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Combating Resistance To Epidermal Growth Factor Recpetor Inhibitors In Triple Negative Breast Cancer

Julie Marie Madden
Wayne State University,

COMBATING RESISTANCE TO EPIDERMAL GROWTH FACTOR RECEPTOR INHIBITORS IN TRIPLE NEGATIVE BREAST CANCER

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JULIE M MADDEN

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Advisor Date

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Co-Advisor Date

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DEDICATION

Vires, Fides, Motum Ducit

This work is dedicated to my unwavering parents. They never questioned when I wanted to stay in school forever and always encouraged me to follow my dreams no matter where they took me. They were always there to offer support when I decided to fly halfway across the world to study (three times) or travel hundreds of miles to see Oasis in concert or watch Man Utd play. Your trials with cancer led me into this field and your strength through it all drove me to help others fight and survive. To my Dad who taught me the importance of education and that science can be cool, your guidance and willingness to help when I didn't understand something made me want to get a PhD because I wanted to be like Daddy and be a scientist. To my Mother who is the most caring and thoughtful person on the planet, thank you for always listening to my ramblings about lab while proclaiming, "that word sounds familiar but I don't know what it means" and inserting commas copiously into all my papers. Without you both I would never be where I am now as an individual or a scientist.

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LIST OF ABBREVIATIONS

4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1
5-FU	5-fluorouracil
ACT	Adriamycin, cyclophosphamide, and taxol
AKT	Protein Kinase B
AML	Acute myeloid leukemia
AR	Amphiregulin
ATP	Adenosine triphosphate
Bad	Bcl-2-associated death promoter
BCL-2	B-cell lymphoma 2
BCL-XL	B-cell lymphoma-extra large
Bcr-Abl	Breakpoint cluster region-Abelson murine leukemia viral oncogene homolog 1 Philadelphia chromosome
BL1	Basal-like 1
BL2	Basal-like 2
BRCA1	Breast cancer 1
BTC	Betacellulin
c-Met	MNNG HOS transforming gene
c-Src	Proto-oncogene tyrosine-protein kinase Src
CI	Combination index
CK5/6	Cytokeratin 5/6
CREB	cAMP response element-binding protein
CTLA4	Cytotoxic T-Lymphocyte Antigen 4

Deptor	DEP-domain-containing mTOR-interacting protein
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
eEF1A	Eukaryotic elongation factor 1A
eEF2	Eukaryotic elongation factor 2
EGFR	Epidermal growth factor receptor
eIF4A	Eukaryotic translation initiation factor 4A
eIF4B	Eukaryotic translation initiation factor 4B
eIF4E	Eukaryotic translation initiation factor 4E
eIF4F	Eukaryotic translation initiation factor 4F
eIF4G	Eukaryotic translation initiation factor 4G
eIFs	Eukaryotic initiation factors
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial to mesenchymal transition
eNOS	Endothelial nitric oxide synthase
EPR	Epiregulin
ER	Estrogen receptor
ErbB	Erythroblast leukemia viral oncogene
eRF1	Eukaryotic release factor 1
eRF2	Eukaryotic release factor 2
eRF3	Eukaryotic release factor 3
ERK	Extracellular signal-regulated kinase
FBS	Fetal Bovine Serum

FGF	Fibroblast growth factor
FGF2	Fibroblast growth factor 2
FGPR3	Fibroblast growth factor receptor-3
FLT3	Fms-like tyrosine kinase 3
GAP	GTPase-activating protein
GBM	Glioblastoma multiforme
GEF	Gefitinib
GI_{50}	Growth inhibition at 50%
GTP	Guanosine-5'-triphosphate
HB-EGF	Heparin-binding epidermal growth factor-like factor
HER2/Neu	Human epidermal growth factor receptor 2
HIF-1α	Hypoxia-inducible factor 1-alpha
hTERT	Human telomerase reverse transcriptase
IGFR-1	Insulin-like growth factor receptor 1
IL12	Interleukin 12
IL7	Interleukin 7
IM	Immunomodulatory
IRES	Internal ribosome entry site
JAK	Janus kinase
Ki67	MK167 antigen identified by monoclonal antibody Ki-67
LAR	Luminal androgen receptor
LC3	Microtubule-associated protein 1A/1B-light chain 3
LumA	Luminal A

LumB	Luminal B
M	Mesenchymal
MAPK	Mitogen-activated protein kinase
Mcl-1	Induced myeloid leukemia cell differentiation protein
Mdm2	Mouse double minute 2 homolog
MDS	Myelodysplastic syndrome
MEK	Mitogen-activated protein kinase kinase
MEM	Minimum Essential Medium
Met	Methionyl
mLST8	Mammalian lethal with Sec13 protein 8
MMP	Matrix metalloproteinases
mRNA	Messenger ribonucleic acid
mSIN1	Mammalian stress-activated protein kinase interacting protein
MSL	Mesenchymal stem–like
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
mTORC2	Mammalian target of rapamycin complex 2
Myt1	Myelin transcription factor 1
NCCN	National Comprehensive Cancer Network
NF-KB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
NRG	Neuregulin
NSCLC	Non-small-cell lung carcinoma

p21Cip/Waf1	Cyclin-dependent kinase inhibitor 1
p27Kip1	Cyclin-dependent kinase inhibitor 1B
p38MAPK	P38 mitogen-activated protein kinase
P70S6K	70kDA Ribosomal protein S6 kinase
P90RSK	90kDa ribosomal s6 kinase
PABP	Poly(A)-binding protein
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly ADP ribose polymerase
pCR	Pathological complete response
PDCD4	Programmed cell death 4
PDGFR	Platelet-derived growth factor receptor
PDK1	3-phosphoinositide-dependent kinase-1
PH	Plekstrin homology
PI3K	Phosphatidylinositide 3-kinases
PIP2	Phosphatidylinositol-4,5-bisphosphate (PI-4,5-P2)
PIP3	Phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P3).
PKA	Protein kinase A
PKC	Protein kinase C
PKCα	Protein kinase C alpha
PKG	Protein kinase G
PR	Progesterone receptor
PRAS40	Proline-rich AKT substrate 40 kDa
Protor-1	Protein observed with Rictor-1

PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene fluoride
R	Arginine
RAF	Rapidly Accelerated Fibrosarcoma
Raptor	Regulatory-associated protein of mTOR
RAS	Rat sarcoma viral oncogene
RHEB	Ras homolog enriched in brain
Rictor	Rapamycin-insensitive companion of mTOR
RNA	Ribonucleic acid
rpS6	Ribosomal protein S6
RTK	Receptor tyrosine kinase
S6K1	Ribosomal protein S6 1
S6K2	Ribosomal protein S6 2
SDS	Sodium dodecyl sulfate
Ser (S)	Serine
SGK1	Serum/glucocorticoid regulated kinase 1
SH2	Src homology 2
siRNA	Silencing ribonucleic acid
STAT	Signal transducer and activator of transcription
STAT3	Signal transducer and activator of transcription 3
STATTIC	STAT three inhibitory compound
TCR	T cell receptor
TCS2	Tuberous sclerosis protein 2

TEM	Temsirolimus
TGF-α	Transforming growth factor alpha
Th1/Th2	Type 1/2 T helper
Thr (T)	Threonine
TKI	Tyrosine kinase inhibitor
TNBC	Triple negative breast cancer
TNM	Tumor, lymph node, metastasis
TOP	Terminal oligopyrimidine tract
TOR	Target of rapamycin
tRNA	Transfer ribonucleic acid
Tyr (Y)	Tyrosine
UNS	Unstable
VEGF	Vascular endothelial growth factor
VEGFA	Vascular endothelial growth factor A
VEGFR	Vascular endothelial growth factor receptor
XIAP	X-linked inhibitor of apoptosis

CHAPTER 1: Introduction

1.1 Breast Cancer: A Review

While billions of dollars have been designated to cancer research, care, and education, the number of people developing this disease is still on the rise. Early detection and better treatments have increased survival for almost all types, but cancer is still the second leading cause of death in the United States, after heart disease. More effective treatment options need to be developed to increase survival rates for patients. The focus of this dissertation is a combination targeted therapy for triple negative breast cancer (TNBC) and the molecular mechanisms of the therapy.

Breast cancer is the second leading cause of cancer related deaths and remains the most diagnosed among American women. It is estimated that in 2013 there were 232,340 new cases and 39,620 deaths attributed to breast cancer in women, accounting for 14% of all female cancer incidence. The lifetime risk for a woman to develop breast cancer is one in eight, with the highest risk of development occurring at 70 years or older (ACS, 2013).

Public awareness of the disease has been greatly increased by organizations such as Susan G Komen for the Cure, and campaigns such as “October is Breast Cancer Awareness Month” and the Pink Ribbon. Many women are routinely getting mammograms and checking for early detection, but even early detection has not greatly reduced breast cancer related mortality. Public awareness and knowledge about breast cancer has increased to the level where women are actively advocating for more effective treatments and better survival rates. The influx of research dollars from fundraising organizations and campaigns has led to many advances in the field but

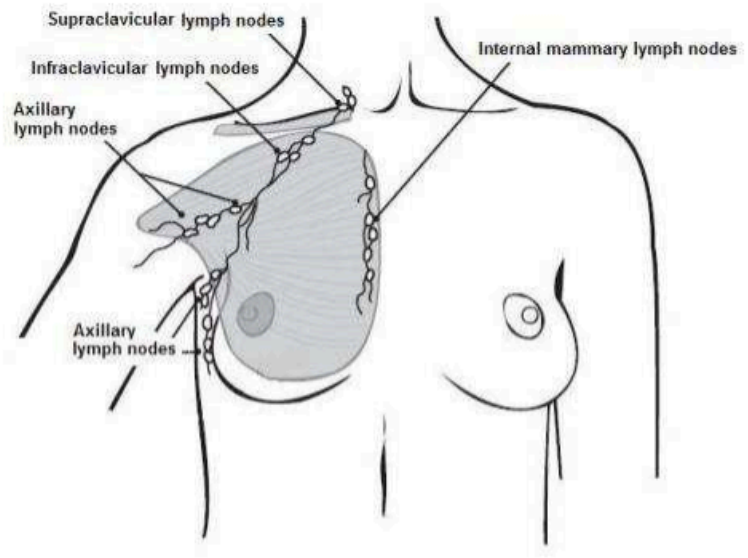
much is still unknown about the etiology of breast cancer and the most effective treatments.

Breast cancer is a very heterogeneous disease and can be characterized into four molecular subtypes based on gene expression profiling: Luminal A, Luminal B, HER2+, and Basal (Goldhirsch et al., 2011; Schnitt, 2010). Table 1 describes these subtypes and the current therapeutic options. The guidelines are meant to serve as a reference for physicians to treat the individual patient based on a variety of other clinical and pathological factors. These include patient age, overall health, and the stage and grade of the tumor (Schnitt, 2010). Age is often a factor in relation to menopause status. Hormone therapy is often reserved for post-menopausal women or as a last option for pre-menopausal women as the side effects from hormone ablation are greater in the pre-menopausal population (ACS, 2013). The subtypes have different incidence and mortality rates. Luminal A cancers comprise 40% of all diagnosed breast cancers and according to data from the Carolina Breast Study, have an 84% survival rate. Luminal B cancers are less prevalent at 20% but have a slightly better prognosis with an 87% survival rate. HER2+ cancers have a good molecular target and drug, trastuzumab, but their survival rate is only 52%, and the subtype comprises 10-15% of all breast cancers. The basal-like subtype is predominantly TNBC but not all basal cancers are TNBC and not all TNBC is basal-like. Basal-like cancers had a prognosis of 75% in the Carolina cohort. It also comprises about 20% of breast cancers (Carey et al., 2006). Basal-like tumors, particularly TNBC, are highly aggressive and have a poor prognosis compared to the most common luminal cancers so we need to find a better treatment option that has low toxicity and combats developed resistance for TNBC patients.

1.1.1 Stage and Grading

Stage and grade information was gathered from the National Comprehensive Cancer Network Clinical Practice Guidelines in Oncology, Breast Cancer, version 2.2012 (NCCN, 2012). Stage and grade are used to classify solid tumors. Both are important indicators for prognosis and are useful in determining how a patient is to be treated. For instance, if a patient has a low grade and stage tumor, that patient is most likely treated with radiation and/or surgical resection, often breast conserving, is proposed. Hormone therapy might also be recommended for the patient. Later stage and grade tumors are often indicators of poor prognosis and must be treated with aggressive chemotherapy when the patient is able to tolerate the high doses. Stage 4 disease indicate the tumor has metastasized to distant lymph nodes and parts of the body, therefore palliative care is the only treatment option in hopes of extending life. Common sites of metastasis for breast cancer include the lungs, liver, bones and brain. Staging is based on TNM where T is the size of the tumor, N is lymph node involvement, and M is the presence or absence of metastasis. T can be subdivided into T0 or the absence of a primary tumor; T1 where the tumor is ≤20mm; T2 where the tumor is >20mm but ≤50mm; a T3 tumor is >50mm; and T4 in breast cancer indicates the tumor has invaded the chest wall and/or skin. Regional lymph node involvement is measured as the N staging where N0 is no node involvement; N1 is detectable metastasis to a movable ipsilateral level I,II axillary lymph node; N2 in clinically fixed or matted ipsilateral level I,II or internal mammary nodes without axillary node detection;

Figure 1: Breast lymph nodes.



Staging is based on TMN system. Lymph node involvement within the breast is an important indicator of stage and can dictate treatment options. Cancer cells detected in the supraclavicular, intraclavicular, and internal mammary lymph nodes is more advanced disease and staged N3. Image reproduced with permission from American Cancer Society.

N3 metastasis in ipsilateral infraclavicular (level III axillary), internal mammary nodes, or ipsilateral supraclavicular lymph nodes (Figure 1). M is used to indicate the presence or absence of metastasis. M0 means there is no detectable distant metastasis in the body where M1 is the detection of metastasis, the extent of metastasis is unnecessary for TMN staging (NCCN, 2012).

Grade is based on the histological characterization of the tumor. G1 indicates a low grade where the cancer cells are more differentiated and generally have a more favorable prognosis; G2 cells have an intermediate histology where cells are less differentiated but the prognosis is still moderately favorable; G3 is the least favorable grade and indicates that the tumor cells are poorly differentiated (NCCN, 2012).

1.1.2 Triple Negative Breast Cancer

TNBC is a highly malignant and aggressive subtype of breast cancer. While it encompasses 12-20% of all diagnosed breast cancers, it is responsible for a disparate number of breast cancer related deaths (Chacon and Costanzo, 2010; Schneider et al., 2008). Premenopausal African American women are likely to develop TNBC at a disproportionate rate compared to white counterparts for reasons that are currently unknown (Stead et al., 2009). TNBC is characterized by a lack of receptor overexpression (ER [estrogen receptor], PR [progesterone receptor], and HER2) and therefore the commonly used hormone targeted and HER2 driven antibody therapies are ineffective against the subtype. Part of the high mortality rate associated with TNBC is due to the aggressive nature of basal-like cancers. A large proportion of TNBC tumors are basal-like and often have higher histological grade, a high Ki67 index, marked cellular pleomorphism, increased mitotic activity, and atypical mitotic figures

Table 1: Molecular subtypes, characteristics, and treatment.

Molecular Subtype	Biomarker Profile	Clinical Features	Treatment
Luminal A	ER+ and/or PR+, HER2-, low Ki67 (<14%)	~40% of invasive breast cancer Luminal A.	Hormone therapy. Radiotherapy. Chemotherapy variable. Prognosis better for LumA than LumB
Luminal B (HER2+ and HER2-)	*Her2-:* ER+ and/or PR+,HER2-,and high Ki67 (>14%) *Her2+:* ER+ and/or PR+,HER2+, any Ki67	~20% of invasive breast cancer Luminal B. Higher histological grade than LumA.	Hormone therapy. Radiotherapy. Chemotherapy better response in LumB.
HER2+	ER-, PR-, and HER2+	HER2 overexpressed or amplified.	Trastuzumab (Herceptin). Lapatinib Radiotherapy. Anthracycline-based chemotherapy. Poor prognosis
Basal	ER-, PR-, HER2-, and CK5/6 and/or EGFR+	~80% overlap between 'TNBC' and intrinsic 'basal-like' subtype. BRCA1 dysfunction Often in African Americans. Very aggressive and highly malignant.	Adriamycin, cyclophosphamide, taxane. Radiotherapy. Platinum-based chemotherapy. PARP inhibitors. Poor prognosis.

The four main molecular subtypes of breast cancer are Luminal A (LumA), Luminal B (LumB), HER2+, and Basal. They are characterized by the presence of the Estrogen Receptor (ER), the Progesterone Receptor (PR), HER2 Receptor (human epidermal growth factor 2), Ki67 (MK167), EGFR, and CK5/6 (cytokeratin 5,6) levels (Cheang et al., 2009; Goldhirsch et al., 2011; Nielsen et al., 2004; Schnitt, 2010).

(Nielsen et al., 2004; Rakha et al., 2007). These are all characteristics of higher proliferative potential and poorly differentiated tumor cells. Genomic instability and increased DNA copy number also contribute to TNBC malignancy (Chin et al., 2006). TNBC can contain detrimental mutations in p53, increased expression of immune response genes, and/or BRCA1 mutations (Schneider et al., 2008). BRCA1 alterations are often associated with TNBC. Mutations in BRCA1 lead to decreased DNA repair mechanisms and therefore increased genomic mutations and genetic instability. BRCA1 and BRCA2 mutations are responsible for 25% of hereditary breast cancers (Easton, 1999). 90% of all BRCA1 associated tumors are triple negative (Chacon and Costanzo, 2010). With such heterogeneous characteristics, TNBC is difficult to treat and the genomic instability and variety of mutations along with the growth signaling pathway alterations make a disease that often develops resistance to many cytotoxic chemotherapy agents.

