin repeat domains as well as a C-terminal HECT domain and was therefore designated Hect domain and ankyrin repeat containing E3 ubiquitin ligase 1 (HACE1).

1.4.2 Function of HACE1

The presence of a HECT domain suggested that HACE1 possessed ubiquitin ligase activity and was capable of degrading proteins through the
ubiquitin proteasome pathway (Figure 1-7). HECT domain containing E3 ligase differ from other types of E3 ligases such as the ring domain containing family of E3 ligases in that the activated ubiquitin is transferred onto a conserved cysteine residue within the HECT domain and then transferred onto the target protein whereas RING domain E3 ligases facilitate the transfer of ubiquitin directly onto the target protein [60]. The E2 carrier protein UbcH7 was shown to transfer ubiquitin onto cysteine C876 that was required for HACE1 ubiquitnation activity [61]. HACE1 was identified to be an E3 ubiquitin-ligase that polyubiquitinlyates the active GTP-bound form of Rac1 at lysine 147 resulting in degradation of Rac1 via the 26S proteasome [62]. In addition to targeting Rac1 for proteosomal degradation, HACE1 has also been reported to interact with Rab proteins controlling Golgi biogenesis during the cell cycle [63].

1.5 Rac1

1.5.1 Rac protein GTPases

Rac is a member of the Rho GTPase family of proteins that includes Rac, Rho and CDC42. There are four Rac isoforms, Rac1, Rac2, Rac3, and the Rac1 splice variant Rac1b. They contain ~90% conservation of sequence identity and possess structural characteristics common to all Rho GTPases (Figure 1-7, [64, 65]).

The tertiary structure of Rac consists of a G-domain fold consisting of a six-stranded β-sheet surrounded by α-helices. Rac contains a switch region that changes conformation as it cycles between GDP and GTP bound isoforms. Rac
Figure 1-7: Schematic overview of the ubiquitin proteasome pathway. Ubiquitin is activated by an E1 ligase and transferred to an E2 ligase, and then transferred onto a HECT containing E3 ligase. The E3 ligase then transferred ubiquitin onto a target protein which is eventually degraded by the 26S proteasome.
also contains a C-terminal CAAX motif that allows interaction with the membrane after isoprenylation [66].

As with all Rho family GTPases, the activation of Rac is achieved by a tightly regulated GDP/GTP bound cycle where (Figure 1-8). The GDP bound inactive form of Rac becomes activated when it loads GTP, a process that is facilitated by guanine nucleotide exchange factors (GEFs). GEF engagement of Rac destabilizes the binding of the GDP nucleotide and Mg\(^{2+}\) within Rac promoting a brief nucleotide-free intermediate. GTP is quickly loaded due to the considerably higher intracellular concentration gradient of GTP than GDP [67, 68].

Upon GTP loading, Rac undergoes a conformational change of the switch I and switch II regions in the GTPase, enabling Rac to bind downstream effector proteins. Rac recognizes multiple effector resulting in broad downstream signaling effects [69]. Rac remains in the activated GTP bound position until the bound GTP becomes hydrolyzed to GDP. While Rho family GTPases have intrinsic GTP hydrolysis ability, this activity is very slow. Instead, GTP hydrolysis is stimulated by GTPase-activating proteins (GAPs). GAP binding to Rac enhances the rate of GTP hydrolysis by a multiple of 10\(^5\) [70].

1.5.2 Rac1 mediated intracellular signaling

Small GTP-binding proteins such as Rac, lie downstream of diverse cell surface receptor signaling (Figure 1-9). Rac1 has been shown to be activated in response to EGFR family receptors, MET, PDGFR, IGFR, GPCRs, chemokine
receptors, integrins, as well as many others [71-73]. Activation between cell surface receptors and Rac1 is mediated through GEFs that catalyze GDP

Figure 1-8: GTP/GDP cycling of Rac1. Rac GEFs facilitate the exchange of GTP for GDP resulting in activated Rac1. GAPs facilitate the ability of Rac to hydrolyze GTP to GDP resulted in the inactive form of Rac.
Figure 1-9: Schematic representation of Rac signaling. Activation of multiple upstream factors result in the activation of Rac GEFs. Activation of Rac GEFs facilitates the exchange of GTP for GDP activating Rac1. GTP-bound Rac results in enhanced gene expression, actin cytoskeleton rearrangements, enhanced protein synthesis, and increased cell survival.
exchange for GTP, thereby activating Rac. Typical GEF recruitment and activation are mediated through SH2/SH3 domains that bind phosphorylated proteins and PH domains that facilitate recruitment to the plasma membrane.

Once Rac1 is activated, it exerts diverse phenotypic effects by activating a multitude of downstream effectors. Rac1 activation modulates the cytoskeleton resulting in lamellipodia formation resulting in cellular migration [74]. Through LIM kinases that regulate coflin mediated turnover of actin filaments [75], Rac1 activation enhances cellular motility [76]. In addition to LIM kinase activation, Rac1 also activates the Arp 2/3 complex via the WAVE/Scar family of proteins which also play a critical role in lamellipodia formation at the leading edge of the cell [77] (Figure 1-10). Taken together, Rac1 enhances cytoskeletal rearrangements that allow cells to migrate.

Through other downstream Rac1 effectors such as the p21-activated protein kinases (PAKs) [78] and components of the NADPH oxidase system [79], Rac1 activation results in reactive oxygen species production, proliferation and gene expression, [80, 81], and resistance to apoptosis [82]. Rac1 has been shown to promote cell growth and/or survival by activating numerous transcription factors such as c-Jun [81], cyclin D1 [83] E2F [84], and nuclear factor (NF)-κB [81]. Thus, Rac1 activation results in cellular proliferation, resistance to apoptosis, and enhanced motility, characteristics that contribute to a cancerous phenotype.
Figure 1-10: **Rac mediated cytoskeletal remodeling.** Activated Rac1 results in the activation of WASP. Together with the ARP 2/3 complex, activated WASP add actin monomers to actin chains resulting in cytoskeletal remodeling.
1.5.3 Rac targeted therapies

The modulation of Rac has been implicated as an anticancer target due to a significant number of human cancers exhibiting aberrant Rac signaling [85]. The use of small interfering RNA (siRNA) technology to knock down Rac1 in breast cancer cell lines has been shown to impair migration and invasion [86]. The use of dominant negative Rac1 variants have also shown to inhibit breast cancer tumor growth, migration and metastasis [87]. These studies show the importance of Rac signaling as a key mediator of breast cancer progression and metastasis.

The observation that bacterial derived toxins have the ability to specifically inhibit a subset of Rho GTPases has laid the groundwork for the development of inhibitors of Rac1 (Table 1-1, [88]. Rac1 contains a surface groove that is known to be critical for GEF specification and activation where activated GEFs bind and facilitate the exchange of GTP for GDP. Computational screens combined with synthetic chemistry have identified NSC23766, a small molecule that fits into a surface groove of Rac1 impairing GEF engagement of Rac1 [89]. NSC23766 has been shown to effectively inhibit GEFs TRIO and TIAM-1 from activating Rac1 in a concentration-dependent manner without targeting other Rho family GEFs RhoA and CDC42. Treatment of cancer cells with NSC23766 has been shown to effectively block migration and invasion in vitro as well as induce cell-cycle arrest.
without altering the growth of normal cells [90-92]. While initial studies of NSC23766 seemed promising, the IC$_{50}$ of NSC23766 studies in vivo suggest

<table>
<thead>
<tr>
<th>Drug</th>
<th>Compound Structure</th>
<th>Mechanism of action</th>
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<tbody>
<tr>
<td>NSC23766</td>
<td><img src="image1.png" alt="Image" /></td>
<td>Blocks Rac1-GEF (Trio and Tiam1) interaction</td>
</tr>
<tr>
<td>EHop-16</td>
<td><img src="image2.png" alt="Image" /></td>
<td>Blocks Rac1-GEF (Vav) interaction</td>
</tr>
<tr>
<td>EHT 1864</td>
<td><img src="image3.png" alt="Image" /></td>
<td>Blocks Rac-Rac effector (PAK1) interaction</td>
</tr>
<tr>
<td>IPA-3</td>
<td><img src="image4.png" alt="Image" /></td>
<td>PAK1 allosteric inhibitor</td>
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</tbody>
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**Table 1-1: List of Rac pathway inhibitors.** Name, structure, and mechanism of action of compounds that target the Rac pathway identified in the literature.
that it may not have sufficient efficacy to be used in human patients [89]. Using
the same targeting strategy to disrupt Rac-GEF interactions have yielded other
promising compounds with lower IC50s than NSC23766 such as EHOP-016 [93].
EHOP-016 was shown to specifically inhibit the interaction between Rac and the
Rac GEF Vav2 without affecting Rac-TIAM-1 interactions at micromolar
concentrations. While compounds that target specific Rac-GEF interactions
appear promising, they require the presence of the specific Rac GEFs that they
target to be present in the cancer. Moreover, if cells express numerous Rac
GEFs, the targeting of one may be overcome by Rac activation by another Rac
GEF.

EHT 1864 is a GEF independent inhibitor is the small molecule, which
inhibits Rac from binding its downstream effectors [94, 95]. Rac inhibition using
EHT 1864 has also shown inhibitory effects on migration and invasion [96]. Small
molecule inhibitors that target the p21-activated kinases (PAKs), which lie directly
downstream of Rac, have also been reported to be appealing targets in cancer
therapeutics [97]. While inhibitors that target the Rac pathway appear promising
as cancer therapy, the compounds as well as utilization may require further fine-
tuning in design [98].

Rac targeted therapeutics may also be advantageous as combinatorial
therapeutic approaches with other anticancer drugs [99]. Dual inhibition of both
the Rac and Ras-Raf pathways has been suggested to reverse the effects of
Ras-mediated transformation in a more efficacious manner as Rac and Ras drive
different aspects of tumorigenesis [89]. Activation of the Rac pathway has also emerged as a critical mediator of resistance to targeted cancer therapies. In HER2 positive breast cancers, Rac1 expression was increased with either inactivation of PTEN or insulin-like growth factor-1 receptor (IGF-1R) overexpression in response to HER2 targeted therapies conferring resistance (ref 55). Reducing Rac pathway activation using a Rac inhibitor (44) or siRNA knockdown of Rac1 (56) was capable of resensitizing HER2 positive breast cancer to HER2 targeted therapies after the emergence of acquired resistance. Based on these results, efforts are underway to explore the ability of Rac inhibition to cooperate with conventional therapies or resensitize other types of cancers to their respective treatments (57).

1.6 Summary

With the incidence of DCIS rates rising, the need to identify better prognostic markers of disease progression is needed. While the overexpression of the human growth factor receptor HER2 is frequently overexpressed at the DCIS stage, there is a significant decrease in HER2+ breast carcinoma. This suggests that HER2 may drive progression to the DCIS stage but additional alterations are required for the full malignant transformation.

Transgenic mice that overexpress wild-type neu under the control of the tissue specific MMTV promoter form focal mammary tumors but only after a prolonged latency period. This delay in tumor onset further supports the hypothesis that additional alterations are required to cooperate with neu to transform normal murine mammary epithelial cells. The MMTV-neu mouse fully
recapitulates the natural progression of HER2/neu driven breast cancers and provides a unique model to screen for HER2/neu cooperating genes.

While many forward based screening platforms are utilized, insertional mutagenesis allows one to indiscriminately identify both gain of function and loss of functions genes simultaneously. In this study, using younger MMTV-neu mice that have not had adequate time to acquire additional genomic alterations, we employed an insertional mutagenesis screen to identify genes capable of cooperating with HER2/neu to drive malignant transformation and identified a list of putative oncogenes/tumor suppressor genes.

Using a bioinformatics and literature based search, we chose to pursue HACE1, a HER2 cooperating tumor suppressor gene. Here, we will show that loss of HACE1 frequently occurs in human breast cancer patients. Additionally, we will show that HACE1 controls ubiquitin mediated degradation of the proto-oncogene Rac1 and that loss of HACE1 results in the accumulation of activated Rac1 driving numerous malignant phenotypes. We will also show that HER2 alone is capable of activating Rac1 and that the loss of HACE1 in HER2 overexpressing cells results in even greater levels of activated Rac1. We will provide new insights into what controls the progression of HER2+ DCIS and allows it to become invasive ductal carcinoma. Moreover, we will show that pharmaceutical intervention of Rac signaling can reverse the transformative effects of HACE1 loss suggesting a possible therapeutic strategy for Rac driven cancers.
CHAPTER 2: MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 General chemicals and solutions

All chemicals were purchased from Sigma unless otherwise indicated.

- 10X TG Buffer: Diluted to 1X with H20 and 20% Methanol for western blot transfer (Biorad)
- 10X TGS Buffer: Diluted to 1X with H20 for SDS-PAGE (Biorad)
- 50X TAE Buffer: Diluted to 1X with H20 for DNA gel electrophoresis (Biorad)
- Agarose: Diluted in 1X TAE Buffer to 1 – 2% for DNA gel electrophoresis (Invitrogen)
- Ampicillin: Bacterial antibiotic
- Beta Mercaptoethanol: Used for protein loading buffer (EMD/Millipore)
- Crystal Violet (1%): Used as a cellular stain (Aqua Solutions)
- DMSO: Used as a vehicle for drugs (Invitrogen)
- DNA smart ladder: Used for DNA gel electrophoresis (Invitrogen)
- PBS: Used to wash cells (Invitrogen)
- EHT1864: pan Rac inhibitor (Tocris)
- Epidermal Growth Factor (EGF): Used as cell culture supplement (Peprotech)
- Ethidium bromide: Used for DNA gel electrophoresis
- Fetal Bovine Serum (FBS): Used for cell culture (Hyclone)
- Heregulin (HRG): Used as cell culture supplement (R&D Systems)
- Isoflourane: Used for animal anesthesia (Butler Schein)
- Isopropanol: Used for molecular biology (VWR)
-L-Agar plates: Bactoagar was added to L-Broth to a final concentration of 1.5%.
-L-Broth: 10g bactotryptone, 5g bactoyeast, 5g NaCl, 50µl 10M NaOH made up to a final volume of 1L with H2O.
-Lipofectamine 2000: used for transfection (Invitrogen)
-Low melting point agarose: Used for DNA gel electrophoresis (Invitrogen)
-Matrigel: Used for tumor inoculation in mice (BD Biosciences)
-Neomycin/G418: Eukaryotic antibiotic (Gibco)
-Non-fat dried milk powder: Used for western blotting (Invitrogen)
-NuPage 4X Loading buffer: Used for western blotting sample preparation (Invitrogen)
-Phosphotase Inhibitor Cocktail: Used for western blot sample preparation (Thermo Scientific)
-Precision Plus Protein Standard: Used for western blotting (Biorad)
-Protease Inhibitor Cocktail: Used for western blot sample preparation (Thermo Scientific)
-Puromycin: Eukaryotic antibiotic (Gibco)
-Qiazol: Used for RNA extraction (Quiagen)
-RNAseA: Used for cDNA conversion (Invitrogen)
-Select Agar: Used for soft agar assay
-Super signal West Pico: Chemiluminescent reagents for western blotting (Thermo Scientific)
-Superscript III: Used for cDNA conversion (Invitrogen)
- Trypan Blue: Used for cell counting (Invitrogen)
- Tryple Express: Used for cell culture (Invitrogen)
- Trypsin 0.25%: Used for cell culture (Invitrogen)
- Tween 20%: Detergent used in wash buffer
- X-gal: Used for molecular biology

2.1.2. Cell lines

MCF-12A cells (ATCC) were grown in DMEM/F12 supplemented with 10mM HEPES, 10 µg/ml insulin, 20 ng/ml EGF, 20 ng/ml cholera toxin, 30, mM sodium bicarbonate, 0.5 µg/ml hydrocortisone, and 5% normal horse serum in a humidified atmosphere containing 5% CO$_2$ at 37°C. BT20, BT549, HCC1937, HCC38, MCF7, MDA-MB-231, MDA-MB-436, MDA-MB-468, and T47D cells (ATCC) were grown in IMEM (Invitrogen) with 10% FBS. BT474 and ZR-75-1 cells (ATCC) were grown in RPMI1640 (Invitrogen) with 10% FBS.

2.1.3. Plasmids/Constructs

Lentiviral shRNA vectors against HACE1 and Rac1 were purchased from open biosystems.

Lentiviral expression vectors Lv105-HACE1, Lv105-HACE1C876S, Lv105-GFP, Lv105-Neg, Lv105-HA-Rac1, Lv105-HA-Rac1K147R, Lv151-HER2 were purchased from Genecopoeia.

VBIM lentiviral expression vector, VBIM1, used for insertional mutagenesis screen was obtained from Dr. George Stark.

pMG2.G lentiviral packaging plasmid is used for packaging viral particles in 293T cells and encodes the VSV-G envelope protein was purchased from Addgene.
psPAX lentiviral packaging plasmid is used for packaging viral particles in 293T cells and encodes the viral GAG and POL proteins was purchased from Addgene.

Constructs were propagated in One-Shot DH5α chemically competent E. coli (Invitrogen) grown in LB broth containing 1mg/mL ampicillin (Sigma).

**2.1.4. Antibodies**

Westerns blots. Primary antibodies: HACE1 (Abcam), HER2 (Cell Signaling), Rac1 (Millipore), β-Actin (Abcam), Ubiquitin (Abcam), HA (Abcam). Secondary antibodies: anti-Rabbit HRP conjugated secondary (Biorad), anti-Mouse HRP conjugated secondary (Biorad).

**2.2. Methods**

**2.2.1 Tissue culture**

**2.2.1.1 Isolation of mammary epithelial cells**

Murine mammary epithelial cells were isolated from 7 week-old female MMTV-neu mice. Mice were euthanized using a lethal dose of isoflourane followed by cervical dislocation. Mammary fat pads 4 and 9 were isolated and pooled together and digested in a solution of collagenase in DMEM/F12 media rotating at 37°C for 1 hour. Digested cells were pelleted by centrifugation and plated into 6-well plates.

**2.2.1.2 Generation of stable cells with lentiviral transduction**

**2.2.1.2.1 Production of viral particles**

HEK293T Packaging cells were plated at approximately 60% confluence in DMEM media w/o antibiotics into a T75 flask. Once cell reached ~80%
confluence, cells were transfected using Lipofectamine 2000 with 10µg lentiviral expression plasmid (Lv-105, Lv-151, GIPZ, TRIPZ, VBIM1), 7µg pPAX and 3µg pMD2.G plasmid DNA in 300µl OptiMEM per T75 flask. Transfection complexes were incubated with cells for 6-8 hours and replaced with DMEM media w/o antibiotics. Viral containing supernatant was collected on days 2, 3 and 4 post transfection. Viral supernatant was filtered using a 0.45µm pore membranes to remove any cells and polybrene was added to a final concentration of 4µg/ml. Virus that was not used for immediate transduction was stored in aliquots at -80°C.

2.2.1.2.2 Viral Titer determination

2.0 x 10^5 NIH3T3 mouse fibroblasts were plated into each well of a 6-well plate. Cells were allowed to adhere here for 24 hours and media was changed to remove non-adherent cells. Lentivirus was then thawed at room temperature and 2 ml ten-fold serial dilutions over a range of 1 to 1 (10)^5 in 15 mL conical tubes were made. 1 ml of each serial dilution was added to one well of the 6-well plate. Viral transduction was carried out for 18-20 hours and virus was replaced with fresh media. 48 hours later, cells were inspected under a fluorescent microscope for GFP expression (due to viral integration). Cells in each of the 6-well plate were trypsinized and transferred to a 5 mL conical tube to be analyzed by flow cytometry. Serial viral dilution was used to determine the multiplicity of infection (MOI) per 1 ml of viral supernatant.
2.2.1.2.3 Lentiviral transduction

Mammalian cells to be transduced were plated at ~60% confluency and incubated overnight with approximately 5 – 6 mL of virus containing supernatant. The following day, viral supernatant was removed and replaced with media that cells were grown in. 72 hours post transduction, cells were selected using the appropriate mammalian drug selection. Overexpression of the transgene/knock-down was subsequently confirmed by immunoblot analysis.