TNBC can be subdivided into further histological categories (see Table 2), the most common type being basal-like. Most TNBC tumors express basal markers such as the EGFR (epidermal growth factor receptor) and cytokeratins. Lehman and colleagues divided TNBC into 7 subcategories based on differential gene expression. The categories can be seen in Table 2 and are as follows: basal-like 1 (BL1); basal-like 2 (BL2); immunomodulatory (IM); mesenchymal (M); mesenchymal stem–like (MSL); luminal androgen receptor (LAR); and unstable (UNS) (Lehmann et al., 2011). The IM subtype has gene expression enriched in the immune cell processes. IM is characterized by immune signaling in addition to immune cell-surface antigens,

Table 2: TNBC Subtypes.

Subtype	Associated Pathways
Basal-like 1 (BL1)	Cell Cycle DNA replication reactome RNA Polymerase
Basal-like 2 (BL2)	EGF Pathway NGF Pathway MET Pathway WNT β-catenin Pathway
Immunomodulatory (IM)	CTLA4 IL12 Pathway Th1/Th2 Pathway IL7 Pathway
Mesenchymal-like (M)	IGF/mTOR Pathway ECM Pathway Regulation of Actin by RHO WNT Pathway
Mesenchymal Stem-like (MSL)	ECM Receptor Interaction TCR Pathway WNT β-catenin Focal Adhesion
Luminal AR (LAR)	Pentose/Glucuronate Interconversion Glutathione Metabolism Tyrosine Metabolism Steroid Biosynthesis
Unstable (UNS)	Cytokeratin Expression Multiple chromosome rearrangements

There are 7 TNBC subtypes that are characterized by differential gene expressions; basal-like 1, basal-like 2, immunomodulatory, mesenchymal, mesenchymal stem–like, luminal androgen receptor, and unstable.

cytokine signaling, complement cascade, chemokine receptors and ligands, and antigen presentation (Lehmann et al., 2011). The M and MSL subtypes are enriched for cell motility, ECM receptor interaction, and cell differentiation pathways (Lehmann et al., 2011). MSL is also enriched for angiogenesis and claudins. The LAR subtype is characterized by increased steroid synthesis, porphyrin metabolism, and androgen/estrogen metabolism (Lehmann et al., 2011). The different TNBC subtypes have significant variability in relapse-free survival. LAR has a significant decrease in relapse-free survival compared to BL1 and IM (Lehmann et al., 2011). The M subtype also had a lower relapse-free survival compared to BL1, and that for MSL was greater than M. The MSL subtype had the greatest relapse-free survival while patients with the LAR subtype had the worst prognosis for relapse-free survival based on Kaplan-Meier analysis following the patients for 10 years. Lehmann and colleagues found that there was no significant difference in tumor size or grade at diagnosis between the TNBC subtypes but women diagnosed with LAR were older compared to the other subtypes (Lehmann et al., 2011).

These following data concerning surgical resection are from cancer.net and Kaviani et al., 2013. Most TNBC tumors are surgically resected with administration of adjuvant chemotherapy and radiation (Gangi et al., 2014). Surgery for the treatment of breast cancer has changed drastically in the last 30 years. While complete removal of the breast and all surrounding tissue was once commonplace and left debilitating scars and largely deformed chests, surgery is now able to effectively remove the tumor without excising a considerable amount of normal surrounding tissue. The former surgery was called a radical mastectomy and the entire breast, muscle, and all

surrounding tissue was completely removed, even up through the armpit and into the back on certain occasions. The surgery left many women disfigured and reconstruction of a new breast was not possible. Surgeons were later able to remove less of the normal breast tissue while still getting clean margins around the edges of the tumor, a necessity to ensure total removal of cancerous cells in the area. A lumpectomy is now a common practice for smaller tumors within the breast allowing for removal of the tumor mass while conserving as much normal tissue as possible. This allows for easier reconstructive surgery with the remaining tissue and enabling many women to keep the appearance of a normal breast. In one study, breast-conserving therapy with whole breast radiation had the same survival rate as a mastectomy for TNBC(Gangi et al., 2014).

Surgery is most often paired with either radiation and/or chemotherapy in a neoadjuvant or adjuvant setting. Neoadjuvant chemotherapy is given to reduce the size of the tumor before surgery. If the oncologist is able to reduce tumor burden, less tissue can be removed leading to less downtime for the patient and an easier recovery. Radiation and adjuvant chemotherapy are often given after surgery to ensure removal of microscopic cancer cells that might have been left behind after the surgery (Kaviani et al., 2013). TNBC has a greater chance of recurrence after resection than the other molecular subtypes of breast cancer, therefore; aggressive chemotherapy and radiation are almost always suggested for these patients (Meyers et al., 2011; Zaky et al., 2011).

Scientists and clinicians are actively working to find a better treatment for women with TNBC that helps combat the high rate of treatment resistance and tumor recurrence. The current chemotherapy standard of care for TNBC patients is the

combination ACT (adriamycin [doxorubicin], cyclophosphamide, and a taxane). Many ongoing clinical trials are aimed at exploiting the signaling pathways upregulated in TNBC or DNA repair mechanisms, as BRCA1 and PARP1 (Poly[ADP]ribose polymerase 1) are often mutated. A current treatment with a relatively good success rate is an aggressive combination of systemic cytotoxic chemotherapy. 5-fluorouracil (5-FU), doxorubicin and cyclophosphamide was shown to have a 45% pathological complete response (pCR) in a 2005 study (Rouzier et al., 2005). Other studies have looked at platinums as some TNBCs express BRCA1 mutations, which confer sensitivity to cisplatin (Byrski et al., 2010; Silver et al., 2010). PARP1 inhibitors have also shown some success in TNBC and many clinical trials are ongoing to assess their efficacy in the clinic in Phase I and II trials (NCT01116648, NCT00516724) (Santana-Davila R, 2010; Tutt et al., 2010). Many chemotherapeutic options exist for TNBC but unfortunately they have not delivered high response rates.

TNBC also expresses receptors that can be inhibited through targeted drugs. Unlike systemic cytotoxic chemotherapy, targeted agents are more effective at selectively killing the cancer while sparing a greater number of normal cells. Side effects of targeted therapies still exist but are often better tolerated than their cytotoxic counterparts. Many targeted therapies work on the premise of oncogene addiction. Oncogene addiction is the theory that cancer cells rely on the overexpression of certain growth factors and receptors, such as EGFR and HER2, therefore when the receptor is inhibited the cancer cells are less able to adapt to the inhibition and subsequently die. When this signaling is reduced, normal cells can better adjust and therefore survive;

opposed to the addicted cancer cells which are more likely to die when the strong growth stimulus is removed (Malina et al., 2011; Weinstein, 2002).

Further trials are ongoing in TNBC using a combination of EGFR inhibitors and other cytotoxic chemotherapies such as docetaxel and carboplatin (NCT00491816). EGFR inhibitors are approved for treatment of non-small-cell lung carcinoma (NSCLC) (Rosenberg et al., 2004). Antibodies such as cetuximab, and tyrosine kinase inhibitors (TKIs) such as gefitinib and erlotinib, are used to inhibit the EGFR and its downstream effectors. As previously mentioned, EGFR is overexpressed (greater than 3-fold) in TNBC, up to 50%, and therefore could be a potential drug target (Nielsen et al., 2004; Rakha et al., 2007). Other targets for TNBC therapies are mTOR (mammalian target of rapamycin) and VEGF (vascular endothelial growth factor). The mTOR inhibitor, everolimus, is being used in clinical trials in metastatic TNBC disease (NCT00827567) and in combination with paclitaxel and cisplatin (NCT00930930). VEGF targeted anti-angiogenic therapy has also been evaluated with bevacizumab with or without paclitaxel and/or carboplatin followed by doxorubicin and cyclophosphamide, or bevacizumab as a single agent (NCT00861705, NCT00528567). Among pathways that are upregulated in TNBC, mTOR is often activated. Studies have shown that EGFR and mTOR inhibitors are effective in preclinical models as a combination but their mechanism of action is still unknown (Bianco et al., 2008; Liu et al., 2011). The combination of EGFR and mTOR inhibitors in TNBC was explored in the clinic, however, all trials were terminated due to slow accrual or funding termination before any clinical results were reported. Such trials included the combination of lapatinib and everolimus (NCT01272141), which was terminated in March 2014. The mTOR inhibitor, rapamycin was shown to sensitize

NSCLC cells that have gained resistance to gefitinib and a similar study showed everolimus, a rapamycin analog (rapalog), had the same effect (La Monica et al., 2009; Liu et al., 2011). Colon, pancreatic, and breast cancer cell lines that were resistant to EGFR TKIs were also sensitized when treated with an mTOR inhibitor but the mechanism of action is unknown (Bianco et al., 2008; Buck et al., 2006). More about EGFR and mTOR will be discussed below in upcoming sections.

1.2 Epidermal Growth Factor Receptor

The epidermal growth factor (EGF) was first discovered in 1962 by Stanley Cohen as an agent that promoted eye opening in newborn mice (Cohen and Carpenter, 1975). Years later Graham Carpenter discovered the receptor (Carpenter et al., 1978). The EGFR is part of the ErbB/HER family of transmembrane growth factor receptors, which include four members: EGFR, also known as ErbB1/HER1, ErbB2/HER2/NEU, ErbB3/HER3, and ErbB4/HER4 (Mitsudomi and Yatabe, 2010) (Figure 2). The deregulation of ErbB (erythroblast leukemia viral oncogene) proteins have been implicated in the tumorigenesis of many epithelial cancers including lung, breast, ovarian, pancreatic, and prostate (Hynes and Stern, 1994).

ErbB proteins have four functional domains including a cysteine-rich extracellular ligand-binding domain, a transmembrane domain, an intracellular tyrosine kinase domain, and a C-terminal regulatory domain (Burgess et al., 2003; Hynes and Lane, 2005; Riese and Stern, 1998). The ErbB family of receptor tyrosine kinases are activated in response to a ligand, homo- or heterodimerize, and activate downstream signaling pathways through tyrosine phosphorylation of different residues on the intracellular domain (Riese and Stern, 1998). The heterodimerization enables the

orphan receptor ErbB2/HER2 and the kinase-dead ErbB3/HER3, to initiate signaling through binding the other family members (Hynes and Lane, 2005). Downstream pathways activated by the ErbB family include the mitogen-activated protein kinases (MAPK) cascade (Figure 3 EGFR Y1148) and phosphatidylinositide 3-kinases (PI3K) activated AKT pathway cascade (Figure 3, EGFR Y1101). Y1148 and Y1101 correspond with the site on the EGFR that when phosphorylated allows for binding and activation of the respective proteins. P70S6K (70kDA Ribosomal protein S6 kinase) can also be activated directly by ErbB3 and ErbB4 dimers and indirectly through ErbB1 and ErbB2 (Yarden and Sliwkowski, 2001).

The four members of the ErbB family can be activated through eight ligands grouped into three different classes (Figure 2). The first class can only bind EGFR and contains EGF and its analogs, transforming growth factor alpha (TGF-α) and amphiregulin (AR), also known as keratinocyte autocrine factor or colorectum-cell derived growth factor. The second class can bind ErbB3 and ErbB4 and contains the neuregulins (NRGs) and the neuregulin-2s (NRG-2s), also known as the cerebellum-derived growth factors. The final group can bind both EGFR and ErbB4 and contain heparin-binding epidermal growth factor-like factor (HB-EGF), epiregulin (EPR); betacellulin (BTC); also known as heregulins (Riese and Stern, 1998). The ErbB family can also be activated through receptors and signals that do not directly interact with EGFR. These include hormones, neurotransmitters, lymphokines, and stress inducer signals demonstrating the diversity of the receptor and its activators (Carpenter, 1999).

The EGFR signaling cascade has been extensively studied as it plays a major role is many aspects of normal cellular processes including apoptosis, migration,

growth, adhesion, and differentiation (Figure 2) (Yarden and Sliwkowski, 2001; Yecies and Manning, 2011). Both the ligand and dimerization partner determines the output signal from ErbB family members. This interaction allows for the autophosphorylation of ErbB, recruiting the specific docking proteins to sites of phosphorylation to begin the signaling cascade (Olayioye et al., 1998). There are three pathways that can be activated by all ErbB dimerization couples. These include the Ras activated MAPK cascade, PI3K/ AKT pathway, and P70S6K/p85S6K (Soltoff and Cantley, 1996; Yarden and Sliwkowski, 2001). While all dimers can activate these pathways, they require certain docking proteins that can only interact with specific dimers. For example, c-Cbl is unable to interact with ErbB3, and the receptor is also unable to bind PLCγ, and Grb2 affecting ErbB3's ability to ubiquitinate and activate the transcription factor Fos (Fedi et al., 1994). ErbBs can also be trans-activated by GPCRs and heterologous signals including hormones, neurotransmitters, lymphokines, and stress inducers. Non-receptor tyrosine kinases such as JAK are also able to directly phosphorylate the kinase part of the receptor resulting in activation of EGFR dependent pathways (Carpenter, 1999; Yarden and Sliwkowski, 2001). Most of what is known about ErbB signaling results in growth and proliferation. Our data suggest that the EGFR pathway also may play a role in translation through a lesser known and explored mechanism involving eukaryotic initiation factor 4B (eIF4B). The EGFR function in translation closely links the pathway with mTOR's involvement in the same process and provides a potential pathway crosstalk that is important in TNBC and that this dissertation explores.

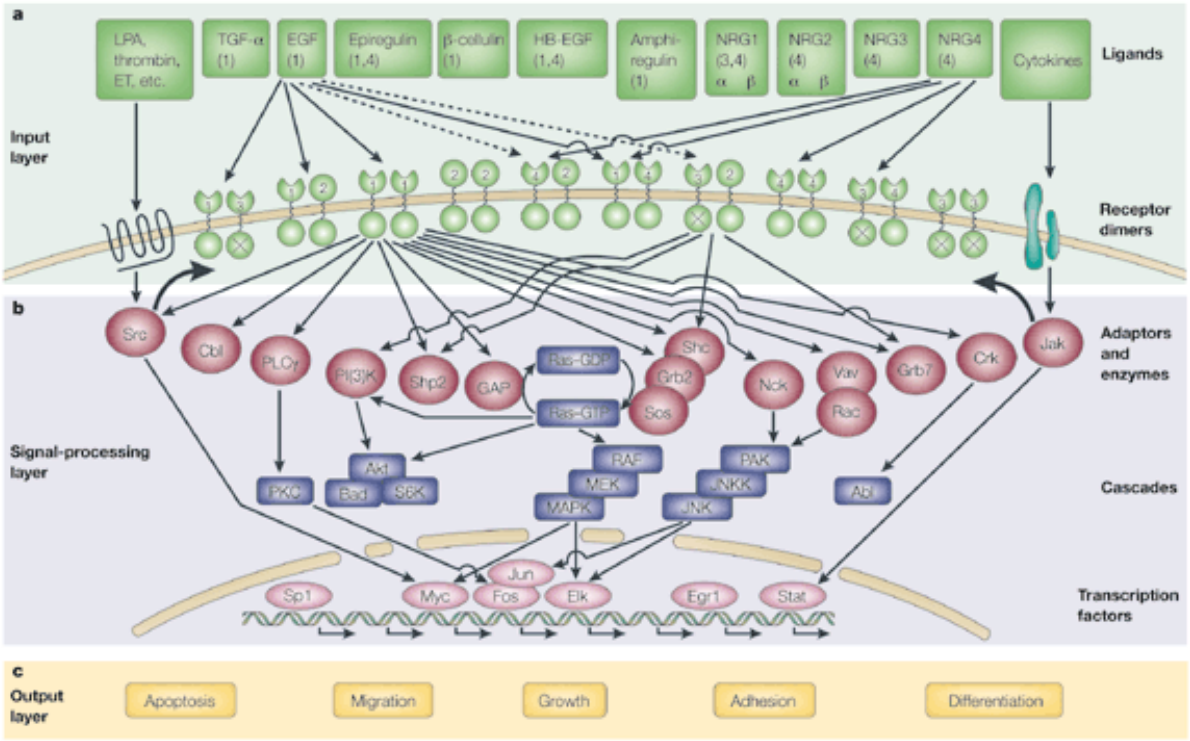
Knockout mice of EGFR have proven its vital role in skin, lungs, and the gastrointestinal tract with knockout of the receptor being embryonic lethal (Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995). Of the many pathways that EGFR is involved in; much research has been done with the EGFR as a typical receptor tyrosine kinase (RTK) and the start of the RAS/RAF/MEK/ERK cascade (Yarden and Sliwkowski, 2001). Studying the activation of proteins within the cascade has helped to understand a substantial amount about RTK signaling through phosphorylation.

HER2 is amplified in 15-30% of invasive ductal carcinomas (Slamon et al., 1987). Tumors with higher levels of HER2 are generally larger at diagnosis, have greater lymph node involvement, higher grade, and contain a greater number of proliferative cells (Yarden and Sliwkowski, 2001). The recognition of HER2 as a druggable target led to the generation of an antibody to HER2 in 1998, known as herceptin (trastuzumab). The antibody has been effective at reducing tumor burden in patients with HER2 overexpression through binding and subsequent inhibition of downstream pathways and internalization. It also induces expression of the cyclin dependent inhibitors, $p27^{Kip1}$ and p130, which inhibit the cell cycle, and recruit immune cells (Clynes et al., 2000; Sliwkowski et al., 1999).