2.2.1.3 Phenotypic assays

2.2.1.3.1 Cell proliferation assay

Mammalian cells were plated into 12-well tissue culture plates at a density of $2.5 \times 10^4$ cells/well. 24 hours after plating, cells were washed with PBS and counted using a hemocytometer and considered Day 0 counts. If drug treatment was to occur, drugs or vehicle controls were added. Media (with drug or control) was changed every 48 hours and cell growth was assessed at different time points as indicated. Growth assays were executed in triplicate and performed at least three times.

2.2.1.3.2 Soft agar assay

Bottom agar layer was made by mixing 1.6% granulated agar in water and 2X cell culture medium yielding a 0.8% agar/1X mixture of cell culture media. 1.5 mL of 0.8% agar/1X mixture of cell culture media was plated into each well of a 6-well plate. 0.4% agar top layer containing $5 \times 10^5$ cells was made by mixing 1.6% agar with 2X cell culture medium yielding a 0.8% agar/1X mixture of cell culture media. 1 $\times 10^5$ cells/ml were mixed with 0.8% agar/1X mixture of cell culture
media to yield a mixture of $5 \times 10^4$ cells/ml in 0.4% agar/1X media and 1 mL was plated on top of the bottom agar layer. Fresh medium was added every 3-4 days, and left to grow for one month. At the end of the assay, colonies were stained with Crystal Violet and counted. Soft agar assays were executed in at least triplicate and performed at least two times.

2.2.1.3.3 Boyden chamber migration assay

Cells were serum starved for 12 – 18 hours. Cells were then trypsinized and suspended in serum free media at a concentration of $1 \times 10^5$ cells/mL. 200 µl (2 x $10^4$ cells) were plated into the upper portion of a migration chamber in triplicate. 750 µl of full cell culture medium supplemented with HRG (10 ng/ml) was added to lower chambers as chemo attractant. Cells are placed at 37°C and cells in the top layer are allowed to migrate through the 8 µM pores. After 20 hours after plating, media and cells from the upper chambers were removed by aspiration and scrubbing of membranes with cotton swabs. Chambers were fixed in 4% PFA and stained with crystal violet. 5 images of the 3 filters were imaged using a light microscope, and cells were counted. Data was normalized to control and fold-migration was calculated. Data were plotted as a fold migration and Student’s t-tests were used to determine significance. Migration assays were performed at least two times.

2.2.1.3.4 Boyden Chamber invasion Assay

Cells were serum starved for 12 – 18 hours. Cells were then trypsinized and suspended in serum free media at a concentration of $1 \times 10^5$ cells/mL. 200 µl (2 x $10^4$ cells) were plated into the upper portion of a invasion chamber (migration
chamber coated with a basement membrane like substrate) in triplicate. 750 µl of full cell culture medium supplemented with HRG (10 ng/ml) was added to lower chambers as chemo attractant. Cells are placed at 37°C and cells in the top layer are allowed to migrate through the 8 µM pores. After 20 hours after plating, media and cells from the upper chambers were removed by aspiration and scrubbing of membranes with cotton swabs. Chambers were fixed in 4% PFA and stained with crystal violet. 5 images of the 3 filters were imaged using a light microscope, and cells were counted. Data was normalized to control and fold-migration was calculated. Data were plotted as a fold migration and Student’s t-tests were used to determine significance. Migration assays were performed at least two times.

2.2.2 DNA manipulation

2.2.2.1 Extraction of plasmid DNA

A single bacterial colony grown on LB agar plates was picked and inoculated in 5ml of LB broth supplemented with the appropriate antibiotic and grown overnight shaking in a 37°C incubator. For small-scale plasmid extraction, the culture was grown overnight and plasmid DNA extracted using the Qiaprep Spin Miniprep Kit (Qiagen) according to the manufacturer’s instructions. For large-scale plasmid extraction, 200 mL of LB broth containing the appropriate antibiotic was grown in overnight culture and extracted using the Maxiprep Plasmid kit (Sigma) according to the manufacturers instructions.
2.2.2.2 Polymerase chain reaction (PCR)

PCR reactions contained 10 – 100 ng of plasmid or genomic DNA, 20 µl of 2X PCR supermix (includes Taq DNA Polymerase), 1µl of each, forward and reverse primer (200 nM) and water in a total volume of 40 µl. PCR protocols were generally as follows, the samples were initially denatured at 94°C for 2 minutes and then underwent 35 cycles of a denaturation step at 94°C for 30 seconds, annealing step at a temperature of 55 to 60°C for 30 seconds, an extension step at 72°C for 1 minute per kb of PCR product and then a final extension period of 5 minutes at 72°C. Following the 5 minute extension period the samples were held at 4°C.

2.2.2.3 3’ Rapid Amplification of cDNA Ends (RACE)

Polyadenylated cDNA was used to perform sequential PCR reactions containing 100 ng cDNA. Fusion, a high fidelity DNA polymerase, was used to amplify 3’ cDNA ends. Fusion mastermix contained 20 µl of 2X mastermix, 1µl of VBIM forward primer 1 (200 nM), 3 µl of oligodT20 reverse primer and water to a total volume of 40 µl. The PCR protocol used for amplification at each step was as follows, the samples were initially denatured at 94°C for 3 minutes and then underwent 35 cycles of a denaturation step at 94°C for 30 seconds, annealing step at a temperature of 55°C for 30 seconds, an extension step at 72°C for 5 minutes and then a final extension period of 5 minutes at 72°C. Following the 5 minute extension period the samples were held at 4°C. After the first PCR amplification step, 5 µl was diluted 1:10 in DNAse free water. Diluted DNA from the first PCR reaction was amplified in the second PCR reaction. 2 µl of the first
PCR reaction was added to 20 µl 2X PCR mastermix, 1 µl of VBIM forward primer 2 (200 nM), 3 µl of oligodT20 reverse primer and water to a total volume of 40 µl. PCR protocol was conducted as described above. A third PCR amplification step was performed exactly as in the second PCR amplification step with the exception that VBIM forward primer 3 was used instead of VBIM forward primer 2.

2.2.2.4 Digestion of DNA with restriction enzymes

1 µg of DNA was incubated with 5 units of the appropriate restriction endonuclease(s) and the respective buffer (1x) in a total volume of 50 µl for 2 hours to over night at 37°C.

2.2.2.5 Agarose gel electrophoresis

0.7 - 2% agarose gels were made up in 1x TAE buffer and 0.1 µg/ml ethidium bromide. Agarose in 1X TAE placed in an Erlenmeyer flask and was heated in a microwave until boiling. Flask was removed from the microwave and then ethidium bromide was added and then poured and allowed to set. DNA samples were mixed with loading buffer (1X), and then loaded in wells of the agarose gel and DNA gel electrophoresis was carried out in 1x TAE buffer at 80 - 120V. For size determination, a 1kb DNA Smart ladder was loaded alongside the samples on the agarose gels. Following electrophoresis, the DNA was visualized on a UV transilluminator (Biorad).
2.2.2.6 Purification of DNA fragments from agarose gel

Following PCR amplification or restriction digestion of DNA, DNA fragments were purified using the QIAquick Gel Extraction Kit according to the manufacturer’s instructions.

2.2.2.7 DNA sequencing

For sequencing, DNA samples (100µg plasmid DNA or 10-100ng PCR product) were sent to the University of Miami DNA sequencing core. There, the DNA was sequenced by capillary sequencing on an Applied Biosystems Prism 3130XL DNA Analyzer. The sequencing data was analyzed using SerialCloner2.1 and 4Peaks software.

2.2.2.8 DNA transformation

One Shot® DH5alpha E. coli cells were thawed on ice. For each transformation, to which 0.2 µg of plasmid DNA was added and mixed by flicking the tube. After 20 minutes incubation on ice, cells were heat shocked at 42°C for 30 seconds and returned to ice for 2 minutes. 500 µl of SOC medium was added, and the cells incubated in a 37°C shaking incubator for 1 hour. The transformation mixture was plated on LB agar plates containing the necessary antibiotic and incubated overnight at 37°C.

2.2.3 RNA manipulation

2.2.3.1 RNA extraction from cells

Cells were grown in a monolayer in tissue culture plates, washed with PBS and RNA was extracted using Qiazol reagent and set at room temperature for 5 minutes. Chloroform was added to the samples followed by isopropanol
precipitation according to the manufacturer’s instructions (Invitrogen). Phase Lock Gel Heavy eppendorf tubes (Eppendorf) were used to facilitate the separation of aqueous and organic phases by centrifugation according to the manufacturer’s instructions. DNaseI was added to eliminate any residual DNA that may have been isolated during the process. RNA concentrations and quality were determined by measuring the UV absorbance at 260nm and 280nm on a nanodrop (Thermo Scientific) spectrophotometer.

2.2.3.2 cDNA synthesis

For cDNA conversion from RNA, 0.1 – 1 µg RNA was used for cDNA synthesis using the Superscript III cDNA synthesis kit (Invitrogen) according to the manufacturer’s instructions. For 3’ RACE (see above), oligodT20 was used as the primer for RT reaction.

2.2.3.3 Quantitative real time PCR (qPCR)

Real-Time qPCR was performed using SYBR Green I Master mix (BioRad) and gene specific primers (see materials) on the LightCycler® 480 (Roche Diagnostics) according to the manufacturer’s instructions. Data was analyzed with the LightCycler® 480 Software (module: absolute quantification) and standard curves were calculated for all reactions with serial dilutions of pooled cDNA from breast cancer cell lines to calculate the reaction efficiency. The expression of genes is shown relative to expression of that particular gene after adjusting for the expression of GAPDH as an endogenous housekeeping control.
2.2.4 Protein analysis and manipulation

2.2.4.1 CK8 staining and flow cytometry

Isolated mammary epithelial cells were washed with PBS and trypsinized. Cells were fixed in 1.5% paraformaldehyde for 10 minutes and then pelleted by centrifugation. Cells were then permeabilized in ice-cold methanol at 4°C for 10 minutes. After permeabilization, cells were washed twice with PBS and then blocked in 1% BSA in PBS for 30 minutes. Cells were stained using a 1:20 dilution of anti-cytokeratin 8 rabbit antibody and incubated for 30 minutes at room temperature. After the 30 minute incubation in primary antibody, cells were washed three times by centrifugation and resuspension in ice-cold PBS. Cells were then incubated in alexaflour 488-conjugated anti rabbit secondary antibody in 5% BSA in PBS for 30 minutes. After secondary antibody incubation, cells were washed three times as described above and analyzed for Cytokeratin 8 positivity using flow cytometry. Cells were compared to secondary only control cells.