1.2.1 EGFR as a Target in Cancer

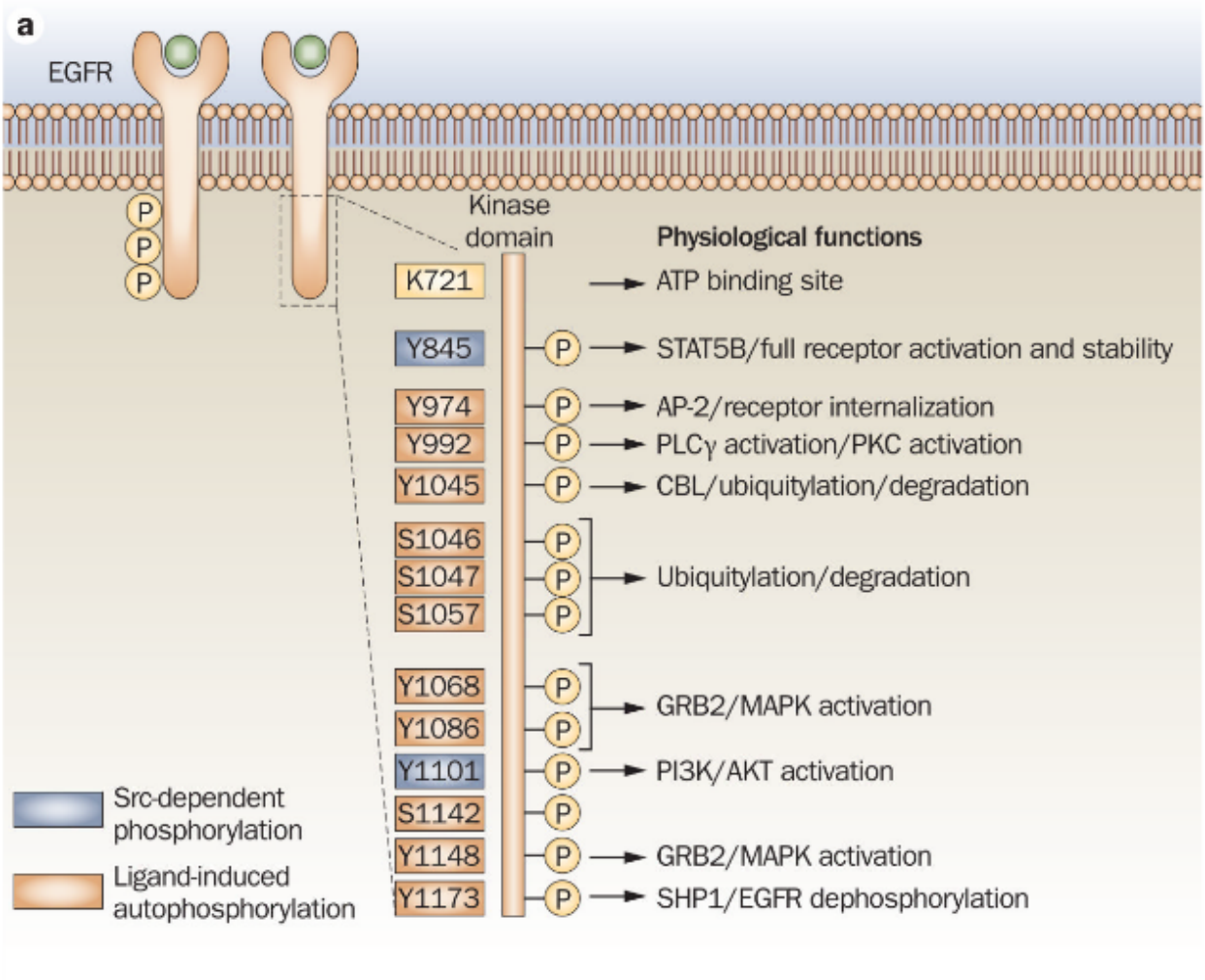
Kawamoto and Sato first explored targeting EGFR in cancer in 1983 when they studied growth inhibition of tumor cells treated with an EGFR antibody (Kawamoto et al., 1983; Sato et al., 1983). Their experiments had promising results and others since have also studied inhibition of the EGFR and its downstream signaling pathways in most solid tumor types (Herbst et al., 2004; Mitsudomi and Yatabe, 2010). The EGFR is involved in

Figure 2: ErbB family members, ligands, and signaling network.



The ErbB family contains four members; EGFR, Her2, Her3, and Her4. Upon ligand stimulation the receptors homo- or heterodimerize, cross phosphorylate and activate downstream signaling pathways through tyrosine kinase activity. Her2 has no ligand but can dimerize with the other family members while Her3 has no kinase activity and therefore must heterodimerize with another family member to signal after ligand binding. This figure is reproduced with permission from Yarden and Sliwkowski, 2001.

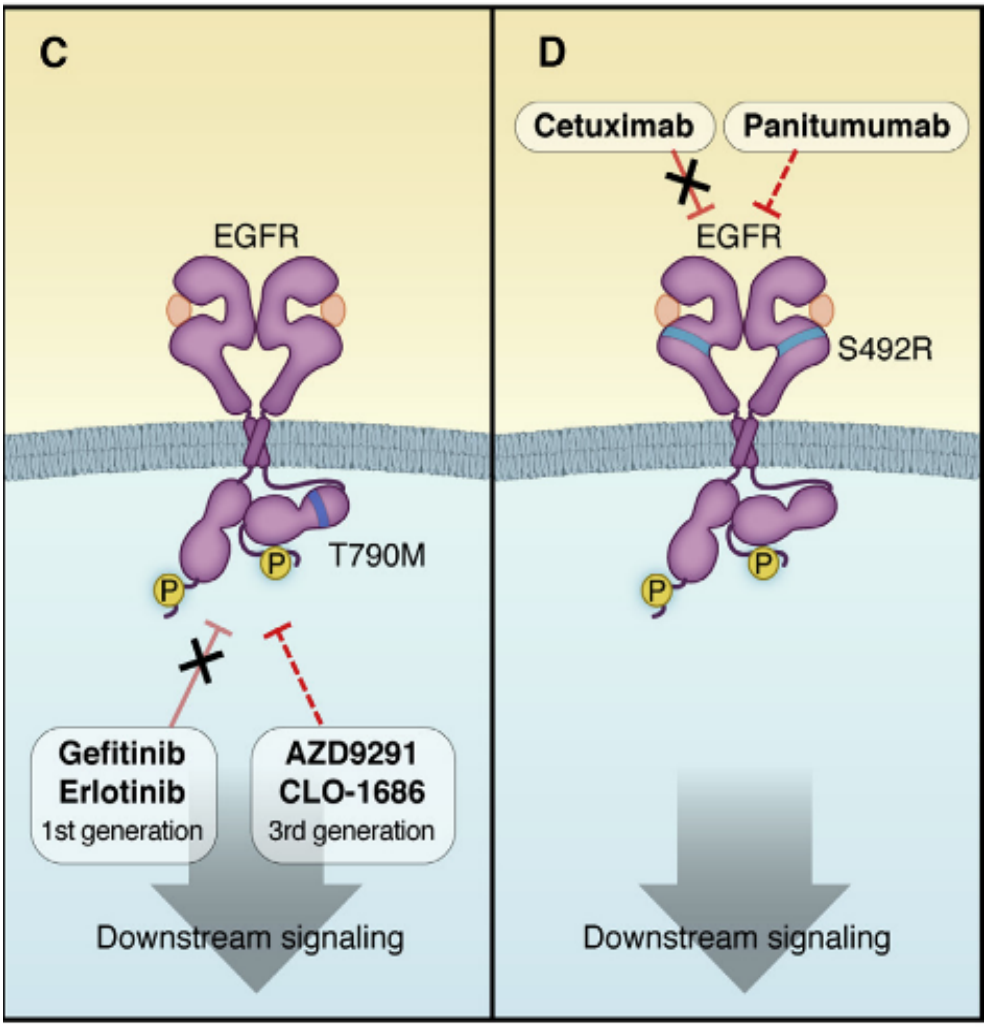
Figure 3: EGFR phosphorylation sites.



Many sites on the EGFR that, when phosphorylated, induce receptor signaling to a variety of known proteins. The signaling is further propagated through kinases leading to pathway activation including cell growth and proliferation. Figure is reproduced with permission from Wheeler et al., 2010.

a complex array of signaling networks. It signals for cell proliferation, apoptosis, adhesion, differentiation, migration, survival, angiogenesis, and tumorigenesis (Wheeler et al., 2010; Yarden and Sliwkowski, 2001). Its overexpression has been found as a driving factor in head and neck, breast, bladder, prostate, kidney cancers, NSCLC, and gliomas (Yarden and Sliwkowski, 2001). It also might be a prognostic indicator in bladder, prostate cancers, and NSCLC (Gorgoulis et al., 1992; Irish and Bernstein, 1993). In breast cancer it has been found to be a predictor of recurrence after surgical resection and associated with a shorter disease free and overall survival. The anti-proliferative ability of EGFR inhibitors was very promising and led the FDA to approve five different EGFR inhibitors within three years (gefitinib 2003 for NSCLC, cetuximab 2004 for colorectal cancer, erlotinib 2004 for NSCLC and pancreatic cancer, panitumumab 2006 for colorectal cancer, laptinib 2006 for breast cancer) (Wheeler et al., 2010). EGFR inhibitors are effective in these cancers as a common mutation in the EGFR confers sensitivity to the drugs (L858R). This same mutation is not seen in breast cancer and therefore leads to resistance to EGFR inhibitors. Since then, EGFR inhibitors have been used to treat a variety of cancers including lung, colorectal, pancreatic, and head and neck (Chong and Janne, 2013). One of the most notable advances is the use of gefitinib and erolitinib to treat metastatic lung cancer patients. The patients treated with gefitinib or erolitinib had a 74% and 83% response rate respectively, compared to the other best treatment with progression free survival and overall survival rates in the 30% (Inoue et al., 2013; Maemondo et al., 2010; Zhou et al., 2011). The effectiveness of inhibiting the receptor has made EGFR inhibitors part of standard treatment for NSCLC with increased expression of EGFR.

Figure 4: Binding sites of EGFR inhibitors.



The EGFR is a transmembrane receptor that, upon ligand binding, dimerizes and cross phosphorylates to signal. The extracellular side of the receptor binds the ligand and anti-EGFR antibodies such as cetuximab and panitumumab. TKIs inhibit the intracellular kinase activty of the receptor. These small molecules include gefitinib (used in this dissertation work), erlotinib, AZD9291, and CLO-1686. This figure was reproduced with permission from Arteaga and Engelman, 2014.

Two different classes of EGFR inhibitors are used in the clinic, TKIs and antibodies. TKIs target the kinase activity of EGFR through ATP competitive binding and include erlotinib, lapatinib, and gefitinib. Monoclonal antibodies, which bind the receptor to inhibit its activity and promote receptor internalization include cetuximab and panitumumab (Chong and Janne, 2013) (Figure 4). Gefitinib is prescribed in the clinic as a 250mg orally available drug that is used daily at a lower concentration than the maximum tolerated dose resulting in less toxicity (Rukazenkov et al., 2009). Like many other targeted therapies, gefitinib and erolitinib have a more favorable side-effect profile then cytotoxics, which often have nausea, diarrhea, and neuropathy as limiting toxicities. Common reported adverse events with gefitinib are rash and diarrhea (Maemondo et al., 2010).

There are a multitude of other EGFR inhibitors available for clinical use and many still waiting for FDA approval. While single agent treatment with EGFR inhibitors in cancers have not proven to be very effective due to their high levels of resistance, discussed below, the combination of EGFR inhibitors along with cytotoxic chemotherapy has proven promising in colorectal and metastatic pancreatic cancers, and especially in lung cancer (Chong and Janne, 2013; Gschwind et al., 2004).

1.2.2 Mechanisms of Resistance

EGFR inhibitors have been used in the clinic for a variety of cancer types. While EGFR is a druggable target, its intricate signaling pathways and wide variety of ligands often allow the cell to remain activated while the EGFR is inhibited. This resistance can either be acquired or *de novo*. Acquired resistance occurs when the cell is able to activate other proteins to compensate for EGFR inactivation. *De novo* resistance is

present at onset enabling the cell to completely be independent of EGFR signaling inhibition with minimal new activation of compensatory mechanisms as seen in acquired resistance. There are many different ways that cells can circumvent EGFR inhibitors for either *de novo* or acquired resistance that are discussed below.

Receptor mutations play an important role in determining sensitivity to EGFR inhibitors. In the clinic, patients with certain EGFR mutations are more sensitive to inhibitors while other mutations render resistance. One resistance mutation was identified in glioblastoma multiforme (GBM) patients who developed insensitivity to gefitinib. The mutation was a constitutively active truncated version of the receptor known as EGFRvIII (Wheeler et al., 2010). Another common mutation that patients develop after prolonged treatment of EGFR TKIs is a substitution in exon 20, T790M. This residue is considered a "gatekeeper" for the ATP-binding pocket of EGFR. Resistance may occur when the TKI is no longer able to bind the pocket on the EGFR due to the larger methionine group and subsequent steric interference (Kobayashi et al., 2005; Pao et al., 2005).

Angiogenesis is activated by EGFR signaling and is a process that is necessary for tumor growth and metastasis. During tumor vascularization, blood vessels grow into the tumor, bringing it nutrients and providing a mechanism for individual tumor cells to circulate through the body and metastasize. VEGF is a ligand that is necessary to support angiogenesis and tumors resistant to EGFR inhibitors are known to upregulate VEGF and its receptor, VEGFR (Viloria-Petit et al., 2001). Activation of the PI3K/AKT pathway was also found in NSCLC patients who were resistant to gefitinib. Researchers found that EGFR was coupled to ErbB3 and could activate AKT only in cell lines that

are sensitive to gefitinib. Gefitinib binds the EGFR/ErbB3 complex and inhibits PI3K association therefore decreasing AKT activity. The association of ErbB3 and PI3K is not seen in resistant cell lines and allows AKT to remain active as it is not inhibited when gefitinib binds the ErbB dimer (Engelman et al., 2005). The receptor c-Met (MNNG HOS transforming gene) is overexpressed in NSCLCs that are resistant to EGFR inhibitors. c-Met also plays a role in breast cancer resistance to EGFR TKIs, as it was found to activate EGFR substrates in the presence of EGFR inhibitors (Mueller et al., 2010; Mueller et al., 2008). AKT is also activated through overexpression of c-Met in lung cancer (Engelman et al., 2007). Ubiquitination is known to mediate resistance to cetuximab as decreased receptors on the cell membrane still elicit a strong EGFR signaling response and ubiquitin levels can determine receptor recycling or degradation (Lu et al., 2007; Wheeler et al., 2008). IGFR-1 (insulin-like growth factor receptor 1) activation is also shown to mediate resistance to EGFR inhibitors through activation of AKT and P70S6K (Chakravarti et al., 2002). Epithelial to mesenchymal transition (EMT) plays a role EGFR inhibitor resistance as mesenchymal type cells rely minimally on EGFR signaling (Wheeler et al., 2010). While EGFR inhibitors are used in the clinic, many studies have found a variety of mechanisms *in vitro* allowing the cell to compensate for EGFR inhibition leading to resistance. These studies can allow researchers and physicians to further explore combination therapies of EGFR inhibitors and a drug that targets one of the known mechanisms of resistance.

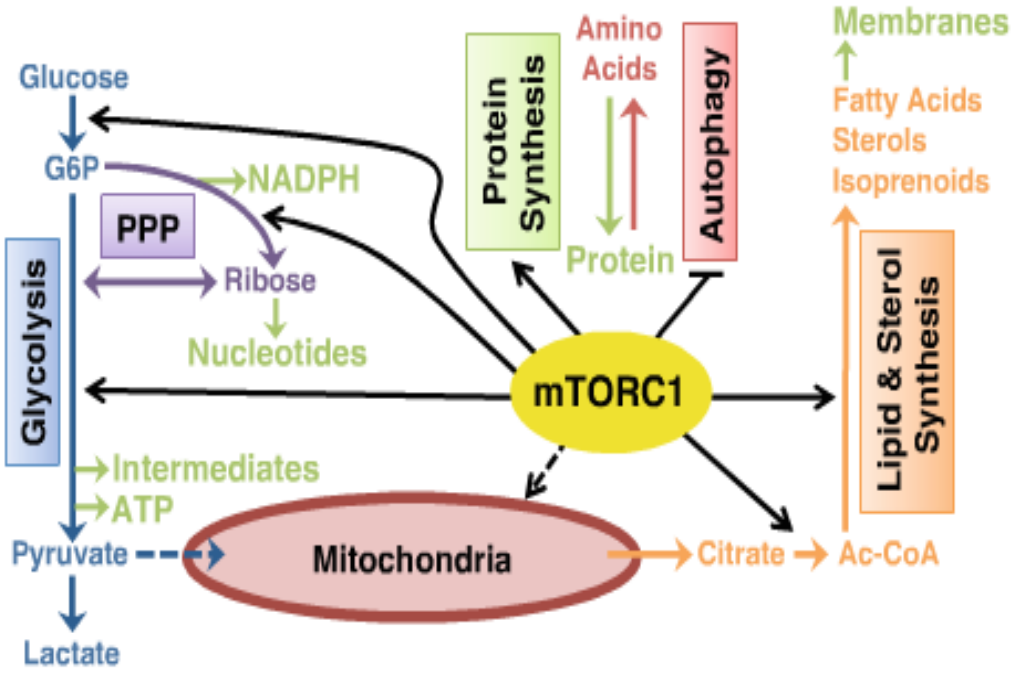
1.3 PI3K/AKT/mTOR

AKT/PKB has been implicated in a variety of processes that lead to tumorigenesis (Faivre et al., 2006; Fresno Vara et al., 2004). Hannahan and Weinberg

designated six different hallmarks of cancer and AKT is involved in all. (1) *Growth signal autonomy:* AKT overexpression or activation leads to signaling with a low amount of growth factors, (2) *Insensitivity to antiproliferative signals:* Recruits Mdm2 (mouse double minute 2 homolog) to the nucleus to inhibit p53, localizes $p21^{Cip/Waf1}$ to promote proliferation, and stabilizes Cyclin D1 to promote cell cycle progression, (3) *Inhibition of apoptosis:* Inactivates Bad (Bcl-2-associated death promoter), procaspase-9, NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), and Fas ligand, *(4) Unlimited replicative potential*: Phosphorylates hTERT (human telomerase reverse transcriptase) to increase telomeres, (5) *Angiogenesis:* Promotes through eNOS (endothelial nitric oxide synthase), (6) *Invasion and metastasis:* Inhibits anoikis and stimulates MMP (matrix metalloproteinases) secretion to increase basement membrane degradation (Fresno Vara et al., 2004).