2.2.4.2 Western blot analysis

Cells were washed with ice cold PBS and lysates were collected with RIPA lysis buffer, supplemented with phosphatase and protease inhibitors. Lysates were vortexed, ran through a 25 gauge syringe, and then cleared by centrifugation. Protein concentrations were measured using a BCA assay (Thermo scientific) according to manufacturer’s specifications. Protein was mixed with NuPage 4x sample buffer containing 10% beta mercaptoethanol and denatured by boiling samples for 5 minutes at 95°C. Samples were loaded into a
Tris-glycine gel (BioRad) and protein was separated by SDS-gel electrophoresis. Protein gels were transferred to an immune-blots PVDF membrane, and blocked in TBST containing 5% milk powder for 1 hour. Membranes were probed with specific primary antibodies according to the manufacturer’s specifications shaking at 4°C overnight. Membranes were washed three times for 5 minutes in TBST. Membranes were then incubated with the appropriate HRP-conjugated secondary antibody at a dilution of 1:10,000 in TBST with 5% milk rocking at room temperature for 1 hour. Membranes were again washed three times for 5 minutes in TBST and then developed using the Supersignal West Pico chemiluminescent reagents according to the manufacturer’s instructions.

2.2.4.3 Rac activity assays

Rac1 pull-down assays were performed on MCF12A and MCF7 cells were grown in a 145-mm dish and serum starved for 12 – 18 hours. Rac1 activation was stimulated with 100 ng/ml EGF and 10 ng/ml HRG for 30 minutes. After 30 minutes cells were lysed to obtain the soluble extract. GTP-Rac1 was pulled down using PAK binding domain (PBD) beads. Beads and lysate were incubated at 4°C for 1 hour rocking and then beads were washed. Active and total Rac1 were identified by western blotting with specific antibodies. Activation of Rac1 GTPase was also determined by G-Lisa Rac1 colorimetric-based kit. Cells were grown and treated as described above. After cells were lysed and solube extract obtained. After correction of total protein concentration according to the manufacturer's instructions, half of the extract volume (40 µl) was mixed with the provided binding buffer, then added to the ELISA plates and incubated on ice for
30 min. Wells were washed and active GTPase detected by specific antibodies according to the manufacturer's instructions in a microplate spectrophotometer. An aliquot was also analyzed by western blot analysis to confirm shRNA-mediated HACE1 depletion. In our hands, the GTPase activation measured in the G-Lisa assays was comparable to that obtained in classical Rac1-PBD pull down assay but with higher sensitivity and thus requiring considerably less cell lysate. All Rac activation assays were repeated at least twice.

**2.2.4 Ubiquitylation assays**

The ability of HACE1 to polyubiquitylate Rac1 was achieved using two different methods. MCF7 cells expressing low levels of endogenous HACE1 were made to overexpress GFP, HACE1, or a ligase dead HACE1 C876S mutant. Cells were serum starved for 16 hours and then stimulated with EGF and HRG while in the presence of the proteasome inhibitor MG132 for 2 hours. Cells were harvested in lysis buffer and polyubiquityted proteins were enriched by ubiquitin pull-down. Cell lysate and anti-ubiquitin agarose were incubated overnight at 4°C. Agarose was washed in TBST and proteins were separated by SDS-PAGE on a 12% polyacrylamide gel. Polyubiquitylated Rac1 was probed by western blot. Additionally, cell expressing GFP or HACE1 were made to also express exogenous HA-tagged WT Rac1 or HA-Rac1 K147R mutant. Cells were serum starved for 16 hours and then stimulated in the presence of MG132 as described above. Protein was harvested in lysis buffer and exogenous HA-Rac1 was enriched using anti-HA beads. Lysate and beads were incubated overnight at 4°C. Beads were then washed in TBST and proteins were separated by SDS-
PAGE on a 12% polyacrylamide gel. Polyubiquitylated Rac1 was detected by western blot analysis using an anti-ubiquitin antibody.

2.2.5 Animal studies

Animal work was done with approval by the Institutional Animal Care and Use Committee (IACUC) and in compliance with protocol #12-199. For orthotopic tumor xenograft assays, 2 x 10^6 cells were resuspended in matrigel (BD Biosciences) then innoculated into mammary fat pad 4 and/or 9 of 24 weeks old female NOD/SCID mice (Jackson Laboratory). Prior to injection, mice were shaved and then anesthetized using an isoflurane vaporizer that mixes isoflurane and oxygen. Tumor growth was measured 3 – 5 times a week by digital caliper and tumor size was calculated by the following formula: (length x width^2)/2.

2.2.6 Bioinformatic and statistical analysis

2.2.6.1 General statistical analysis

Statistical analysis was performed using using either Microsoft Excel or GraphPad Prism. Experimental mean ± SE of at least three triplicate samples were reported. All p-values were calculated using 2-sided Student t-test unless stated otherwise. The cut-off for statistical significance was set at p < 0.05 unless stated otherwise.
CHAPTER 3: INSERTIONAL MUTAGENESIS SCREEN FOR HER2 COOPERATING GENES

Studies have suggested that ER negative breast cancers arise from ER positive cells that have ceased to express the estrogen receptor. ER is highly expressed in all atypical ductal hyperplasias, benign breast lesions with significant premalignant potential [100]. ER expression decreases as atypical ductal hyperplasia progresses to ductal carcinoma in-situ and further to invasive breast cancer, with 75% and 60-70% of these lesions expressing ER, respectively. By contrast, because some ER negative cells exist in all pre-malignant lesions, it is possible that these cells are the precursors of ER negative breast cancers. If these cells were to become independent of the estrogen-induced paracrine support from their ER positive brethren and achieve a growth advantage, they could presumably progress to ER negative breast cancer [101].

Breast cancer develops through a multi-step process driven by genetic and epigenetic changes that gradually transform normal breast epithelium into pre-invasive (pre-cancerous) lesions such as ductal carcinoma in situ (DCIS) and finally culminate in invasive breast cancer. While the transition from DCIS to invasive disease has been implicated as the key transition in breast cancer progression, it is a non-obligate step. [102, 103]. Therefore, the elucidation of alterations that regulate cellular transformation are required to further understand disease progression.

Previous clinical studies identified HER2 (also known as neu) overexpression in DCIS lesions as a predictive indicator for the transition of in situ to invasive breast cancer [29]. However, while HER2 overexpression is seen in up to 50% of
all DCIS lesions less than 25% of invasive breast carcinomas overexpress HER2 [28] suggesting that HER2 alone is not sufficient for full malignant transformation.

Genetically engineered mice (GEM) that express the wild-type neu proto-oncogene under the control of the mouse mammary tumor virus (MMTV) promoter spontaneously develop focal mammary tumors after a prolonged latency period suggesting additional alterations are required for tumorigenesis [32]. Interbreeding of MMTV-neu mice with other GEM models has identified numerous neu cooperating genes [104]. This process is both costly and time consuming.

Utilization of unbiased forward genetics screening approaches has identified novel cancer associated genes as well as validating known cancer genes. One selection system: validation based insertional mutagenesis (VBIM), employs a lentiviral based random insertion of a strong promoter into the genome [59]. This study aimed to perform a VBIM screen on partially transformed murine mammary epithelial cells obtained from MMTV-neu mice.

3.1 Generating VBIM library from isolated mammary epithelial cells

MMTV-neu mice express wild-type neu, the rat homologue of human ErbB2/HER2, under the control of the tissue specific MMTV promoter. These mice form spontaneous focal mammary tumors after a tumor latency period (t50) of 205 days, with the earliest onset of tumors occurring around day 150 [32]. Ductal hyperproliferation in MMTV-neu mice has been observed at 7 weeks of age suggesting expression and activation of the neu proto-oncogene (Figure 3-1A,[105]). At this time point, MMTV-neu mice were euthanized and mammary fat
Figure 3-1: Characterization of MMTV-*neu* mammary epithelial cells. (A) Brightfield image of isolated primary isolated MMTV-*neu* mammary epithelial cells, (B) Flow cytometry analysis of unstained MMTV-*neu* mammary epithelial cells, (C) CK8 staining of MMTV-*neu* mammary epithelial cells.
pads #4 and #9 were isolated and digested to obtain MMTV-\textit{neu} mammary epithelial cells (Figure 3-1B). Primary mammary epithelial cells were verified by CK8 positivity, a marker of epithelial cells (Figure 3-1C).

Upon verification of MMTV-\textit{neu} mammary epithelial cells, cells were transduced with the VBIM lentivirus to generate a library of random mutants. Cells were transduced at a multiplicity of infection rate of 0.3 (33\%) to reduce the risk of multiple viral integrations in the same cell.

### 3.2 Insertional mutagenesis screen for acquisition of clonogenicity in soft agar

The ability of cancer cells to grow in an agar medium under anchorage-independent conditions has been a model of transformation for many years [106]. While untransformed cells undergo anoikis, a form of programmed cell death, transformed cell are capable of surviving and proliferating while in suspension [107]. To screen for HER2 cooperating genes, we screened a library of VBIM transformed MMTV-\textit{neu} mouse mammary epithelial cells for clones that have gained the ability to grow colonies in soft agar.