AKT has three homologous isoforms (AKT1, AKT2, and AKT3) each with four domains: a plekstrin homology (PH) domain, an N-terminal domain, kinase domain, and a C-terminal domain (Alessi et al., 1996; Hay and Sonenberg, 2004). Full activation of AKT requires phosphorylation of Thr308 and Ser473 through amino acid, glucose, and oxygen and/or mitogen (hormone and growth factor) stimuli (Alessi et al., 1996; Engelman, 2009). Activated AKT can then signal through the mTOR complexes by binding to TSC2 and acting as a GAP (GTPase-activating protein) for the GTPase Rheb (Hay and Sonenberg, 2004). Two complexes contain the mTOR protein, mTORC1 (mammalian target of rapamycin complex 1 [containing Raptor]) and mTORC2 (mammalian target of rapamycin complex 2 [containing Rictor]). Not much is known about the regulation and function of mTORC2 but it contains 6 subunits; mTOR,

Figure 5: mTOR is involved in multiple cellular processes.



mTOR is involved in many components of the cell. It signals for energy and protein homeostasis within the cell. This figure is reproduced with permission from Yecies and Manning, 2011.

rapamycin-insensitive companion of mTOR (Rictor), mammalian stress-activated protein kinase interacting protein (mSIN1), protein observed with Rictor-1 (Protor-1), mammalian lethal with Sec13 protein 8 (mLST8, also known as GbL), and DEP-domain-containing mTOR-interacting protein Deptor (Laplante and Sabatini, 2009). Data suggest that it phosphorylates the AGC kinase family, including AKT leading to a feedback loop, protein kinase C alpha (PKCα), and the serum/glucocorticoid regulated kinase 1 (SGK1) (Guertin and Sabatini, 2007). It also leads to actin regulation, cytoskeleton formation and cell survival (Sarbassov et al., 2004). mTORC1 (known as simply mTOR for the purposes of this dissertation) is involved in growth, proliferation, autophagy, and translation (Ganley et al., 2009; Laplante and Sabatini, 2009). It is comprised of five subunits: mTOR, regulatory-associated protein of mTOR (Raptor), mLST8, proline-rich AKT substrate 40 kDa (PRAS40), and (Deptor) (Laplante and Sabatini, 2009).

mTOR activates P70S6K, which phosphorylates 4E-BP1 (eukaryotic translation initiation factor 4E-binding protein 1) to remove it from eIF4E, (eukaryotic translation initiation factor 4E) releasing the translation initiation factor allowing it to complex with eIF4A (eukaryotic translation initiation factor 4A), and eIF4G (eukaryotic translation initiation factor 4G) to form eIF4F (eukaryotic translation initiation factor 4F) and stimulate translation. A more extensive review of translation initiation will follow in Section 1.4. mTOR is also able to stimulate translation through phosphorylating eIF4B allowing it to facilitate eIF4A helicase activity. Important tumorigenic proteins translated through this mechanism are cell cycle regulating proteins, HIF-1α (hypoxia-inducible factor 1-alpha), FGF (fibroblast growth factor), VEGF (vascular endothelial growth

factor), STAT3 (signal transducer and activator of transcription 3), and c-Myc (Strimpakos et al., 2009). mTOR is also involved in lipid biogenesis in the mitochondria (Laplante and Sabatini, 2009; Schieke et al., 2006). Within the mitochondria, mTOR affects mitochondrial membrane potential, oxygen consumption and cellular ATP levels (Schieke et al., 2006). A complex signaling network activates mTOR and it can sense a plethora of stimuli such as amino acids, mitogens, oxygen, stress, and inflammation to generate a cellular response (Guertin and Sabatini, 2007; Hardie et al., 1998; Wouters and Koritzinsky, 2008) (Figure 5).

Upstream of AKT is PI3K. The protein is a heterodimer comprising a catalytic subunit (p110) and a regulatory subunit (p85) (Hay and Sonenberg, 2004). PI3K is responsible for converting phosphatidylinositol-4,5-bisphosphate (PI-4,5-P2) to phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P3). PIP3 then activates AKT through the PH domain and phosphorylation by 3-phosphoinositide-dependent kinase-1 (PDK1). The dephosphatase PTEN converts the second messenger, PIP3, back to the PIP2 form (Fresno Vara et al., 2004).

1.4 Protein Translation

All cells need the production of proteins for normal cellular functions. Cancer cells have a larger requirement for proteins in order to maintain their high metabolic rate and uncontrolled growth and proliferation. It was first observed that cancer cells have larger and more numerous nucleoli, the location of ribosome assembly in 1976 (Gani, 1976). Scientists then found that translation is hyperactive in most cancer cells and the proteins that control the process are often deregulated (Johnson et al., 1976; Silvera et al., 2010). Due to the greater need for more numerous proteins in cancer cells, efforts

are underway trying to target translational control in cancer. If the protein pool within the cell could be decreased through inhibiting translation, then it is possible that the cell would be unable to grow and proliferate even in the presence of tumorigenic stimuli due to a lack of effector proteins. There are three steps in protein translation: initiation, elongation, and termination. Most of the regulation occurs at the initiation step and many drugs have been synthesized to target specific components of the process. There are two types of translation: cap-dependent, which will be discussed immediately below, and cap-independent, which will follow.

Cap-dependent translation is used for the synthesis of 95-97% of all proteins in eukaryotes (Merrick, 2004). The name derives from the mRNA cap of a guanine nucleotide attached to the mRNA via a 5′ to 5′ triphosphate bond. It requires the eukaryotic initiation factors (eIFs). Translation initiation is controlled through the eIF2, eIF3, and eIF4 families (Figure 6). Step 1 begins with the 80S ribosome dissociating and binding to the ternary complex of a 60S ribosomal subunit, eIF3 and eIF41A, and the 40S small ribosomal subunit. This 43S complex then binds with methionyl tRNA (Met-tRNAi) as Step 2 (Gingras et al., 1999; Silvera et al., 2010). Step 3 is binding to the mRNA 5' end through ATP hydrolysis and to the eIF4F complex containing eIF4E, eIF4G, and eIF4A (Gingras et al., 1999; Merrick, 2004). The RNA then binds the 43S complex as Step 4. eIF4B facilitates eIF4A helicase activity. Step 5 is the release of eIF4 family by a GTPase-activating protein (GAP), eIF5 and eIF2, and scanning to find the AUG start codon as the 48S. In Step 6 all the initiation factors are released and the 60S subunit joins the 40S and starts elongation as the 80S initiation complex (Gingras et al., 1999; Merrick, 2004). At the end of initiation the 80S ribosome

is attached to the start codon (Dever and Green, 2012). The second codon is in the A site on the ribosome and a GTP is needed to attach the tRNA through the eukaryotic elongation factor 1A (eEF1A). Elongation is the process of attaching all the aminoacyl-tRNA, which match with the RNA codons. The process repeats itself until reaching a stop codon.

The codon recognizes its respective aminoacyl-tRNA then the hydrolyses of a GTP moves the codon to the next site, P, in the ribosome leaving eEF1A to release and allowing a peptide-peptide bond to form between the peptide in P, currently the start codon and the A site (Dever and Green, 2012). eEF2 moves the peptides along to the E and P sites and the A position opens with a new codon ready to attach to the tRNA. The process continues until a stop codon UAA, UGA, or UAG. Termination requires the factors eRF1 and eRF3. eRF1 recognizes the stop codon and causes peptidyl-tRNA hydrolysis while eRF3 is a GTPase allowing for separation of the ribosome from the newly synthesized peptide and RNA (Atkinson et al., 2008; Dever and Green, 2012).

Translation is a highly regulated process, which cancer cells often misregulate in order to achieve the high number of proteins they require to sustain high metabolic and proliferative rates. While translation initiation is the most regulated step in translation and most drugs that target translation aim at the initiation factors, it is important to understand the whole process of translation and how cancer is able to exploit it. Ribosomal disorders are linked with an increased risk for developing certain types of cancers (Loreni et al., 2013). Table 3 describes common ribosomal disorders, the altered genes leading to the disease, and common cancers resulting from the mutation. The most common cancers associated with ribosomal disorders include

leukemias and lymphomas. For example, Diamond Blackfan anemia is associated with an increased risk for many different types of cancer including MDS (myelodysplastic syndrome), AML (acute myeloid leukemia), colon adenocarcinoma, osteogenic sarcoma, and genital cancer. This disorder is characterized by mutations in many ribosomal proteins (RPS), which comprise the 40S ribosome (Loreni et al., 2013). When the 40S ribosome is improperly formed, it affects hematopoietic cell lineage resulting in red blood cells that are immature and unable to properly bind iron leading to anemia and an increased risk for leukemias (Boria et al., 2010). Cartilage hair hypoplasia is another ribosome disorder that causes abnormal bone growth resulting in dwarfism. Patients also have brittle and sparse hair, weak nails, and immune deficiency. In comparison to Diamond Blackfan anemia, patients with Cartilage hair hypoplasia have a deficiency in RMRP, which produces a noncoding RNA (Loreni et al., 2013). RMRP is part of an enzyme complex called mitochondrial RNA-processing endoribonuclease, or RNase MRP. RNase MRP is thought to be involved in mitochondrial DNA replication and process ribosomal RNA. These patients often develop Non-Hodgkin's lymphoma and basal cell carcinoma along with gastrointestinal problems including celiac disease (Hermanns et al., 2005).

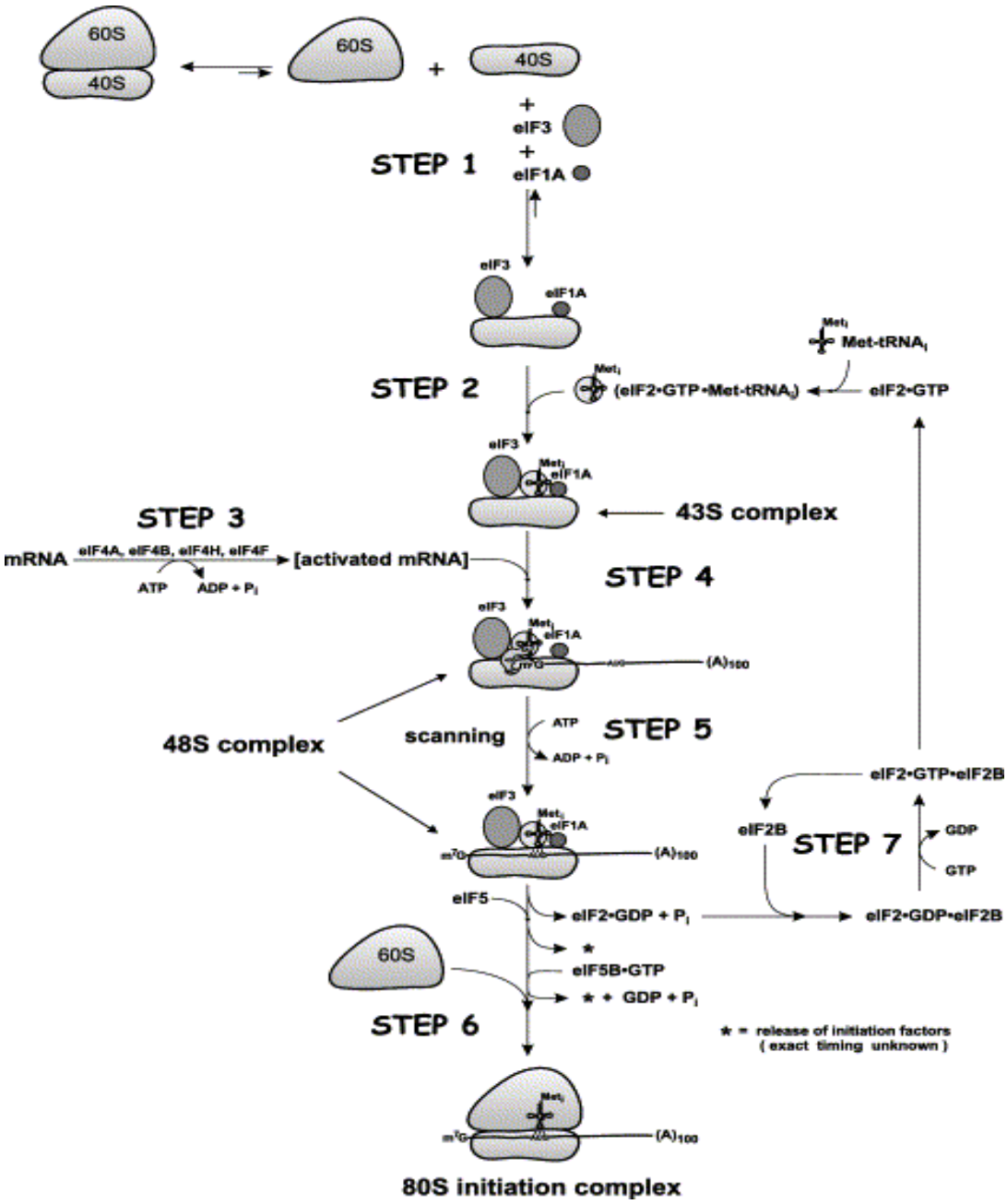
Cap-independent translation (internal ribosome entry site; IRES) does not require the use of the eIF4G mediated RNA binding cap (Silvera et al., 2010). Some mRNAs have IRES sequences where the translation initiation factors can bind without the help of the highly regulated eIF4F complex (Barna et al., 2008). Some of the mRNAs that contain the IRES are involved in tumor progression and metastasis including vascular endothelial growth factor A (VEGFA), fibroblast growth factor 2 (FGF2), BCL-2, X-linked

inhibitor of apoptosis (XIAP), and HIF-1α (Braunstein et al., 2007). IRES-mediated translation can also confer resistance to radiation through upregulation of the antiapoptotic protein XIAP (Holcik et al., 2000). BCL-2 and XIAP are upregulated during chromosomal instability caused by radiation and chemotherapy, and can support cancer development, leading to drug resistance and increased tumorigenesis (Silvera et al., 2010). Translational control is important in regulating cellular proteins that can support a proliferative phenotype.

1.4.1 eIF4

Important to this dissertation is the eIF4 family of initiation factors and their role in regulating translation. As mentioned above they are required for cap-dependent translation. There are six members in the eIF4 family eIF4A, eIF4A1, eIF4B, eIF4E, eIF4G, and eIF4H. eIF4A and eIF4A1 are helicases that are responsible for unwinding the mRNA during translation (Parsyan et al., 2011). eIF4B, a major protein of interest in this dissertation, facilitates eIF4A helicase activity making it much more efficient and promotes the interaction of mRNA-rRNA-Met-tRNAi at the start codon (Gingras et al., 1999). eIF4E has been the subject of extensive investigation (Gingras et al., 1999; Wendel et al., 2004). It is considered an oncogene and is the rate limiting protein and step in translation initiation. eIF4E is often sequestered by 4E-BP1 rendering it inactive and only when 4E-BP1 is phosphorylated by activated mTOR does eIF4E release and allow it to bind to eIF4G and eIF4A to form the eIF4F complex binding it to the mRNA and starting translation initiation (Gingras et al., 1999; Wendel et al., 2004). eIF4G is a scaffolding protein for the eIF4F complex and eIF4H has similar homology to eIF4B but little is known about its function (Gingras et al., 1999).

Figure 6: Cap-dependent translation initiation.



The eIF4 family is required to assemble the translation initiation complex in cap-dependent translation. Translation is comprised of three steps; initiation, elongation, and termination. Cap-dependent translation requires the eIF4 family including eIF4B while cap-independent does not. Figure reproduced with permission from Merrick, 2004.

Table 3: Ribosomal disorders linked to cancer.

Disease	Altered gene	Cancer Association
Diamond Blackfan anemia	RPS 7,10,17, 19,24,26 ,RPL5, 11, 35A	MDS, AML, colon adenocarcinoma, osteogenic sarcoma, genital cancer
X-linked dyskeratosis congenita	DKC1	AML, head and neck tumors
Sq-syndrome	RPS14	AML
Shwachman-Diamond syndrome	SBDS	MDS, AML
Cartilage hair hypoplasia	RMRP	Non-Hodgkin lymphoma, basal cell carcinoma

Many ribosomopathies confer an increased risk for developing cancer. Aberrant translation can lead to abnormal amounts of available protein within the cell leading to increased proliferation and genetic instability. Abbreviations: AML, acute myeloid lymphoma; MDS, myelodysplastic syndrome. Table reproduced with permission from Loreni et al., 2013.

eIF4B is important in the translation initiation process. While it is not the rate-limiting step or considered an oncogene like eIF4E, it is responsible for the efficiency of the eIF4A helicase and is important to translation initiation (Gingras et al., 1999; Shahbazian et al., 2010b). eIF4B is phosphorylated by two kinases on Ser422, P70S6K and P90RSK (90kDa ribosomal s6 kinase, RSK) (Raught et al., 2004). P70S6K is directly activated through mTOR while P90RSK is activated downstream of EGFR and the MAPK cascade (Serra et al., 2013). eIF4B seems to be a point of convergence between EGFR and mTOR further giving evidence to a complex network of signaling cascades (Raught et al., 2004). The extensive crosstalk gives further validation that multiple pathways must be inhibited for cancer to respond to treatment.