1,000 cells were suspended in soft agar in each well of a 96-well plate and allowed to grow (Figure 3-2A). On day 27, any colonies that formed were checked for the expression of GFP (Figure 3-2B) to confirm VBIM integration and extracted from the soft agar. Total RNA was extracted from isolated colonies and reverse transcriptase and an OligodT20 was used to convert polyadenylated mRNA into cDNA (Figure 3-3B). Insertional loci are identified by performing 3' rapid amplification of cDNA ends (RACE), a series of sequential PCR
Figure 3-2: VBIM soft agar assay design. (A) Library of random VBIM transductants are plated into soft agar and allowed to grow for 31 days, (B) Isolated colonies expressed GFP indicating viral integration.
Figure 3-3: Sequencing of MMTV-neu VBIM clones. (A) Schematic of VBIM provirus inserted into cellular DNA, (B) Total RNA is converted into cDNA using oligodT20 reverse primer, (C) BLAST search of PCR amplification sequence downstream of viral insertion site yields putative genes.
amplifications that use a forward primer against the proviral backbone and a non-specific primer against the poly(A)-tail (Figure 3-3C, [108]). PCR amplicons are gel extracted and TA cloned [109] into a shuttle plasmid and transformed into bacteria for rapid amplification of the plasmid. Plasmid DNA is sequenced upon extraction and aligned against the murine genome using BLAT alignment tool [110] yielding putative oncogenes and/or tumor suppressor genes that are capable of cooperating with HER2/neu. Sequencing results revealed a candidate list of putative oncogenes and tumor suppressor genes as well as canonical tumor suppressor genes such as Caspase-9 [111] as well as genes shown to be associated with HER2+ breast cancer such as the mitochondrial transporter protein SLC25A43 [112] (Table 3-1).
<table>
<thead>
<tr>
<th>Putative Oncogene/Tumor Suppressor Gene List</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase 9</td>
</tr>
<tr>
<td>ASS1</td>
</tr>
<tr>
<td>Slc25a43</td>
</tr>
<tr>
<td>HACE1</td>
</tr>
<tr>
<td>Rragc</td>
</tr>
</tbody>
</table>

Table 3-1: List of identified putative HER2 cooperating oncogenes/tumor suppressor genes. Putative HER2 cooperating genes identified by BLAST search against the mouse genome.
CHAPTER 4: STRATIFICATION OF IDENTIFIED GENES BASED ON PUTATIVE CLINICAL CORRELATIONS OF HER2 COOPERATIVITY

Breast cancer results from the accumulation of genetic and epigenetic changes that provide ductal epithelial cells to proliferate and avoid cell death. Recent data from whole-exome sequencing of breast tumors have identified a mean somatic mutation frequency of 1.2 per Mb of DNA [113]. However, not all of these mutations are relevant to the phenotype of the tumor. Only a small subset of these mutations, known as driver mutations, actually confers a selective advantage for the tumor whereas the remainders are called passenger mutations [114]. While the accumulation of genomic data from large scale studies may be difficult to attribute causal genetic links due to vast (intra)tumoral heterogeneity, these efforts allow researchers to draw significant correlations about breast cancer as a whole.

4.1 Bioinformatic stratification of putative HER2 cooperating genes

Recently, large-scale projects aimed at discovering and cataloging genetic, transcriptomic, and proteomic alterations in cancer such as The Cancer Genome Atlas (TCGA) project [115, 116]. Oncomine, a cancer-profiling database, contains such datasets and allows simple annotated analysis in one standard program [117]. Experimental data are normalized and analyzed using standard protocols, and presented to the end-user of Oncomine through a web-based interface. Oncomine contains large datasets including the TCGA breast dataset that allows the comparison of normal breast and invasive ductal breast carcinoma, as well as receptor subtypes (ER, PR, and HER2) of invasive ductal
breast carcinoma. Using the comparison between normal breast and HER2+ invasive ductal breast carcinoma, our initial gene list was narrowed down to genes that had a statistically significant correlation between progression from normal breast to HER2+ invasive ductal breast carcinoma (Figure 4-1). Stratification using Oncomine resulted in a list of clinically actionable genes.

4.2 Literature based investigation of putative HER2 Cooperating Genes

A number of genes have been shown to be capable of cooperating with HER2 to enhance spontaneous tumor formation in mice [39]. Angiogenesis, cell cycle progression, and cell survival mechanisms have all been shown to cooperate with HER2/neu [118-121] signaling. Given the broad nature of the types of genes that were capable of cooperating with HER2 in the literature, not only did we look for genes that had a direct publication history in HER2+ breast cancer but also genes with an association with breast cancer or cancer of other disease sites. Putative HER2 cooperating genes were searched using PubMed and Google Scholar and quarried for both number of scientific publications and the content of the publications as it pertains to (breast) cancer (Table 3-2). Genes that have a clear connection to cancer progression were excluded from consideration. Genes that had few publications were also excluded due to a lack of a mechanistic foundation and/or limited availability of reagents (antibodies, etc.).
Figure 4-1: TCGA analysis of putative cancer associated genes. TCGA gene expression analysis of putative HER2 cooperating genes in normal breast and Invasive Ductal Breast Carcinoma.
<table>
<thead>
<tr>
<th>Gene</th>
<th># of published articles</th>
<th>Articles related to cancer?</th>
<th>Articles related to breast cancer?</th>
<th>Articles related to HER2+ breast cancer</th>
<th>(putative) function</th>
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<tbody>
<tr>
<td>Caspase-9</td>
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<td>Yes</td>
<td>Yes</td>
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<td>No</td>
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<td>No</td>
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</tr>
</tbody>
</table>

Table 4-1: Literature based investigation of putative HER2 cooperating genes (as of March 2012).
CHAPTER 5: RATIONALE FOR CHOOSING HACE1 AS INITIAL GENE OF INTEREST

5.1 Literature based search for previous HACE1 publications

A literature search of previous studies showed that HACE1 was lost in Wilm’s tumors as well as multiple other cancer types due to allelic loss or promoter methylation [122-125]. Moreover, HACE1 knockout mice have been shown to develop spontaneous tumors at multiple locations, including the breast, after a prolonged latency period suggesting the role of HACE1 as a putative breast cancer tumor suppressor gene [125].

5.2 Bioinformatic analysis of HACE1 in the patient sample database ONCOMINE

While looking at ONCOMINE to determine if HACE1 loss has a clinical correlation with breast cancer disease progression, HACE1 was found to be highly significantly under-expressed in HER2+ invasive ductal breast carcinoma compared to normal breast epithelium in multiple clinical patient datasets including The Cancer Genome Atlas (TCGA) breast dataset (Figure 5-1). HACE1 was also found to be significantly under-expressed when looking at all invasive ductal breast carcinoma compared to normal breast in the TCGA dataset suggesting that HACE1 loss is capable of cooperating with other drivers of breast cancer progression (Figure 5-2). Furthermore, allelic loss of HACE1 in invasive ductal breast carcinoma was also observed in the TCGA breast dataset (Figure 5-3). Thus, the under-expression of HACE1 in breast cancer can be attributed in part to allelic loss of the HACE1 locus.
Figure 5-1: HACE1 expression analysis in The Cancer Genome Atlas (TCGA) breast dataset. HACE1 mRNA expression analysis in HER2+ invasive ductal breast carcinoma and total invasive ductal breast carcinoma.
Figure 5-2: HACE1 DNA copy number analysis in The Cancer Genome Atlas (TCGA) breast dataset.
To determine if HACE1 loss also occurs in other cancer types, we queried HACE1 expression and/or copy number in multiple clinical datasets. Notably, HACE1 was underexpressed or underwent allelic loss in cancer compared to respective normal tissues in glioblastoma, melanoma, lymphoma, lung, and pancreatic cancers (Figure 5-3). Taken together, these data show that HACE1 mRNA expression is significantly decreased during the transformation from the normal to malignant state in breast cancer as well as other many other types of cancer.
Figure 5-3: Pan cancer analysis of HACE1 in Oncomine. HACE1 mRNA expression in normal breast and breast carcinoma in (a) Gluck Breast, (b) Curtis Breast, (c) Sorlie Breast, and (d) Radvanyi Breast clinical datasets. HACE1 mRNA expression in (E) normal brain and glioblastoma, (F) normal lung and lung adenocarcinoma. HACE1 DNA copy number in (G) blood, normal pancreas, and pancreatic ductal adenocarcinoma, (H) blood and cutaneous melanoma, and (I) blood and diffuse large B-Cell lymphoma. All figures are modified from Oncomine™ (Compendia Bioscience, Ann Arbor, MI) was used for analysis and visualization. P-value determined by Student’s t-test.
CHAPTER 6: HACE1 LOSS RESULTS IN THE ACCUMULATION OF RAC1 TRANSFORMING MAMMARY EPITHELIAL CELLS

6.1 Validation that HACE1 knock-down transforms MMTV-neu mammary epithelial cells

To identify genes that are capable of cooperating with HER2/neu to complete the process of malignant transformation of mammary epithelial cells, we isolated partially transformed murine mammary epithelial cells from MMTV-neu mice and performed an insertional mutagenesis screen to look for mutants that gain the ability to clone in soft agar. In doing so, we identified HACE1 as a putative tumor suppressor gene capable of cooperating with HER2/neu. To ensure that the anchorage independent growth observed in our screen was due to the out of frame insertion into the murine HACE1 gene, HACE1 was stably knocked down in isolated MMTV-neu murine mammary epithelial cells (Figure 6-1A). HACE1 knock down in the MMTV-neu MMECs allowed anchorage independent growth whereas control cells were not able to form colonies, thus confirming the results of the initial screen (Figure 6-1B). These data support the notion of HACE1 as a putative breast cancer tumor suppressor gene that is capable of cooperating with HER2/neu.

6.2 HACE1 knockdown in human mammary epithelial cells leads to transformation

To further validate HACE1 as a potential human tumor suppressor gene, we determined if HACE1 ablation in the normal human mammary epithelial cell line MCF12A increased its malignant potential. Two independent shRNAs targeting
Figure 6-1: HACE1 knockdown in MMTV-neu MMECs enhances anchorage independent growth. (A) BLAST results maps VBIM insertion into murine HACE1 exon 22, (B) RT-PCR analysis of murine HACE1 in parental MMTV-neu epithelial cells and clone SD1.10, (C) RT-PCR analysis of murine HACE1 in control MMTV-neu cells transduced with control shRNA and and HACE1 shRNA, (D) Soft agar assay of parental MMTV-neu mammary epithelial cells, control shRNA, and shHACE1 cells.
HACE1 (shHACE1 1 & 2) as well as a non-silencing control (shNSC) were introduced into the MCF12A cells by lentiviral infection resulting in HACE1 knockdown (Figure 6-2A). Cells expressing the shRNAs were plated in soft agar, allowed to grow for 28 days, and colony numbers were determined. While the control MCF12A cells were unable to form colonies in soft agar, cells expressing both shRNAs against HACE1 were capable of forming robust colonies (Figure 6-2B). To confirm that these results were not cell line specific, we knocked down HACE1 in another normal mammary epithelial cell line, HME3, which are normal mammary epithelial cells that have been immortalized using hTERT (Figure 6-2C). Again, the knockdown of HACE1 using two independent shRNAs resulted in increased anchorage independent growth compared to the shNSC control cells (Figure 6-2D). Together, these data show that HACE1 knockdown in normal untransformed mammary epithelial cells is sufficient to give them the ability to grow under anchorage-independent conditions.