1.4.2 Ribosomal S6 Kinases

P70S6K is a serine/threonine kinase that is phosphorylated by mTOR and has two homologs, S6K1 and S6K2 (Shima et al., 1998). It is a major protein in mTOR control of translation through activating required proteins including elongation factors, and poly(A)-binding protein (PABP) (Meyuhas, 2000). It also phosphorylates ribosomal protein S6 (rpS6), tumor suppressor protein PDCD4, eIF4B, and translation elongation factor eEF2 kinase (Korets et al., 2011). The kinase therefore plays a role in both the translation initiation and elongation stages. One of the most extensively studied proteins activated by P70S6K is rpS6. Knockout studies *in vitro* and *in vivo* demonstrate that the protein, a member of the 40S ribosomal subunit, is important in binding the mRNA to tRNA between the large and small ribosome (Nygard and Nilsson, 1990). Also of note, eIF4B is activated by P70S6K and P90RSK in response to a multitude of extracellular stimuli, which promote cell growth and proliferation such as serum, insulin, and phorbol

esters (Duncan and Hershey, 1985). rpS6 and eIF4B are two examples of proteins that demonstrate the extensive crosstalk between signaling pathways within the cell. They are regulated by both the mTOR and MAPK pathways and therefore can detect signals from a wide range of inputs.

P90RSK is a kinase that is directly phosphorylated by MAPK downstream of the Ras-MAPK pathway and therefore EGFR. It has a similar motif to S6K and AKT phosphorylating proteins with a basophilic motif RxRxxS/T (R, arginine; S, serine; T, threonine; and x, any amino acid) (Manning and Cantley, 2007). The similar motif explains the crosstalk mentioned above. P90RSK is also capable of activating rpS6 at Ser235/236 and eIF4B at Ser422 independent of mTOR activity, opening the door for EGFR control of translation through recruitment of the initiation complex (Roux et al., 2007). Another example of P90RSK and mTOR crosstalk is the ability of P90RSK to phosphorylate TSC2 thereby inactivating it and modulating mTOR activity (Roux et al., 2004).

P90RSK has four isoforms in mammals RSK1-4 which are activated by extracellular signal-regulated kinase-1 and -2 (ERK1/2) in response to many extracellular signals including growth factors, hormones, neurotransmitters, and chemokines (Chen et al., 1992). RSK1 and 2 are known to be activated in breast cancer (Clark et al., 2005). RSK contains kinase domains, a linker region, and N- and C-terminal tails (Anjum and Blenis, 2008). The N-terminal kinase is homologous to ACG family kinases including PKA, PKG, and PKC and phosphorylates substrates, while the C-terminal domain is similar to the calcium/calmodulin-dependent protein kinases and is responsible for the auto-phosphorylation ability of RSK (Bjorbaek et al., 1995; Fisher

and Blenis, 1996). P90RSK is primarily activated by EGFR through the Ras-MAPK cascade but it can also be activated by p38 MAPK, the ERK5 MAPK and fibroblast growth factor receptor-3 (FGFR3) (Anjum and Blenis, 2008).

P90RSK is involved in translation as mentioned above through phosphorylation of rpS6 and eIF4B but is also plays a role in transcriptional regulation, cell-cycle regulation, and cell survival (Roux and Blenis, 2004). P90RSK is known to activate transcription factors including CREB, ERα, NF-κB, and transcription initiation factor TIF1A (Frodin and Gammeltoft, 1999; Roux and Blenis, 2004). It also regulates Fos and Jun and through CREB regulation, P90RSK also controls Signal transducer and activator of transcription 3 (STAT3) activation (Anjum and Blenis, 2008; Chen et al., 1993). P90RSK has a hand in cell survival through the phosphorylation and inactivation of Bad, disabling Bad's ability to inhibit the pro-survival protein, BCL-XL (Shimamura et al., 2000). P90RSK also controls cell cycle progression through regulating p27, a cyclin-dependent kinase inhibitor, allowing G1 progression (Anjum and Blenis, 2008). G2-M phase also is regulated through P90RSK and its ability to inhibit Myt1 kinase allowing progression into meiosis as demonstrated in *Xenopus laevis* oocytes (Palmer et al., 1998).

1.5 STAT3

STAT3 is a transcription factor that can be activated by a variety of signaling proteins including membrane receptors, i.e., EGFR, and other kinases, i.e., mTOR (Figure 7). The STAT family has seven members including STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6 (Quesnelle et al., 2007). Janus kinases (JAKs) are intermediary kinases that often activate STATs. Cell surface receptors such as

PDGFR, EGFR, and FLT3 can activate the transcription factors through phosphorylation. They can also be activated through non-receptor protein tyrosine kinases such as c-Src, Bcr-Abl, and mTOR (Buettner et al., 2002). Once activated, STATs dimerize and translocate to the nucleus where they recruit cofactors to then bind the DNA at STAT3 specific binding sites resulting in transcription. The wide range of interacting proteins and different mechanisms of activation demonstrate the broad network to which the transcription factors are connected.

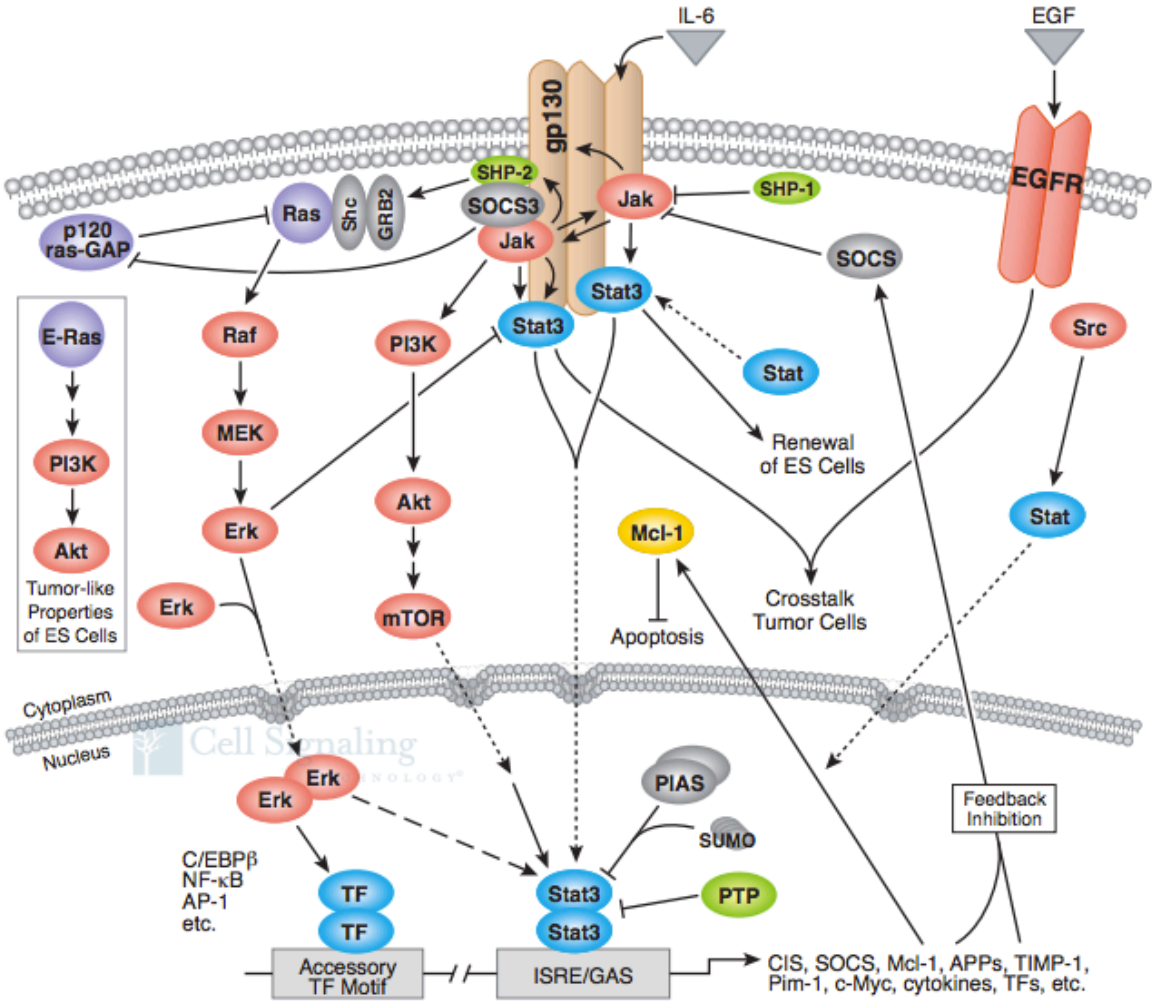
While most is known about STAT5 and STAT3 as they are associated with cancer, the STAT family shares common features (Buettner et al., 2002). Once phosphorylated STATs can either homo- or heterodimerize and translocate to the nucleus where they exert their control on gene expression (Furqan et al., 2013). Each STAT is transcribed by a separate gene but they have six conserved domains: an N-terminal oligomerization domain, a coiled coil, a DNA binding domain, a linker domain, an SH2 domain, and a C-terminal transactivation domain (Furqan et al., 2013). STAT2, 4, & 6 are known to regulate immune response while STAT1, 3, and 5 are involved in cell cycle, survival and angiogenesis (Furqan et al., 2013).

STAT3 is often considered an oncogene due to its ability to drive tumor formation in mice when constitutively activated (Bromberg et al., 1999). STAT3 has been implicated in cancer progression for many years and is the most researched member of the STAT family. Its overexpression has been found in lung, gastric, breast and colorectal cancers and is associated with a poor prognosis (Haura et al., 2005; Kusaba et al., 2005; Sheen-Chen et al., 2008; Yakata et al., 2007). STAT3 is involved in cell cycle regulation through activation of transcription of CyclinD1, CyclinD3, c-Myc,

p21waf1, and p27, angiogenesis through VEGF, and invasion and metastasis through MMP-2 and MMP-9 (Furqan et al., 2013; Quesnelle et al., 2007). STAT3 target genes also include anti-apoptotic genes such as Survivin, Mcl-1, and Bcl-XL (Leeman et al., 2006). There is significant overlap between the genes whose transcription is regulated by STAT3 and STAT5. STAT5 also mediates c-Myc, CyclinD1, CyclinD2, and Mcl-1 transcription (Page et al., 2012). Conversely, STAT1 has not been linked to tumor progression but might be a tumor suppressor and activates transcription of similar proteins involved in cell survival (Chan et al., 2004; Ferbeyre and Moriggl, 2011; Watanabe et al., 2001) (Table 4).

The oncogenic potential of STAT3 is through its control of important cell cycle regulators leading to cell cycle progression, survival, and malignant progression (Bowman et al., 2000). Figure 8 shows how STAT3 plays a role in many of the Hannahan and Weinberg hallmarks of cancer. STAT3 is involved in *1. Inhibiting apoptosis:* regulation of BCL-XL and survivin, *2. Cell cycle activation*: Myc, CyclinD1, and Cdc25A, 3*. Telomere length*: upregulation of telomerase, *4. Metastasis*: MMP-9, *5. Angiogenesis*: VEGF. Many drugs have been developed to control STAT3 signaling. STATTIC (STAT Three Inhibitory Compound), a drug used in this project was the first non-peptide small molecule inhibitor for STAT3. It works by obstructing the dimerization of the transcription factor and therefore prevents its activity. It has been studied in a variety of cancers including breast, hepatocellular carcinoma, pancreatic, colon, glioblastoma, and multiple myeloma (Furqan et al., 2013).

Figure 7: STAT3 signaling pathway.



STAT3 is a transcription factor that can be activated by a variety of signaling cascades including EGFR and mTOR. This figure is reproduced with permission from Cell Signaling.

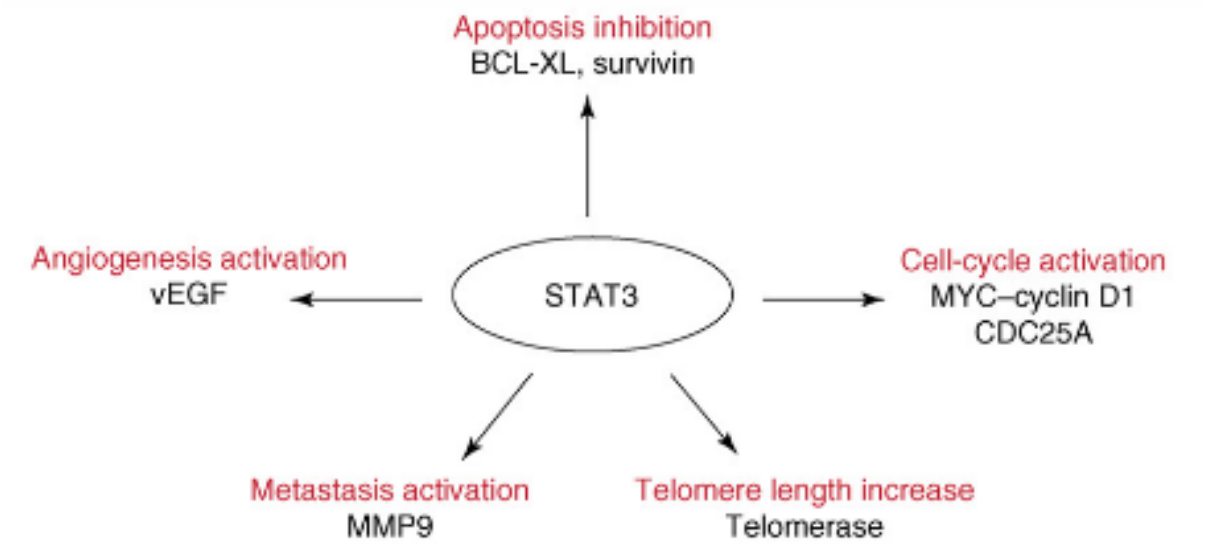
Table 4: STAT activation in tumors.

Solid tumor type	STAT activation
Breast	STAT1, STAT3, STAT5
Head and neck	STAT1, STAT3, STAT6
Lung	STAT3, STAT5
Prostate	STAT3
Colon	STAT3
Glioma	STAT3
Melanoma	STAT3
Ovarian	STAT3
Pancreatic	STAT3
Renal	STAT3
Liver	STAT3

STATs are activated in a variety of solid tumors, most frequently STAT3 and STAT5. Table reproduced with permission from Quesnellle et al., 2007.

STAT3 is phosphorylated on two residues required for full activation, Tyr705 and Ser727. Of particular note, in relation to this dissertation, is that STAT3 can bind to the EGFR and be phosphorylated on Tyr705 through EGFR sites Y1086 and Y1068, and mTOR on Ser727, which increases the transcription activity (Quesnelle et al., 2007). One interesting article noting the crosstalk between EGFR, mTOR, and STAT3 found that when all three proteins are upregulated in gastric cancer, there is a significant correlation with higher tumor stage, lymph node involvement and invasion (Inoki et al., 2005; Inokuchi M et al., 2011). The combination of EGFR and mTOR inhibitors was found to decrease tumorigenesis in GBM cell lines through inhibition of STAT3 phosphorylation (Rajan et al., 2003). The ability of EGFR and mTOR to converge upon STAT3 in different types of cancer suggests that the drug combination of EGFR and mTOR inhibitors should further be explored in combination for their ability to inhibit p-STAT3.

Figure 8: STAT3 is involved in tumorigenesis.



STAT3 is involved in many aspects of tumorigenesis leading to a decrease in apoptosis, and an increase in replicative potential, metastasis, and angiogenesis. Figure is reproduced with permission from Barre et al., 2007.

CHAPTER 2: Exploring Resistance Pathways to EGFR Inhibitors

2.1 Hypothesis and Specific Aims

The overall hypothesis for the project described in this dissertation is that mTOR inhibition can sensitize TNBC cells to EGFR TKIs through the inhibition of eIF4B and STAT3 phosphorylation. To test this hypothesis we addressed three specific aims:

Aim I: To determine the effect of abrogating both EGFR and mTOR signaling on growth and survival in TNBC cell lines. The working hypothesis for this aim is that the combination of mTOR and EGFR inhibitors abrogates cell growth and colony formation in TNBC cells. We tested this hypothesis by measuring the effect of temsirolimus and gefitinib on cell growth, colony formation, and viability using BT20, MDA-MB-231, and MDA-MB-468 TNBC cells. We also determined the mechanism by which viability is decreased by measuring apoptosis, autophagy, and cytostasis.

Aim II: To investigate the role eIF4B plays in the synergistic effect of EGFR and mTOR dual inhibition in TNBC cell lines. The working hypothesis for this aim is that the phosphorylation of eIF4B represents a common mediator of survival in TNBC and that this phosphorylation needs to be abrogated to decrease cell growth. To test this hypothesis we used siRNA and small molecule inhibitors to decrease eIF4B phosphorylation and expression. We also investigated the role of the two kinases responsible for phosphorylating eIF4B, P70S6K and P90RSK. Finally, we determined the significance of cap-dependent versus cap-independent translation in the mediation of cell survival in TNBC cell lines.

Aim III: To identify the role of STAT3 in the treatment of TNBC cell lines with EGFR and mTOR inhibitors. The working hypothesis for this aim is that STAT3 phosphorylation is co-regulated by EGFR and mTOR signaling and that inhibition of STAT3 phosphorylation is required for efficacy of the combination of gefitinib and temsirolimus. To test this hypothesis we abrogated STAT3 activation and phosphorylation using an established small molecule inhibitor, STATTC. We also constitutively activated STAT3 through plasmid transfection (Stat3c) to determine if STAT3 phosphorylation is a common mediator for EGFR and mTOR signaling.

We think the studies proposed here outlined EGFR and mTOR inhibitors as an effective *in vitro* combination that warrants further investigation in the treatment of TNBC. In addition, these studies defined the eIF4B and STAT3 signaling pathways as activated by EGFR and mTOR that need to be abrogated to mediate the synergistic effects of gefitinib and temsirolimus treatment. Clinically, many women who develop TNBC ultimately fail treatment and therefore better drug regiments need to be developed.