### 6.3 HACE1 attenuates anchorage independent growth of human breast cancer cells by reducing levels of activated Rac1

The Rho GTPase Rac1 plays a critical role in cancer progression and has been known to be polyubiquitylated and degraded via the proteasome [126, 127]. Recent studies aimed at elucidating the E3 ubiquitin-ligase responsible for ubiquitylating active Rac1 identified HACE1 as the E3 ubiquitin-ligase that binds preferentially to GTP-bound Rac1 and catalyzes its polyubiquitylation and subsequent degradation via the proteasome [62, 128]. Consequently, the loss of HACE1 resulted in the accumulation of GTP-bound Rac1 resulting in hyperactive Rac signaling. In breast cancer, the Rho family GTPase Rac1 has been reported
Figure 6-2: HACE1 knockdown in normal human mammary epithelial cells enhances anchorage independent growth. (A) HACE1 knockdown in MCF12A mammary epithelial cells, (B) Soft agar assay of MCF12A shNSC and shHACE1 cells, (C) HACE1 knockdown in HME3 mammary epithelial cells, (C) Soft agar assay of HME3 shNSC and shHACE1 cells.
to be overexpressed and hyperactive [129]. Upon activation by guanine nucleotide exchange factors (GEFs), active (GTP-bound) Rac1 plays a significant role in disease progression by increasing cancer cell migration, proliferation, transcription and survival by activating a multitude of downstream effectors [73]. To verify that HACE1 is capable of controlling Rac1 protein turnover via the proteosome in human breast cancer, HACE1 was overexpressed in MCF7 breast cancer cell line (Figure 6-3A). Rac activation was achieved using EGF and HRG, ligands known to potently activate Rac1 in breast cancer cell lines. Upon ligand driven activation of Rac1, control MCF7 cells showed potent activation of Rac1 as determined by Rac1-GTP pull-down. However, the overexpression of HACE1 in MCF7 cells resulted in a dramatic decrease in levels of GTP-bound Rac1 (Figure 6-3A). The decrease in Rac activation in the HACE1 overexpressing cells indicates that HACE1 controls Rac1 turnover in breast cancer cells and attenuates the levels of GTP-bound Rac1 upon activation. To verify that the reduction of GTP-bound Rac1 was due to polyubiquitylation by HACE1 and subsequent proteosomal degradation, we performed ubiquitin assays on control MCF7 cells, HACE1 overexpressing cells, and cells that overexpress the ubiquitin dead HACE1 C876S mutation. Concordant with what has been previously reported, the overexpression of HACE1 resulted in enhanced Rac1 polyubiquitylation compared to control cells while the expression of the HACE1 C876S mutant had no effect on Rac1 polyubiquitylation (Figure 6-3C), [61]). These data confirm that the E3 ligase HACE1 controls levels of activated Rac1 by targeting it for proteasomal degradation.
Figure 6-3: HACE1 expression attenuates breast cancer clonogenicity by attenuating levels of Rac1 by proteosomal degradation. (A) Rac1-GTP Pull-down in control MCF7 and HACE1 overexpressing MCF7 cells, (B) Control and HACE1 overexpression soft agar assay, (C) Ubiquitin pull-down in control MCF7 cells, HACE1 overexpressing, and HACE1C876S overexpressing cells, (D) HA-Rac1 pull-down in control MCF7 and HACE1 overexpressing cells that also express ectopic HA-Rac1 or HA-Rac1K147R.
Previous studies have indicated that Rac1 polyubiquitylation occurs on lysine 147 upon activation and is subsequently degraded via the 26S proteasome (53). To confirm that HACE1 ubiquitylates Rac1 at lysine 147, ectopic HA-tagged WT Rac1 and HA-tagged Rac1K147R were introduced into control MCF7 cells or MCF7 cells that overexpress HACE1. Upon stimulation activation with EGF and HRG, MCF7 control cells that express low levels of HACE1 showed low levels of HA-Rac1 polyubiquitylation while the HA-Rac1K147R mutant was not ubiquitylated. As expected, the overexpression of HACE1 enhanced wildtype HA-Rac1 polyubiquitylation but had no effect on the HA-Rac1K147R mutant (Figure 6-3D). Taken together, these data confirm that HACE1 polyubiquitylates activated Rac1 at lysine 147 and facilitates its turnover via the 26S proteasome.

While breast cancer cell lines have reduced levels of HACE1 compared to their normal counterparts, we were interested to see whether the reduction of HACE1 may have contributed to the their initial transformation. To assess if HACE1 can mediate anchorage independent growth in breast cancer cell lines, HACE1 was expressed in breast cancer cell lines that are capable of growing in soft agar and express low levels of HACE1. Over-expression of HACE1 in MCF7 breast cancer cells reduced colony formation in soft agar compared to control cells (Figure 6-3B). Together, these data indicate that breast cancer cells lose HACE1 during their transformative process allowing cells to accumulate activated Rac1. Additionally, HACE1 re-expression in breast cancer cells can attenuate the levels of activated Rac1 resulting in diminishing their clonogenic potential.
6.4 Loss of HACE1 leads to accumulation of active Rac signaling in normal mammary epithelial cells

The overexpression of HACE1 in breast cancer cell lines resulted in decreased activated Rac1. To determine if HACE1 loss leads to enhanced Rac1 activity in mammary epithelial cells, MCF12A cells that have HACE1 knocked down were tested for Rac activation by Rac1-GTP pull-down assays. Control and shHACE1 knockdown MCF12A cells were serum starved and stimulated with EGF and HRG to induce Rac1 activation. EGF and HRG stimulation resulted in activation of Rac1 in the control MCF12A cells (Figure 6-4A). Moreover, the knockdown of HACE1 in the MCF12A cells resulted in enhanced Rac1 activation levels as determined by Rac1-GTP pull-down. Levels of activated Rac1 were also measured by a Rac1-GTP ELISA. Again, corroborating the results of the Rac1-GTP pull-down assay, the knock-down of HACE1 enhanced Rac1-GTP levels over that of the control cell (Figure 6-4B). Together, these results show that HACE1 loss in normal mammary epithelial cells results in enhanced levels of Rac1 signaling.

Growth factor induced Rac activation has long been associated with actin-cytoskeletal rearrangements resulting in enhanced cell motility [130]. We reasoned that the accumulation of active Rac1 due to HACE1 loss would result in an increased migratory ability of normal mammary epithelial cells. To investigate if HACE1 loss can enhance the migratory ability of normal breast epithelial cells, HACE1 depleted MCF12A cells were placed in the upper portion of a Boyden chamber and allowed to migrate through the semi-permeable membrane towards a chemoattractant. While the control cells showed only a modest ability to
Figure 6-4: HACE1 loss in normal mammary epithelial cells results in enhanced migration and invasion due to accumulation of active Rac1. (A) Rac-GTP Pull-down in serum starved MCF12A control and HACE1 knockdown cells and cells that are stimulated with HRG and EGF, (B) Rac1 GTP ELISA on serum starved MCF12A control and HACE1 knockdown cells and cells that are stimulated with HRG and EGF, (C) Boyden chamber migration assay of control MCF12A cells and HACE1 knockdown cells, (D) Boyden chamber invasion assay of control MCF12A cells and HACE1 knockdown cells.
migrate, the HACE1 knockdown cells showed significant increase in migratory ability (>3-fold) (Figure 6-4C). Although migratory ability is an important trait of cancer cells, the key step in the initiation of breast cancer is the transition from DCIS to invasive disease, where the confined precancerous cells gain the ability to breach the ductal membrane and invade the surrounding tissue. Furthermore, once these invasive cells have overcome the basement membrane barrier, invasive cancers may enter the vasculature and metastasize to distant sites. To determine if HACE1 loss increases invasiveness, HACE1 depleted MCF12A cells were plated in the upper portion of a modified Boyden chamber coated with matrigel. While the control MCF12A cells were non-invasive, loss of HACE1 gave the non-invasive MCF12A cells the ability to invade through matrigel (Figure 6-4D). Therefore, HACE1 loss resulted in enhanced Rac1 activation in normal mammary epithelial cells enhancing both migratory and invasive potentials.

6.5 Rac1 is required for transformation in HACE1 depleted cells

Due to the exquisite spatial, temporal and substrate specificity requirements for E3 ligases to properly ubiquitylate their target substrates, the number of target substrates is limited. However, E3 ubiquitin ligases have been shown to have multiple target substrates. While HACE1 has been shown to be capable of ubiquitylating activated Rac1 for proteasomal degradation, HACE1 has also been reported to be involved in ubiquitylating a Rab GTPase [63]. To confirm that accumulation of activated Rac1 is the major driver of HACE1 loss in transforming mammary epithelial cells, MCF12A cell lines that have HACE1 knocked down as well as their shNSC counterparts were additionally engineered
to have a Tet-On-inducible shRac1 system. Upon doxycycline induction of RAC1 shRNA expression, endogenous RAC1 levels were significantly down-regulated (Figure 6-5A). To determine if Rac1 knockdown could reverse the effects on anchorage-independent growth of the MCF12A cells that have HACE1 knocked down, cells were plated into soft agar with [+DOX] and without [-DOX] the presence of doxycycline. Without doxycycline induction of the Rac1 shRNA, MCF12A HACE1 knock-down cells continued to form robust colonies in soft agar Conversely, doxycycline induction of Rac1 shRNA diminished the HACE1 knockdown cells’ ability to grow colonies (Figure 6-5B).