CHAPTER 3: Materials and Methods

3.1 Human Breast Cancer Cell Lines and inhibitors

Gefitinib (Iressa) was provided by AstraZeneca (London, UK). Temsirolimus was purchased from LC Labs (Woburn, MA, USA). STATTIC was purchased from Abcam (Cambridge, UK). BI-D1870 and AT7867 were purchased from Selleck Chemicals (Boston, MA, USA). MDA-MB-231, MDA-MB-468, and BT20 cells were purchased from ATCC (Manassas, VA, USA). HEK293T cells were purchased from Invitrogen (Carlsbad, CA, USA). MDA-MB-231, MDA-MB-468, and HEK293T cells are grown in DMEM+10% FBS media (Dulbecco's modified Eagle's medium supplemented with 10% Fetal Bovine Serum). BT20 cells are grown in Eagle's + NEAA media (Eagle's MEM [Minimum Essential Medium] with 2 mM L-glutamine and Earle's Balanced Salt Solution adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 10% FBS). All other reagents were purchased from Thermo Fisher (Houston, TX, USA) or Sigma (St. Louis, MO, USA), unless indicated. A genomic profile of the cell lines with common mutations can be found in Supplemental Table 2.

3.2 Phospho Mass Spectrometry

The Mass Spectrometry methods were generated by Dr. Paul Stemmer and can be found in a recently submitted manuscript with the title "Abrogating phosphorylation of eIF4B is required for EGFR and mTOR inhibitor synergy in triple-negative breast cancer" of which he is a co-author with the author of this dissertation work and in Supplemental Methods. The samples were prepared under the guidance of Dr. Stemmer by J. Madden after which he took control of the samples for further analysis. Methods that this author performed are included.

BT20 cells were treated with 0.5 μM gefitinib or a DMSO vehicle control for 24 hours. Cells were washed with ice-cold HANK's solution then proteins precipitated with 100% EtOH before cell proteins were scraped from plates and transferred to microcentrifuge tubes. Samples were then taken to the Proteomics Core and further analysis was performed by Dr. Paul Stemmer. Experiment was done two times with the first experiment containing 14 samples per treatment and the second containing 4 samples.

3.3. Ingenuity® Pathways Analysis

The methods were performed by Dr. Aliccia Bollig-Fischer for a recently submitted manuscript with the title "Abrogating phosphorylation of eIF4B is required for EGFR and mTOR inhibitor synergy in triple-negative breast cancer" of which she is a co-author with the author of this dissertation work. As the author of this work did not do the bioinformatics work, detailed methods have been excluded.

3.4 Cell Viability Assays

Cells were plated in triplicate at 2,000 cells /well of a 96-well plate on Day 0. Cells were treated with 0.001 μM, 0.01 μM, 0.1 μM, 1 μM, 10 μM, 20 μM, and 50 μM gefitinib and/or temsirolimus, BI-D1870 and/or AT7867, and STATTIC on Day 1. The MTS reagent was added per manufacturer's directions after 72 hours (Day 5) (Promega, Madison, WI, USA) and was read using a Dynex spectrophotometer. GraphPad Prism was used to generate GI_{50} curves at inhibitory growth curves with top=1 and bottom=0. GI_{50} values were generated by the program from the data of at least three experiments performed in triplicate.

3.5 Cell Growth Analysis

Cells were plated in triplicate at 30,000/ well of a 6 well plate on Day 0. Treatment with gefitinib (1 μM), temsirolimus (1 μM), BI-D1870 (10 μM), AT7867 (10 μM), alone or in the combinations specified in the text began on Day 1 and continued every other day for 8 days. Day 1 untreated cells were counted using a hemocytometer. On Days 4 and 8 the respective plates were counted again using a hemocytometer. Experiments were repeated at least three times. Graphs were prepared and statistical analysis was performed in GraphPad Prism (La Jolla, CA, USA) using an ANOVA.

3.6 Clonogenic Survival Assays

BT20 cells (40,000 cells/ 35-mm dish) or MDA-MB-231 and MDA-MB-468 cells (30,000 cells/ 35-mm dish) were plated on Day 0. Cells were treated with gefitinib ([1 μM BT20] [10 μM MDA-MB-231 and MDA-MB-468]) and/or temsirolimus ([1 μM BT20] [10 μM MDA-MB-231 and MDA-MB-468]) as specified in the text. Treatment began on Day 1 and continued every other day for 10 days. On day 10, cells were trypsinized and replated at 5,000 cells/ 35-mm dish (BT20) or 3,000 cells/ 35-mm dish (MDA-MB-231 and MDA-MB-468) without treatment for 7 days. Different plating densities allowed for the longer doubling time in BT20s compared to MDA-MB-231 and MDA-MB-468, which replicate more quickly. Colonies were stained with crystal violet for 20 minutes, washed with water to remove excess dye, and counted using the Gelcount colony counter the following day (Oxford Optromix; Abingdon, United Kingdom). Counts were normalized to the untreated control for each experiment. Experiments were done in triplicate and

repeated at least three times. Graphs were prepared and statistical analysis was performed in GraphPad Prism using an ANOVA.

3.7 Immunoblotting

Cells were lysed in CHAPS lysis buffer (10 mM CHAPS, 50 mM Tris [pH 8.0], 150 mM NaCl, and 2 mM EDTA with 10 μM Na_3VO_4 and 1x protease inhibitor cocktail [EMD Biosciences, Rockland, MA]) at 4°C. Proteins were separated using sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) 7.5% or 12% gel and transferred onto polyvinylidene fluoride (PVDF) membranes for 1 hour at 23V. Blots were blocked in 5% milk or 5% BSA according to manufacturer's instructions and then were placed in primary antibody overnight at 4°C shaking, washed with TBS-T for 10 minutes x3, and incubated with secondary antibody for 1 hour at room temperature. After secondary antibody incubation, blots were washed with TBS-T for 10 minutes x3 and developed using Amersham ECL Prime Western Blotting Detection Reagent solution (GE Healthcare, Amersham, United Kingdom). Relevant antibodies can be found in Table 4. Antibodies used in this study were purchased from Cell Signaling Technologies (Beverly, MA, USA), BioSouce (Grand Island, NY, USA), or Millipore (Billerica, MA, USA).

3.8 siRNA silencing

siRNA constructs were purchased from Thermo Scientific Dharmacon®. Constructs to eIF4B are non-overlapping and described as #7 (catalog number J-020179-07 sequence 5' AAACCUACCCUAUGAUGUU 3') and #8 (catalog number J-020179-08 sequence 5' GCAGUGCGUUUACCACGUG 3') in the text. Non-silencing off-target siRNA (non-silen) was used as a control (catalog number D-0018810-01-05).

Plates were treated with 0.7 µg/ well siRNA (96 well) or 2.5 µg siRNA / well (6 well) siRNA using the Lipofectamine LTX transfection system. siRNA was combined with Lipofectamine LTX and PLUS in Opti-MEM supplemented with antibiotic free growth media to a final volume (96 well LTX 0.3 µL, PLUS 0.01 µL, 6 well LTX 2 µL, PLUS 0.5 µL).

3.9 Bicistronic luciferase Assay

A dual luciferase plasmid was purchased from Addgene (Cambridge, MA, USA). HEK293T cells were transfected with 11510:pFR_HCV_xb (Supplemental Figure 2). After 24 hours, media were removed and replaced with media containing gefitinib and/or temsirolimus at 1 µM. Cells were then harvested after an additional 24 hours and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) and read using the BioTek Synergy 2 machine (Winooski, VT, USA) according to the manufacturer's instructions. Relative luciferase units were plotted in GraphPad Prism as normalized firefly (cap-dependent translation) over normalized renilla (cap-independent translation).

3.10 Stat3c plasmid

The EF.STAT3C.Ubc.GFP plasmid (Stat3c) to express constitutively activated STAT3 was purchased from Addgene, plasmid number 24983 (Hillion et al., 2008). Cells were treated with 1 µg/well plasmid DNA (96 well) or 1.5 µg DNA/well (6 well) using the Lipofectamine LTX transfection system. Stat3c was combined with Lipofectamine LTX and PLUS in Opti-MEM supplemented with antibiotic free growth media to final volume (96 well LTX 0.3 µL, PLUS 0.08 µL, 6 well LTX 3 µL, PLUS 1 µL) and harvested or read through MTT cell viability assay after 72 hours.

3.11 STAT3 DNA Binding ELISA

The STAT3 Transcription Factor Assay Kit (catalog number 45696) was purchased from Active Motif (Carlsbad, CA, USA). Protocol was followed according to manufacturer's instructions. Briefly, the kit detects and quantifies the transcription factor activity when active STAT3 in the sample binds to a STAT3 consensus sequence that is bound to the kit plate (5' TTCCCGGAA 3'). A STAT3 primary antibody detects the STAT3 bound to the plate containing the STAT3 consensus site and a secondary HRP-conjugated antibody provides a colormetric readout that is then quantified using spectrophotometry. 10 µg of cell lysate was used for each assay. Experiments were repeated at least two times. Treatments were 1 µM of the indicated drug for 24 hours.

Table 5: Relevant antibody information.

Target	Company	Catalog number	Dilution used
AKT	Cell Signaling	9272	1:1000
Caspase 3	Cell Signaling	9665	1:1000
Caspase 7	Cell Signaling	9494	1:1000
Caspase 9	Cell Signaling	9508	1:1000
Cleaved Caspase 3	Cell Signaling	9501	1:1000
Cleaved Caspase 7	Cell Signaling	9491	1:1000
Cleaved Caspase 9	Cell Signaling	9664	1:1000
EGFR	Cell Signaling	2232	1:1000
eIF4A	Cell Signaling	2013	1:1000
eIF4A1	Cell Signaling	2490	1:1000
eIF4B	Cell Signaling	3592	1:1000
eIF4E	Cell Signaling	2067	1:1000
eIF4G	Cell Signaling	2469	1:1000
eIF4H	Cell Signaling	2444	1:1000
Anti-mouse IgG HRP linked	Cell Signaling	7076	1:1000
Anti-rabbit IgG HRP linked	Cell Signaling	7074	1:2000
LC3*	Cell Signaling	4599	1:1000
MAPK	Cell Signaling	9102	1:1000
P21Waf1*	Cell Signaling	2946	1:2000
P38	Cell Signaling	9212	1:1000
P53	Millipore	OP09	1:1000
P70S6K	Cell Signaling	2708	1:2000
phospho-AKT (Ser473)	Cell Signaling	4060	1:2500
phospho-EGFR (Thr669)	Cell Signaling	3056	1:1000
phospho-EGFR (Tyr1045)	Cell Signaling	2237	1:500
phospho-EGFR (Tyr1068)	Cell Signaling	2234	1:5000
phospho-EGFR (Tyr1086)	Cell Signaling	2220	1:500
phospho-EGFR (Tyr1173)	Cell Signaling	4407	1:500
phospho-EGFR (Tyr845)	Cell Signaling	2231	1:750
phospho-EGFR (Tyr992)	Cell Signaling	2235	1:500
phospho-eIF4E (Ser209)	Cell Signaling	9741	1:1000
phospho-eIF4G (Ser1108)	Cell Signaling	2441	1:2000
phospho-MAPK (Thr20/Try204)	BioSource	44-680g	1:2000
phospho-P38 (Thr180/Tyr182)	Cell Signaling	9211	1:500
phospho-P70S6K (Thr389)	Cell Signaling	9205	1:1000
phospho-P90RSK (Ser380)	Cell Signaling	9335	1:500
phospho-PKCpan	Cell Signaling	9371	1:1000
phospho-STAT3 (Y705)*	Cell Signaling	9138	1:500
phospho-STAT3 (S727)	Cell Signaling	9134	1:1000
RSK1/2/3	Cell Signaling	9355	1:500
β-actin*	Sigma	A5441	1:10000

Antibodies used in this work were purchased from Cell Signaling, BioSource, or Millipore. Catalog numbers and concentrations used are provided. All antibodies are used with secondary rabbit HRP-linked IgG unless noted with * indicating use of mouse HRP-linked IgG.

CHAPTER 4: Identification of proteins remaining phosphorylated after gefitinib treatment in TNBC

4.1 Introduction

As previously mentioned 50% of TNBC patient tumors express high levels of EGFR but are resistant to inhibitors (Liu et al., 2011). EGFR inhibitors are approved in the clinic for colon and NSCLC but have proven ineffective for breast cancer due to high levels of developed and *de novo* resistance. Therefore, they are not used as a single agent therapy but are given in combination with cytotoxics (Chong and Janne, 2013). An emerging technology is the use of proteomics to better understand complex signaling networks within the cell. The therapeutic potential of phospho-proteomics is rapidly advancing (Lopez et al., 2012). Since many signaling proteins are considered active based on the presence or absence of a phosphate(s) group (PO_4^{3-}), phospho-proteomics is an excellent tool to study protein activation on a massive scale. Interpreting the data through bioinformatics can then allow for identification of important signaling pathways specific for the samples. Other phospho techniques, such as antibody detection of protein levels through immunoblotting are limited in their ability to identify only one protein at a time, but phospho-mass spectrometry is able to detect and measure the phosphorylation of tens of thousands of proteins simultaneously. Mass spectrometry based assays can assist in drug development through (i) clarification of the mechanism of drug action, (ii) identification of proteins related to a signaling network, (iii) discovering novel drug targets for diseases (Lopez et al., 2012; Lopez et al., 2011). While phospho-proteomics can be a good tool to identify drug targets, it also

has promise to be used on a patient level to diagnose and better understand the individual disease and help predict treatment response (Lopez et al., 2012).

Phospho-mass spectrometry is able to capture and identify proteins based on the presence of the phosphate group. Once proteins have been precipitated in ethanol, they are digested, run through a titanium dioxide (TiO_2) column to select for peptides containing a phosphate. TiO_2 was used as the selecting agent in this work as it is able to select proteins phosphorylated at serine, tyrosine, and threonine sites, therefore, giving a complete profile of the phospho-proteome (Chen and Chen, 2005). The phosphorylated proteins are then identified through mass-spectrometry.

4.2 Results

4.2.1 Summary of proteins remaining phosphorylated in the presence of gefitinib

In order to identify potential mechanisms of resistance to EGFR inhibitors in TNBC, we utilized a phospho-proteomics approach. We treated the TNBC cell line, BT20 with gefitinib, an EGFR TKI and performed Phospho Mass Spectrometry with the help of the Proteomics Core at Wayne State and Dr. Paul Stemmer. BT20s were chosen for their TNBC status, high levels of EGFR, and intrinsic resistance to EGFR TKIs. Briefly, after 24 hours treatment with gefitinib or a vehicle treated DMSO control, proteins were harvested in ethanol. The samples were then sent to the Proteomics Core where they were lysed in deoxycholate and digested in trypsin. The phospho-peptides were enriched at all three phosphosites, tyrosine, serine, and threonine using TiO_2. The Proteomics Core was able to identify 279 proteins whose phosphorylation statues did not significantly change in the presence of gefitinib. All identified proteins can be found in Supplemental Table 1.

4.2.2 mTOR signaling remains activated in the presence of EGFR inhibitors in TNBC

With the help of the Bioinformatics Core and Dr. Aliccia Bolig-Fischer we utilized Ingenuity® Pathway Analysis to interpret the phospho-proteomic data. Bioinformatics are a useful tool to identify commonalities within a data set and have been used in many studies to discover relevant signaling pathways from a library of proteins. The analysis found many pathways containing multiple proteins that remained activated after gefitinib treatment. The top scored pathways are found in Table 6. Molecular transport, RNA trafficking, and protein synthesis had the highest number of associated proteins. This analysis also led us to discover many of the phosphorylated proteins from the proteomic data are involved in the mTOR pathway, particularly translation initiation (Table 7).

4.3 Conclusions

mTOR is often activated in many types of cancer and has been studied in relation to resistance to EGFR inhibitors (Buck et al., 2006; Wheeler et al., 2008). EGFR and mTOR pathways have been implicated in cancer progression and linked as having extensive crosstalk for many years (La Monica et al., 2009; Rini et al., 2007). Therefore, the bioinformatics data led us to further explore the role of mTOR as a mechanism of resistance in TNBC due to the high number of mTOR related proteins that remained activated after EGFR inhibition.

Of note many proteins remained phosphorylated after gefitinib treatment in the Mass Spec data as seen in the Supplemental Table 1. Many of these proteins fit into a variety of pathways that we could have explored as mechanisms of resistance for this project. mTOR is only one of what can be many other pathways that contribute to EGFR

inhibitor resistance in TNBC. Many of the pathways related to mTOR had the top hits in the proteomics data. eIF2, eIF4, and P70S6K had the most number of proteins involved that fit into the pathway. However, other pathways were also enriched in high numbers including Integrin signaling, RhoA, and CHK mediated cell cycle control. Phospho-proteomics are a good resource to identify pathways that can contribute to inhibitor resistance and generate hypothesis driven research. It is a tool that can lead to interesting pathways and proteins that otherwise would have remained undiscovered.

Table 6: Ingenuity® Pathway Analysis Top Functional Scores.

Score	Focus Molecules	Top Functions
54	27	Molecular Transport, RNA Trafficking, Protein Synthesis
44	23	Cardiovascular System Development and Function, Organismal Development, Cellular Assembly and Organization
41	22	Cell Cycle, DNA Replication, Recombination, and Repair, Gene Expression
39	21	Cellular Compromise, Cellular Function and Maintenance, Protein Synthesis
23	15	Infection Mechanism, Reproductive System Disease, Cellular Assembly and Organization
23	14	Cellular Assembly and Organization, Cellular Function and Maintenance, Nervous System Development and Function
17	11	Cellular Development, Hematological System Development and Function, Connective Tissue Development and Function
15	10	Cellular Compromise, Cellular Growth and Proliferation, Cellular Assembly and Organization

Ingenuity based pathway analysis sorted proteins based on the phospho-proteomic data and scored the most commonly activated pathways. Analysis is based on proteomic data from BT20 cells treated with 0.5 μM gefitinib compared to a DMSO vehicle control.

Table 7: Proteins involved in translational control that remained phosphorylated after gefitinib treatment involved in mTOR pathway.