To determine if Rac1 was the main mediator of HACE1 loss induced cellular migration and invasion, Rac1 knocked down was induced in the MCF12A cell lines and subjected to in vitro migration and invasion assays. Rac1 depletion in the MCF12A cells that have HACE1 knocked down reduced their ability to migrate and invade (Figure 6-5C&D). The ability of Rac1 depletion to reduce HACE1 loss induced motility and migration indicates that the enhanced potential is Rac1 mediated. Together, these results support the role of Rac1 as a key mediator in HACE1 loss mediated transformation.

6.6 Inhibition of Rac1 reverses HACE1 loss induced transformation

The molecular knockdown of Rac1 using an inducible shRNA resulted in the rescue of HACE1 loss mediated transformation proving Rac1 to be a viable therapeutic target. However, the notion that Rac1 can act as a scaffolding partner with numerous protein complexes [131] suggests that small molecule
Figure 6-5: Rac1 is required for HACE1 loss mediated transformation. (A) Doxycycline induction of Rac1 shRNA in control MCF12A and HACE1 knockdown cells, (B) Soft agar assay of control MCF12A and HACE1 knockdown cells with and without doxycycline induction of Rac1 shRNA, (C) Migration assay of control MCF12A and HACE1 knockdown cells with and without doxycycline induction of Rac1 shRNA.
intervention of the activated from of Rac1 may be less toxic than genetic ablation \[132\]. Because of this, we employed a small molecule inhibitor EHT1864 that inhibits Rac1 by promoting the loss of the nucleotide bound Rac1 in an inert and inactive state thus, inhibiting downstream signal transduction \[94\].

MCF12A cells, cells were treated with EHT1864 and then stimulated with EGF and HRG to induce RAC1 activation. Pretreatment with the Rac inhibitor EHT 1864 at a concentration of 25 µM inhibited Rac signaling in the control MCF12A cells as well as MCF12A cells with HACE1 knocked down while the vehicle control had no suppression of RAC1 activation (Figure 6-6A). These results support previous studies that EHT 1864 is a potent Rac inhibitor and indicates that EHT1864 can be used to inhibit Rac1 signaling of human mammary epithelial cells. To rule out non-specific toxicity we performed a proliferation and cell viability assay on MCF12A cells treated with the Rac inhibitor. While EHT 1864 caused a marked decrease in cellular proliferation (Figure 6-6B), cell viability was unaffected to (Figure 6-6C).

Just as the doxycycline induced knockdown of Rac1 halted anchorage-independent growth, the inhibition of Rac by EHT 1864 eliminated the ability of the normal MCF12A cells with HACE1 knocked down to grow colonies in agar (Figure 6-7A). These results indicate that EHT 1864 blockade of Rac1 signaling is as efficacious as Rac1 ablation by RNAi in inhibiting the growth hyperactive Rac1 driven cells.
Figure 6-6: EHT 1864 inhibits Rac1 downstream signaling without effecting cellular viability. (A) Rac1-GTP pulldown in control MCF12A and HACE1 knockdown cells treated with vehicle control or EHT 1864, (B) Proliferation assay of MCF12A shNSC and shHACE1 cells treated with vehicle control or EHT 1864, EHT was removed on day 4 from treated cells, (C) Cell viability as of MCF12A shNSC and shHACE1 cells treated with vehicle control and EHT1864.
Figure 6-7: EHT1864 reverses the effects of HACE1 loss in MCF12A cells. (A) Soft agar assay of MCF12A shNSC and shHACE1 cells treated with vehicle control or EHT 1864, (B) Boyden chamber migration assay of MCF12A shNSC and shHACE1 cells treated with vehicle control or EHT 1864.
In order to determine the efficacy of EHT 1864 on migration, cells were treated with the Rac inhibitor or vehicle control and plated in a Boyden chamber. Treatment of cells with the Rac inhibitor recapitulated the results of Rac1 knockdown resulting in a 2-fold reduction in migratory ability (Figure 6-7B). These results suggest that the inhibition of Rac signaling has the ability to reduce migratory gains caused by HACE1 loss similar to that of the molecular knockdown of Rac1. These results emphasize the notion that EHT 1864 is a potent Rac inhibitor and reinforce the concept that Rac activation is a key component in cellular motility.
CHAPTER 7: HER2 OVEREXPRESSION COOPERATES WITH HACE1 LOSS RESULTING IN HIGHER LEVELS OF ACTIVATED RAC1

We identified HACE1 as a candidate tumor suppressor gene that is capable of cooperating with HER2 by screening for genes that enhanced the clonogenicity of HER2 overexpressing murine mammary epithelial cells. Rac1 activation has been reported to play a key role in the progression of breast cancer progression. The overexpression of HER2 in mammary epithelial cells has been previously reported to activate Rac1 by activating RAC1 GEFs such as DOCK1, PREX1, and VAV2 [133-136], which in turn activate Rac1, by facilitating the exchange of GTP for GDP.

7.1 HER2 overexpression results in increased activated Rac1 enhancing cell motility

To verify that HER2 overexpression increases Rac1 activation in our normal mammary epithelial cells, MCF12A cells were constructed to stably overexpress HER2 (MCF12A-HER2) (Figure 7-1A). HER2 overexpressing cells and control cells were serum starved for 18 hours and stimulated using HRG and EGF. Activated Rac1 levels were determined by Rac1-GTP ELISA. Confirming what has been previously reported in the literature, HER2 overexpression resulted in enhanced GTP-Rac1 (Figure 7-1B). Because Rac activity results in enhanced cellular motility, HER2 overexpressing and control cells were tested for migratory ability using a Boyden chamber. HER2 has been previously been shown to enhance migratory and invasive ability of mammary cells by activating Rac1 [137]. Concordant with previous literature, HER2 overexpression in MCF12A cells resulted in enhanced migratory ability (Figure 7-1C).
Figure 7-1: HER2 overexpression cooperates with HACE1 loss to further transform mammary epithelial cells. (A) HACE1 expression in MCF12A cells and MCF12A-HER2 cells after treatment with two independent HACE1-specific shRNAs (shHACE1 (1) and shHACE1 (2)) as determined by western blot analysis. Non-silencing control (NSC) shRNA is shown as a control. (B) Rac1 fold activation of MCF12A shHACE1 (1 & 2), shNSC, HER2 shHACE1 (1 & 2) and HER2 shNSC cells as determined by Rac1 G-LISA. Cells were stimulated for 30 minutes with 100 ng/ml EGF and 10 ng/ml HRG after overnight starvation. Data from triplicates (fold increase relative to NSC in the absence of stimuli) are presented as mean ± SEM of three independent experiments. (C) Migration (20 h) of MCF12A shHACE1 (1 & 2), shNSC, HER2 shHACE1 (1 & 2) and HER2 shNSC cells. 100 ng/ml EGF and 10 ng/ml HRG was used chemoattractant. Data are expressed as mean ± SEM of three separate experiments. (D) Soft agar colony formation of MCF12A shHACE1 (1 & 2), shNSC, HER2 shHACE1 (1 & 2) and HER2 shNSC cells. Data are expressed as mean ± SEM of three separate experiments.
7.2 HACE1 knockdown in HER2 overexpressing cells further enhances Rac1 activation and migratory ability

To verify if HER2 was capable of cooperating with HACE1 loss, two independent HACE1 shRNAs were used stably knocked down HACE1 as described above. Activated Rac1 levels were determined using a Rac1-GTP ELISA. Control MCF12A-HER2 overexpressing cells as well as shHACE1 MCF12A-HER2 cells were serum starved for 18 hours and stimulated with HRG and EGF. Just as in MCF12A cells that do not overexpress HER2, the knockdown of HACE1 results in higher levels of activated Rac1 compared to the control cells (Figure 7-1B). The combination of HER2 overexpression resulted in higher levels of GTP bound Rac1 than either the HACE1 knockdown or HER2 overexpressing cells alone supporting the hypothesis that HER2 overexpression combined with HACE1 loss has cooperative effects to further enhance Rac1 downstream signaling.

To determine if the increase in Rac1 activation in the MCF12A-HER2 shHACE1 cells correlated with an enhanced cellular phenotype, migration assays were performed using a Boyden chamber. While the HER2 overexpressing MCF12A control cells were capable of migrating through the Boyden chamber, the knockdown of HACE1 resulted in a 3-fold increase in migration suggesting synergistic effects of HER2 overexpression and HACE1 loss in terms of migratory potential (Figure 7-1C). To determine if HACE1 loss could also cooperate with HER2 to enhance clonogenicity, MCF12A derivatives were plated in soft agar. While the overexpression of HER2 enhanced the number of colonies formed in soft agar over the control MCF12A cell, the combination of HER2
overexpression and HACE1 loss resulted in even greater numbers of colonies (Figure 7-1D). These data support the role of HACE1 loss having cooperative effects towards the transformation process of normal mammary epithelial cells.

7.3 Rac1 is necessary for HER2 mediated enhancement in cell migration and clonogenicity

Knockdown of Rac1 using a Rac1 shRNA or inhibition of Rac1 using a pharmaceutical inhibitory was shown to attenuate the effects of HACE1 loss in MCF12A cells. To determine if Rac1 was required for the increase in cell motility and anchorage-independent growth in MCF12A-HER2 shHACE1 cells, Rac1 was knocked down using a doxycycline inducible shRNA as described above.