Proteins involved in translational control that remained phosphorylated after gefitinib treatment involved in mTOR pathway	
Protein	**Function in Translation**
eIF1B	Enhance rate and accuracy of Translation
eIF3B	Step 1 of Translation initiation 40S subunit binding
eIF3D	Step 1 of Translation initiation 40S subunit binding
eIF3G	Step 1 of Translation initiation 40S subunit binding
eIF3J	Step 1 of Translation initiation 40S subunit binding
eIF3C	Step 1 of Translation initiation 40S subunit binding
eIF2A	Step 2 Bind initiator tRNA and 40S subunit
eIF4G1	Step 3 of Translation initiation activation and binding of mRNA to 40S
Raptor	Major subunit of mTORC1
Rictor	Major subunit of mTORC2
GSK3A	Glycogen synthase kinase
4EBP1	Directly binds and activates eIF4E- rate limiting step in Translation initiation
FKHR (FOXO3)	Forkhead Transcription factor- glucose homeostasis, cell-cycle progression, and apoptosis

Cells were harvested in ice cold ethanol, lysed in deoxycholate, trypsin digested, and phospho-peptides were enriched at tyrosine, serine, and threonine sites using Ti_2O. Phospho-mass spectrometry on BT20 cells showed 279 proteins remained activated in the presence of 0.5 μM gefitinib. Ingenuity® based pathway analysis found many components of the mTOR pathway and translation initiation factors to be of interest. A complete list of phosphorylated proteins can be found in Supplemental Table 1.

CHAPTER 5: EGFR and mTOR Inhibitor Synergy in TNBC

5.1 Introduction

TNBC cells with high levels of EGFR have an intrinsic resistance to EGFR TKIs. Phospho-proteomic data indicated that the mTOR pathway may be responsible for EGFR inhibitor resistance and suggest that inhibition of mTOR may circumvent this resistance and sensitize cells to EGFR treatment. Rapamycin (sirolimus) was first discovered in the soil of Easter Island as a macrolide antibiotic produced from *Streptomyces hygroscopicus* and was used as an immune suppressant after patients received organ transplants (Albert et al., 2010; Vezina et al., 1975). Analogs of the drug have been developed, coined rapalogs, also inhibit mTOR.

5.2 Results

5.2.1 Inhibiting mTOR activity sensitizes TNBC cells to EGFR Inhibitors

In our studies we have chosen to use the mTOR inhibitor temsirolimus, which is used in the clinic to treat renal cell carcinoma (Albert et al., 2010). MTT assays were used to determine the GI_{50}s of temsirolimus for the TNBC cell lines. The cell lines used in this project were chosen due to their triple negative status, the fact that they contain high levels of EGFR, and have an increased resistance to EGFR inhibitors (μM GI_{50} values, Table 8). As seen in Table 8, the temsirolimus GI_{50} values for the BT20, MDA-MB-231, and MDA-MB-468 TNBC cell lines were all >9.5 μM, a value considered to indicate resistance to mTOR inhibition.

We further used growth assays to determine the effect gefitinib and temsirolimus had individually and in combination on TNBC cells. Cells were plated on Day 0, treated on Day 1, and every other day until Day 8, with gefitinib, temsirolimus, or the

combination at the indicated doses (Figure 9A). On Days 1, 4, and 8, cells were counted using a hemocytometer from triplicate wells and plotted using GraphPad Prism. In support of the MTT GI_{50} data in Table 8, gefitinib (GEF) and temsirolimus (TEM) did not have a significant effect on cell growth in any of the cell lines (Figure 9A, red and green lines). However, the 1:1 combination of gefitinib and temsirolimus at 1 μM had a significant decrease ($p<0.01$ for MDA-MB-231, and $p<0.001$ for BT20 and MDA-MB-468) in cell growth over an 8 day period that was not seen with single agent treatment at the same concentration in all cell lines (Figure 9A). There was also a significant decrease in growth comparing GEF (red line) and GEF+TEM combination (blue line) in all cell lines ($p<0.05$). EGFR and mTOR dual inhibition has a significant effect at decreasing TNBC cell growth that is not observed with individual treatment. Both gefitinib and temsirolimus are approved for the treatment of cancers. Using already approved drugs in a new setting allows for easier study and transition into patients, as the toxicity profile is known. FDA approval for drugs already in the clinic is much easier if they show efficacy in a new disease. Therefore, it is a significant finding that our studies found a synergistic effect at decreasing TNBC cell growth using approved drugs.

Clonogenic survival assays were then utilized to analyze the ability of EGFR and mTOR inhibitors to decrease cell survival over an extended period of time. The assay is a way to look at resistance as the cells are grown with drug treatment for a 10 days, replated and grown with normal growth media for another week to see if they were able to recover from the drug treatment. An effective cancer treatment needs to efficiently and permanently inhibit cell growth while circumventing resistance, a major problem for

TNBC patients in the clinic. While single agent treatment with gefitinib and temsirolimus had minimal effects at decreasing colony formation, the combination showed a significant decrease (Figure 9B p*<0.05, **0.01, ***0.001, ****0.0001). One cell line, BT20, had a significant decrease in colony formation when treated with single agent temsirolimus treatment. Our results suggest that mTOR inhibition has a greater effect at decreasing cell growth and colony formation than single agent EGFR inhibition but not to the extent of the combination. Our data suggest that mTOR inhibition can sensitize TNBC cells that are resistant to EGFR inhibitors and decrease cell growth and colony formation.

5.2.2 EGFR and mTOR inhibitors are synergistic in TNBC

Other studies have found that EGFR and mTOR inhibitors in combination are effective experimentally in NSCLC, pancreatic, colon and breast cancers (Buck et al., 2006; Chacon and Costanzo, 2010). Our results demonstrate a significant decrease in growth and colony formation with the combination treatment of gefitinib and temsirolimus (Figure 9). As mentioned previously, MTT data on three TNBC cell lines shows that they are resistant to single agent treatment of gefitinib and temsirolimus (Table 8). The growth and colony assays suggest a combinatorial effect. Figure 10A shows GI_{50} growth curves for all cell lines. The combination of gefitinib and temsirolimus (GEF+TEM) shifts the curves to the left, indicating a lower GI_{50} value than with the individual treatments alone. The raw MTT data were then used to evaluate the synergy of the two drug combination using the Chou-Talalay method (Chou, 2006).

Table 8: Gefitinib and temsirolimus GI_{50} and CI values in TNBC.

Cell Line	GI_{50} GEF	GI_{50} TEM	GI_{50} GEF+TEM	CI value
BT20	5.3 μM	9.79 μM	0.94 μM	0.21
MDA-MB-231	>100 μM	>100 μM	4.1 μM	0.28
MDA-MB-468	6.8 μM	16.4 μM	3.7 μM	0.56

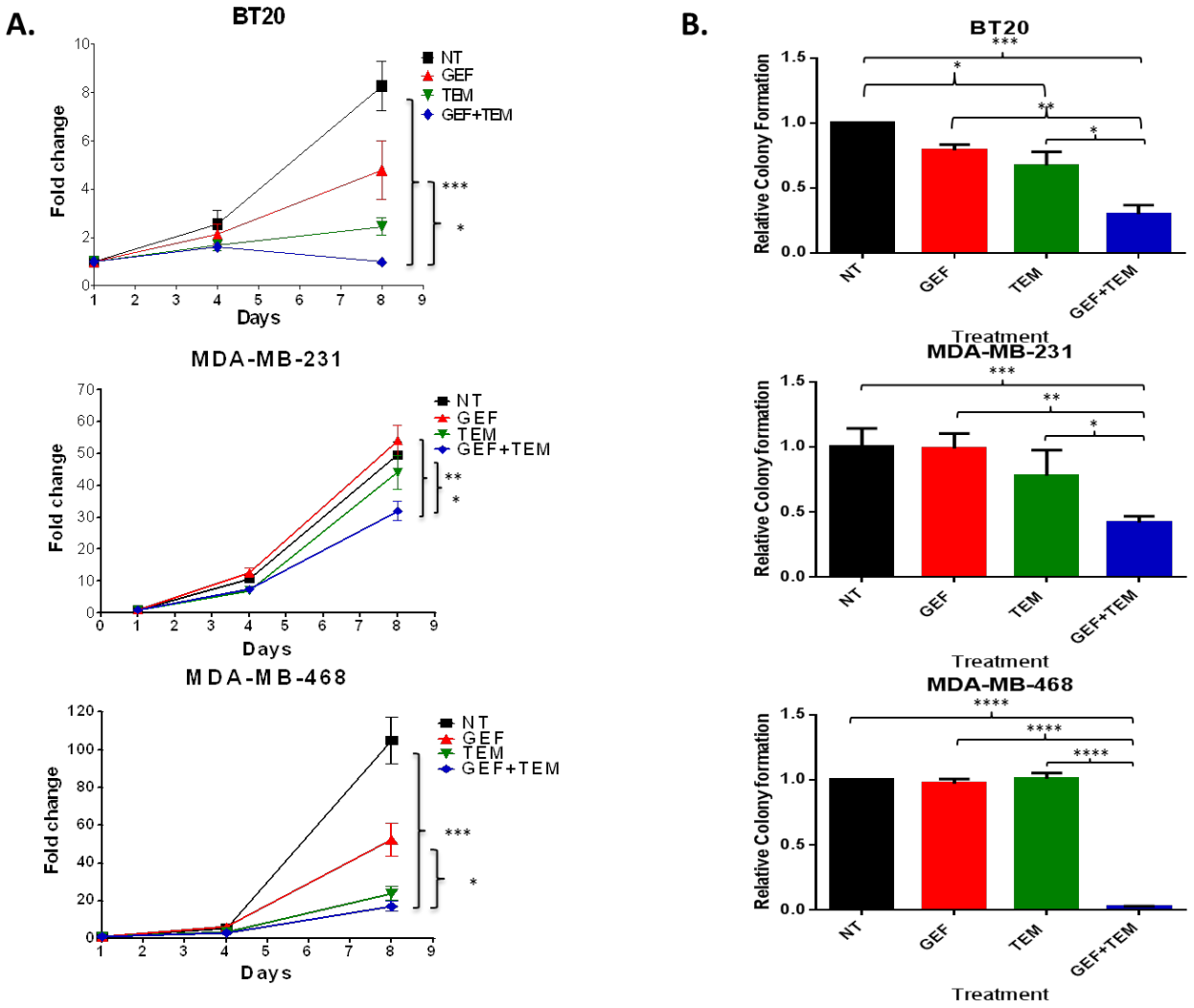
The GI_{50} values for each TNBC cell line were considered resistant for individual drug treatments. The combinations had lower GI_{50} values for all cell lines and the CI values were calculated using CalcuSyn. All CI values <1.0 indicate a synergistic effect with the combination of gefitinib and temsirolimus in BT20, MDA-MB-231, and MDA-MB-468 cell lines.

Using CalcuSyn software we calculated the GI_{50} value for each cell line and drug from MTT assays done with increasing concentrations of gefitinib and temsirolimus at constant ratios. The GI_{50} for each individual drug alone is plotted on a graph, gefitinib on the Y-axis and temsirolimus on the X-axis (Figure 10B). By drawing a line between the GI_{50} values and plotting the calculated drug concentration in the presence of the other drug on the same graph (Figure 10B, triangles) for a given drug concentration, we can observe synergy. Any points falling under the line indicate the drug combination is synergistic. In all three cell lines, all points fall below the line indicating synergy. The calculation of CI (combinatorial index) values was done to further confirm the synergy of gefitinib and temsirolimus in TNBC cell lines (Table 8). CI values less than 1.0 are another indicator of synergy and all cell lines had calculated CI values of less than 1.0. Together, our data confirm what others have seen and suggest that EGFR and mTOR inhibition results in a significant and synergistic decrease in cell growth and colony formation in TNBC cell lines.

5.2.3 Cell death mechanism

The synergy observed with the gefitinib and temsirolimus combination along with the decreases in growth and colony formation suggest changes in TNBC cell line survival, viability, and proliferation. In order to further understand how the treatment combination has led to the observed effects we explored cell death mechanisms to understand these decreases and synergy. Many cancer therapeutics decrease cancer cell growth by initiating apoptosis or cell suicide when the genetic material becomes damaged enough to trigger death. When apoptosis is initiated, pro-caspases are cleaved to their activated state and start the cascade leading to cell death.

Figure 9: Gefitinib and temsirolimus combination decreases TNBC cell growth and colony formation.



A. Cell growth assays were done on BT20, MDA-MB-231, and MDA-MB-468 cells over 8 days in triplicate. Cells were treated every other day with 1 μM and counted on days 1,4, and 8 with a hemacytometer. ANOVAs were performed on GraphPad Prism software with a significant decrease with the combination of gefitinib and temsirolimus (GEF+TEM 1 μM) in all three cell lines. **B.** Colony formation assays were plated in a 6 well plate in triplicate. Treatments were done every other day at 1 μM for two weeks in BT20 cells and 10 μM in MDA-MB-231 and MDA-MB-468. Cells were trypsinized, replated at a low density, and allowed to grow for another week in normal growth media. Colonies were counted on a Cell Counter. ANOVAs were done in GraphPad Prism software and found a significant decrease in colony formation in the gefitinib and temsirolimus (GEF+TEM) combination (blue bars). p*<0.05 **<0.01 ***0.001 ****0.0001

Cleaved caspases are therefore a good marker for induction of apoptosis, along with cleavage of another late stage apoptosis associated protein, PARP. We used immunoblotting to look at three cleaved caspases 3, 7, and 9 and cleaved PARP as indicators of apoptosis (Figure 11). There was no increase in apoptotic markers observed in the MDA-MB-231 or MDA-MB-468 cells after 72 hour of treatment. Treatment time courses from 0 to 72 hour were performed to ensure markers of apoptosis were not missed in these studies.

mTOR is known to play a role in autophagy, another mechanism of cell death (Laplante and Sabatini, 2009). mTOR controls autophagy in times of cell stress and can initiate the recycling of cellular components when nutrients are scarce or in response to certain chemotherapy drugs (Albert et al., 2010). A marker for autophagy is the observance of increased compartments within the cell called autophagosomes that can be observed under the microscope and the increased levels of converted LC3. After treatment with the inhibitors, there appeared to be no increase in autophagosome formation across the treatments (observed data not shown). Further immunoblotting shows no increase in LC3 conversion suggesting that autophagy plays a minimal role in the observed synergy (Figure 11).

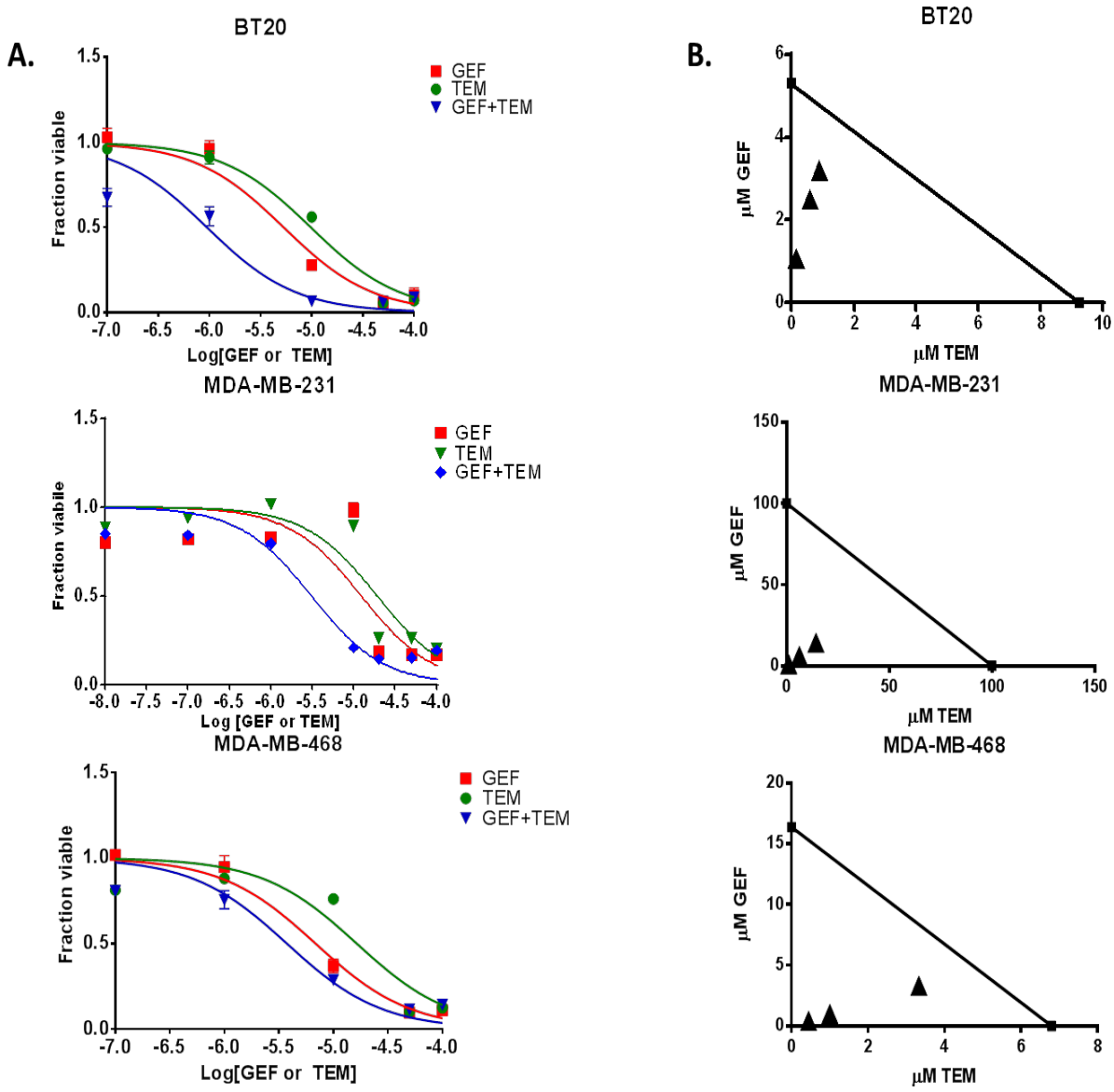
The data suggest only a decrease in cell growth and colony formation, not a complete inhibition (Figure 9). The growth curves slow over time (8 day time point) suggesting a potential role for a cytostatic effect of gefitinib and temsirolimus combination. Cytostasis is the process by which cells are removed from the cell cycle therefore they are unable to proliferate. Cellular markers of cytostasis include the accumulation in p21 and p53, two proteins that, when increased, stop progression of the

cell cycle. Interestingly, there was an increase in both proteins with the combination treatment. p53 levels increase after 24 hours while p21 levels take 48 hours to increase, as shown through immunoblotting (Figure 11). Our results are in agreement with other studies that have found the combination of EGFR and mTOR inhibitors is cytostatic (Boffa et al., 2004; La Monica et al., 2009).