MCF12A-HER2 shHACE1 and control cells with and without doxycycline were plated in soft agar to assess their ability to grow under anchorage independent conditions. Without doxycycline induction of the Rac1 shRNA, MCF12A-HER2 shNSC and shHACE1 cells behaved as previously described where the knockdown of HACE1 enhanced colony formation (Figure 7-2a). Doxycycline induction of Rac1 diminished the ability of HER2 overexpressing control cells as well as the HACE1 knockdown cells suggesting that Rac1 is required not only in cells that have both HER2 overexpression and HACE1 knocked down but also the control HER2 overexpressing control cells (Figure 7-2A). The effects of Rac1 knockdown on cellular migration was also tested on these cell lines. Without doxycycline induction of the Rac1 shRNA, MCF12A-HER control cells showed enhanced migration over control MCF12A cells as determined by migration through the boyden chamber as previously seen. The knockdown of HACE1 enhanced migration in the HER2 overexpressing cells
Figure 7-2: Rac1 is required for HACE1 loss mediated transformation in HER2 overexpressing cells. (A) Soft agar assay of MCF12A derivatives that express a doxycycline inducible Rac1 shRNA with and without doxycycline, (B) Migration assay of MCF12A derivatives that express a doxycycline inducible Rac1 shRNA with and without doxycycline.
compared to the control cells (Figure 7-2B). When the Rac1 shRNA was induced with doxycycline, Rac1 knockdown reduced the migratory ability of the MCF12-HER2 control cells down to levels of the control MCF12A cells. Additionally, the knockdown of Rac1 in the MCF12A-HER2 shHACE1 cells also reduced migratory rates to that of the MCF12A control cells (Figure 7-2B). Taken together, these experiments indicate that Rac1 is a crucial component in the ability of HER2 as well as the combination of HER2 and HACE1 loss to transform mammary epithelial cells.

The pharmacologic inhibition of Rac1 using the Rac inhibitor EHT1864 also attenuated the cooperativity of HER2 overexpression and HACE1 loss in terms of clonogenicity as well as migratory ability (Figure 7-3A &B) further supporting the role of Rac1 as a key signaling component of HER2 overexpression and HACE1 loss.

### 7.4 HER2 cooperates with HACE1 loss to allow tumor formation in mice

To see if the in vitro tumor formation assay translates to tumor formation in vivo, MCF12A and MCF12A-HER2 cell line that have HACE1 knocked down in them were implanted orthotopically into the mammary fat pads of NOD-SCID mice. Concordant with our initial hypothesis, the overexpression of HER2 in the MCF12A cells was not enough to allow full malignant transformation (Figure 7-4A). The MCF12A cells that had enhanced Rac1 activation due to HACE1 knockdown were also unable to form tumors. Interestingly, only the combination of HER2 and HACE1 loss were able to generate tumors in (6 of 12) NOD-SCID
Figure 7-3: Rac1 inhibition reverses the effects of HACE1 loss mediated transformation in HER2 overexpressing cells. (A) Soft agar assay of MCF12A derivatives in the presence of vehicle or EHT1864, (B) Migration assay of MCF12A derivatives in the presence of vehicle or EHT1864.
mice (Figure 7-4A & B). These results establish HACE1 as a tumor suppressor gene that cooperates with HER2 overexpression. HER2 overexpression in mammary epithelial cells results in activation of Rac1 signaling, signaling that is then prolonged when HACE1 is absent. This hyperactivation of Rac1 signaling results in enhanced anchorage-independent growth, migration, and tumorigenicity in NOD/SCID mice.

Given that we have generated a model of Rac1 hyperactivation due to HER2 overexpression and HACE1 loss, we wanted to evaluate the antitumor activity of EHT 1864 in orthotopic-implanted MCF12A-HER2 shHACE1 cells. Mice were pre-randomized to either drug or vehicle control group. Once tumors reached 100 mm$^3$ in volume, a bi-weekly treatment regimen was begun. As shown in figure 7-5A., bi-weekly dosing for 21 days of the MCF12A-HER2 shHACE1 xenograft mice resulted in significant reduction of tumor size and tumor weight (Figure 7-5B) relative to vehicle-treated controls while having no obvious toxicity to the mice. These results support the role of Rac in mammary epithelial cell transformation and demonstrate the in vivo utilization of the Rac inhibitor EHT 1864 to suppress tumor growth.
Figure 7-4: HER2 overexpression cooperates with HACE1 loss fully transforming normal epithelial cells to form tumors in mice. (A) In vivo tumor formation of MCF12A-shHACE1 (1 & 2), MCF12A-shNSC, MCF12A-HER2 shHACE1 (1 & 2) and MCF12A-HER2 shNSC cells. Cells were injected into NOD/SCID mice and allowed to grow for 30 weeks. n = 6 per group (B) time course of in vivo tumor formation of MCF12A-HER2 shHACE1 (1 & 2) and MCF12A-HER2 shNSC cells. Cells were injected into NOD/SCID mice and allowed to grow for 20 weeks. n = 10 per group.
Figure 7-5: EHT 1864 reduces tumor growth of Rac driven tumors in vivo. (A) Schematic overview of in vivo EHT 1864 dosing schema, (B) MCF12A-HER2 shHACE1 tumor volumes from mice treated with i.p. injections of 30 mg/kg EHT 1864 or vehicle. Graphs show mean ± SEM of tumor volume at indicated days. n = 4 or 5 mice per group. (C) Graph shows tumor weight after 21 days of vehicle or EHT 1864 treatment of MCF12A-HER2 shHACE1 tumors (**P < 0.00001 between groups, Student’s t-test). Graphs show mean ± SEM of tumor weight. n = 4 or 5 mice per group.
CHAPTER 8: DISCUSSION AND FUTURE DIRECTIONS

Over the course of the last few decades the incidence of pre-cancerous DCIS has increased in part due to improved imaging techniques [11]. However, the increased incidence rate has plausibly led to over-treatment of patients whose DCIS will not progress to IDC. [138]. There is a clear need for predictive markers of disease progression [139, 140].

While the observation that HER2/neu occurs in up to 50% of in situ and only 25% of invasive ductal carcinomas [30] may seem a bit paradoxical given the poor prognostic outlook of HER2+ breast cancers [25], it suggests that additional alterations are required to transform DCIS into invasive disease.

Using an insertional mutagenesis screen on primary mammary epithelial cells that express wild-type neu, we identified and established HACE1 as a breast cancer tumor suppressor gene that attenuates levels of activated Rac1 by its ability to target Rac1 for ubiquitin mediated proteasomal degradation. We have shown that that HACE1 deficiency results in the accumulation of activated Rac1 resulting in enhanced downstream signal transduction resulting in enhanced clonogenicty, migration/invasion, and tumorigenesis.

Expression of the 6q21 HACE1 gene is downregulated in human breast cancer compared to normal breast epithelium. Using multiple bioinformatics tools to analyze multiple cancer datasets, HACE1 was found to undergo allelic loss in breast cancer as well as many other cancers such as glioblastoma, lung, renal, ovarian, and prostate cancers. Hypermethylation of the HACE1 promoter has also been noted in Wilms' tumor, hepatocellular, gastric, and colorectal
carcinomas resulting in loss of gene expression [125, 141-143]. The gene inactivation of Hace1 in mice results in spontaneous cancers after long latency periods that develop tumors in similar sites, including the breast further supporting the notion of HACE1 being a breast cancer tumor suppressor gene [125].

The tightly regulated balance between active GTP bound and inactive GDP bound Rac1 is governed by GEFs and GAPs, respectively. Over expression of numerous Rac1 GEFs in breast cancer have been shown to shift the equilibrium towards active GTP-bound Rac1 [73]. Growth factor activation of ErbB family receptor members has been shown to activate Rac1 and its subsequent downstream effectors leading to enhanced mitogenic and motility signaling effects through the activation of Rac exchange factors. HER2, in particular, has been shown to activate numerous Rac exchange factors including P-Rex1, VAV2, and DOCK1 resulting in Rac activation in breast cancer [134-136]. Constitutively active Rac1 variants have also been identified in breast cancers such as the splice variant Rac1b [129, 144] and activating mutational variants [145] suggesting that Rac1 signaling is a major component in breast cancer.

In this study, we found that sustained knockdown of HACE1 in normal breast epithelial cells resulted in enhanced Rac1 activation causing enhanced migratory and invasive ability as well as anchorage-independent growth. Moreover, chronic activation of Rac1 via HER2 overexpression paired with HACE1 knockdown further enhanced the in vitro transformation of these cells
While knockdown of HACE1 alone was not sufficient for normal mammary epithelial cells to become tumorigenic in mice, HER2 overexpression and subsequent activation of Rac1 coupled with knockdown of HACE1 allowed robust tumor formation in vivo. The ability to identify the active state of Rho GTPases histochemically makes it difficult to identify tumors driven by these Rho GTPases. Here, the overexpression of upstream transmembrane receptors or RacGEFs that can either directly or indirectly activate Rac1 coupled with HACE1 loss leads to Rac hyper-activation and may identify tumors that have higher levels of Rac signaling.

Our findings demonstrate that while sustained Rac1 signaling may be a potent driver of transformation in human breast cancer, HACE1 is capable of tempering its downstream signaling capacity. As with previous studies, the Rac inhibitor EHT 1864 was able to inhibit Rac1 activation nullifying the phenotypic effects of Rac signaling in vitro [94, 95]. While Rac1 knockout mice are embryonic lethal raising concerns about Rac1 inhibition in vivo, tissue specific Rac1 knockout mice are viable suggesting that Rac1 inhibition in the adult animal may be tolerable [146]. Our findings using the Rac inhibitor in an in vivo setting demonstrate that EHT 1864 can mitigate tumor burden while having no adverse effects on the host. We have shown that the blockade of Rac1 activity by pharmacological means can affect breast cancer cell lines without affecting the growth of normal mammary epithelial cells. Though the inhibition of Rac was on tumors that formed by due to Rac hyperactivation, our studies indicate that
Figure 8-1: Schematic overview of HACE1 loss in mammary epithelial cells.
systemic administration of a Rac inhibitor can be biologically active and may be tolerable to the host, although further investigation is merited.

In summary, we performed a screen to identify genes capable of cooperating with HER2 allowing malignant transformation and identified HACE1 as a breast cancer tumor suppressor gene. We found that HACE1 loss in mammary epithelial cells and breast cancer leads to enhanced Rac1 signaling resulting in enhanced migration, invasion and anchorage-independent growth. HACE1 loss coupled with the overexpression of a Rac1 activator such as HER2 results in hyperactivation of Rac signaling and is sufficient to transform normal mammary epithelial cells allowing tumor formation in mice. Because aberrant Rac signaling drives these tumors, treatment with the Rac inhibitor diminishes the oncogenic addiction thus hauling the Rac driven phenotypes both in vitro and in vivo. HACE1 loss may therefore be utilized as a surrogate for Rac activation thus identifying patients at risk of disease progression as well as those susceptible to Rac targeted therapy.
REFERENCES