5.3 Conclusions

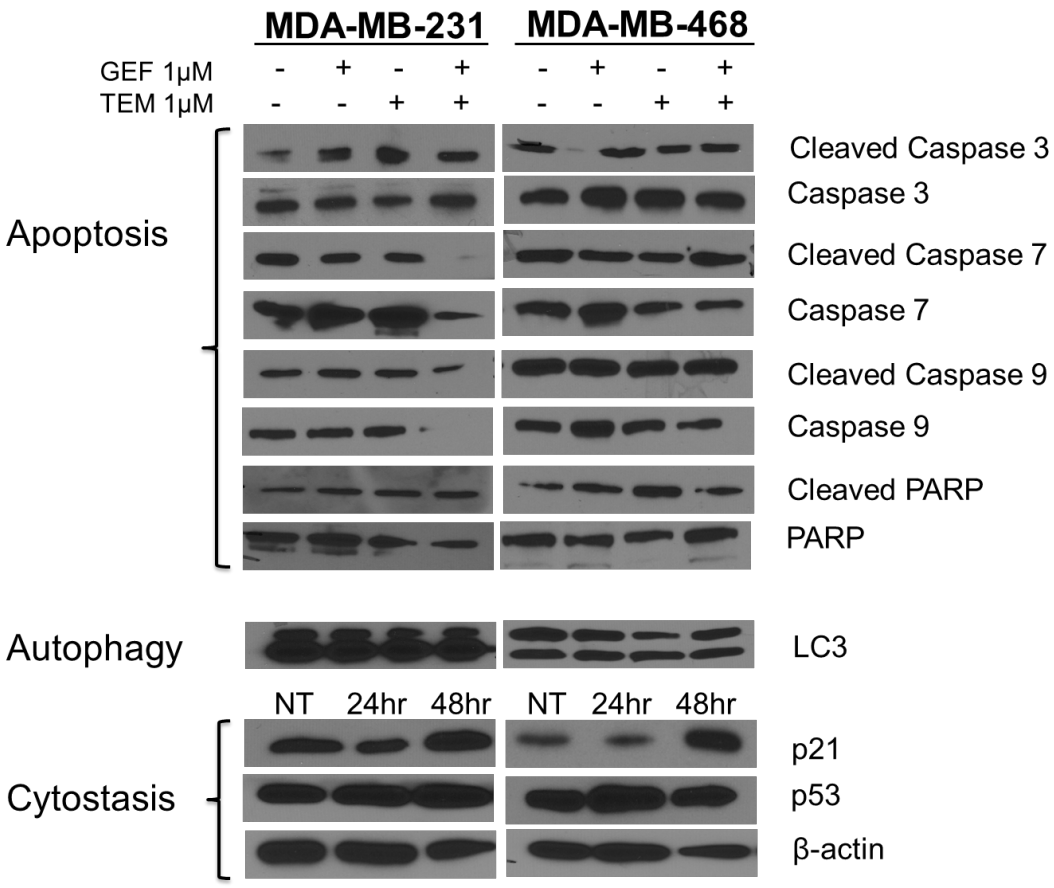
The phospho-proteomic data suggest a role for the mTOR pathway in EGFR inhibitor resistance in TNBC cell lines. The data also propose that TNBC cell lines are resistant to single agent mTOR inhibition (Albert et al., 2010; Buck et al., 2006). Further, MTT viability found that the combination of EGFR and mTOR inhibitors was synergistic and significantly decreased cell growth and colony formation compared to single agent treatment. The lack of activation of caspases and PARP cleavage suggests a minimal role of apoptosis and a lack of LC3 conversion supports a minimal role for autophagy as being a mechanism of the observed decrease in cell growth and colony formation. However, the data suggest the cells are undergoing cytostasis and stalling their growth instead of triggering cell death. Further, the addition of an mTOR inhibitor can sensitize TNBC cell lines to EGFR inhibitor treatment and needs to be further explored in EGFR overexpressing TNBC as a treatment option. As the effect appears to be cytostatic, the addition of a cytostatic drug along with EGFR and mTOR inhibition also needs to be explored as a treatment option. While these results confirm what others have seen with the combination of an EGFR and mTOR inhibitor in cancer cell lines, the mechanism of action is still unknown, which we wanted to further explore.

Figure 10: Gefitinib and temsirolimus synergize in TNBC cells.



A. The GI_{50} curve for BT20 cells was plotted in the presence of gefitinib (GEF), temsirolimus (TEM) or the combination (GEF+TEM). The combination treatment decreases the GI_{50} for the three cell lines (blue lines compared to red or green). Calculations done in CalcuSyn **B.** Isobolograms were made by plotting the GI_{50} of GEF on the Y axis and TEM on the X axis. Points falling below the drawn line indicate a synergistic effect of the drug combination at a constant ratio.

Figure 11: Cell death and senescence in gefitinib and temsirolimus treated TNBC.



After 72 hour 1 μM treatment of gefitinib (GEF) and/or temsirolimus (TEM) in MDA-MB-231 and MDA-MB-468 cells, there is no increase in cleavage of common apoptosis proteins including Caspases 3, 7, 9, and PARP. Autophagy associated protein LC3 also has minimal fluctuation after 72 hours indicating apoptosis and autophagy do not play a major role in the reduction of TNBC viability, cell growth, and colony formation. Increased p21 and p53 are markers for cytostasis. MDA-MB-231 and MDA-MB-468 cells have increased p21 and p53 after 1 μM treatment with GEF+TEM after 24 and 48 hours.

CHAPTER 6: Signaling in the presence of EGFR and mTOR inhibitors

6.1 Introduction

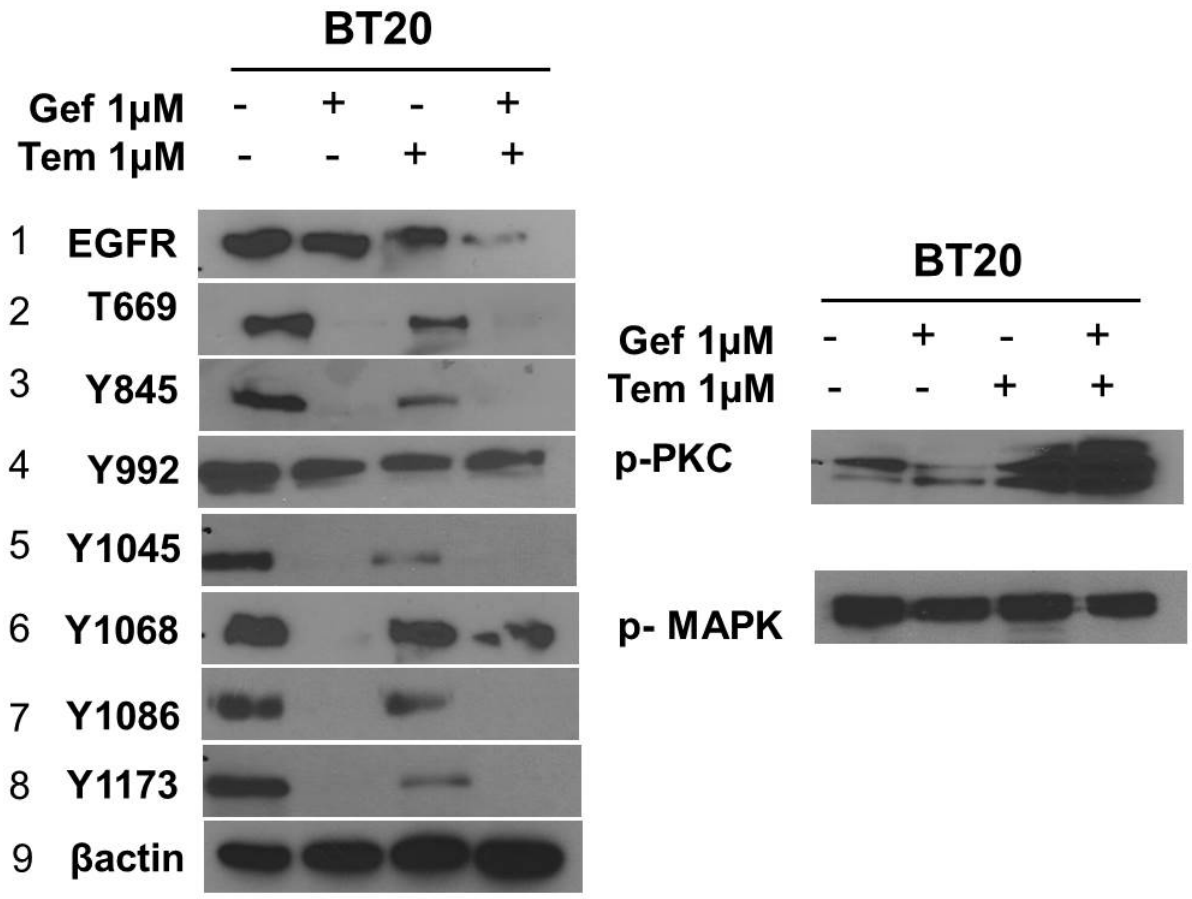
After observing the synergy and significant decrease in growth and colony formation from the combination of gefitinib and temsirolimus, we sought to find the protein(s) that are responsible for regulating the observed effects. The EGFR has many sites that can be phosphorylated to induce growth signaling and stress response pathways, and sites that, when phosphorylated induce receptor internalization. While the TNBC cells are resistant to EGFR inhibitor treatment, they still respond to EGFR inhibitors through receptor dephosphorylation but not at all sites. This dephosphorylation is a good indicator of drug potency and brings up the question of signaling crosstalk within the cell since, even though the receptor itself no longer contains the signaling phosphate, downstream effector proteins are still activated. While a detailed analysis of the different phosphorylation sites on the EGFR and their signaling output is beyond the scope of this work, an excellent review on the subject was written by Jorissen et al., 2002. A diagram of EGFR sites and their signaling effector molecules can be seen in Figure 3.

6.2 Results

6.2.1 EGFR remains phosphorylated in the presence of gefitinib and temsirolimus

Immunoblot analysis on BT20 cells showed that many of the common phosphorylation sites on the EGFR are dephosphorylated when treated with gefitinib (1 μM [Figure 12]). While some sites are dephosphorylated, known downstream proteins may still be activated by other mechanisms e.g., Y1068 and MAPK (Figure 12, row 6).

Figure 12: EGFR phosphorylation sites after EGFR and mTOR inhibitor treatment.



Treatment with gefitinib is able to abrogate EGFR phosphorylation on many sites. Resistance to gefitinib in BT20 cells has allowed for alternate activation of effector proteins even when the phosphorylation site is inhibited (Row 6 Y1068 and MAPK). Gefitinib is unable to dephosphorylate Y992 and there is subsequent activation of the corresponding effector protein PKC (row 4). Cells were treated with 1 μM gefitinib and/or temsirolimus for 24 hours, whole cell lysates were separated on a SDS-PAGE gel.

It is of note that total EGFR levels decrease when treated with GEF+TEM suggesting the combination is able to stimulate internalization of the receptor, possibly in a manner similar to EGFR antibodies. When an EGFR antibody (e.g., cetuximab, panitumumab) binds the receptor, it triggers a conformational change, which often leads to receptor internalization. This makes the action of the drug two fold, it inhibits the ability of the receptor to phosphorylate downstream effector proteins, and it promotes internalization leading to decreased EGFR levels on the cell membrane able to bind growth factor signals.

6.2.2 Activation of MAPK signaling pathways in the presence of temsirolimus and gefitinib

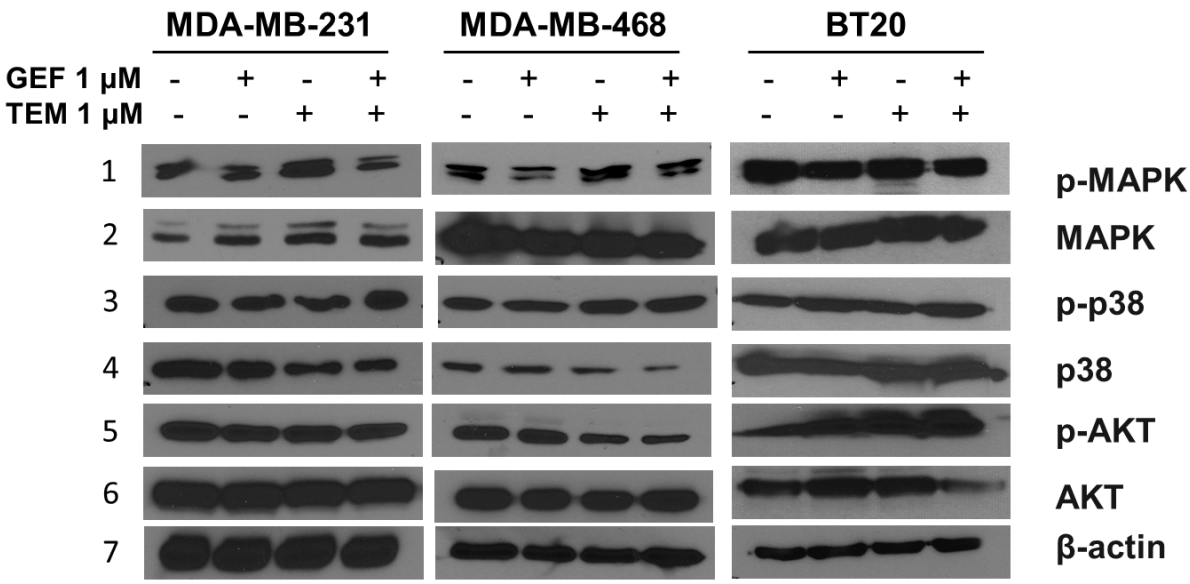
We further performed immunoblot analysis on BT20, MDA-MB-231, and MDA-MB-468 cell lines to determine if classical proteins involved in EGFR and mTOR signaling were affected by dual inhibition of EGFR and mTOR (Figure 13). Specifically, phosphorylation of MAPK, p38MAPK, and AKT along with total protein controls were blotted for after 24 hour treatment with gefitinib and/or temsirolimus. EGFR classical pathway activation is through the Ras-Raf-MEK-MAPK cascade or stress signaling through p38MAPK. mTOR is part of the AKT/PI3K pathway. Unexpectedly, MAPK and p38MAPK phosphorylation was not changed with the single or combination treatments (Figure 13, rows 1 and 3). In contrast, p-AKT increased with temsirolimus treatment in BT20 cells while remaining fairly constant in MDA-MB-231 and MDA-MB-468 while gefitinib had no effect compared to untreated (Figure 13, row 5). mTOR inhibitors are known to be ineffective as single agents as AKT has an activating feedback loop when mTOR is inhibited as seen in BT20s (Hennessy et al., 2005; Wang et al., 2008). Our

results show that, taken together, the classical pathways involved in EGFR and mTOR signaling remain activated with gefitinib and temsirolimus treatment and are likely not involved in the synergy we observe with the combination in TNBC cell lines.

6.3 Conclusions

Both the EGFR and mTOR pathways are linked with signaling cascades that can lead to tumorigenesis. Our results indicate that the most commonly associated pathways linked with EGFR and mTOR remain activated with gefitinib and temsirolimus combination treatment suggesting they are not responsible for the observed synergy. Our data indicate that MAPK, p38, and AKT are not the major players that are inhibited with treatment leading to the decrease in growth and colony formation and further experiments need to be done to elucidate the proteins responsible for the observed synergy.

Figure 13: MAPK and AKT signaling remain activated in the presence of EGFR and mTOR inhibitors.



Immunoblot analysis of TNBC cell lines. Cells were treated with 1 μM GEF and/or TEM for 24 hours. Lysates were electrophoresed on a SDS-PAGE gel and transferred to a PDVF membrane. There is minimal change in phosphorylation across all treatments in TNBC cell lines with the main signaling proteins associated with EGFR and mTOR signaling, MAPK, p38, and AKT, respectively. No decrease in phosphorylation with the combination of GEF+TEM suggests a minimal role for these proteins in regulating the observed synergy.

CHAPTER 7: Role of eIF4B

7.1 Introduction

The common signaling proteins involved in EGFR and mTOR signaling did not offer an explanation for the synergy we observe with gefitinib and temsirolimus dual treatment. Upon returning to the phospho-proteomic data, it was seen that many of the proteins that remained activated are involved in translation. mTOR controls translation initiation through downstream effector proteins P70S6K and 4E-BP1. Translation is often overactive in cancer and while it is usually a highly controlled process, when mTOR is misregulated as often happens in cancer, oncogenic proteins are translated at a greater rate leading to increased carcinogenesis (Loreni et al., 2013; Silvera et al., 2010).

Upon activation, mTOR phosphorylates two effector proteins involved in translation, P70S6K and 4E-BP1 (Raught et al., 2004; van Gorp et al., 2009). P70S6K as described above, controls the phosphorylation of initiation factor eIF4B and 4E-BP1. The latter sequesters eIF4E rendering it inactive. eIF4B facilitates eIF4A helicase activity while eIF4E is the rate limiting factor in formation of the cap-binding complex, eIF4F (Raught et al., 2004; Roux et al., 2007; Silvera et al., 2010). Translation begins when the 40S ribosome binds the newly formed eIF4F complex containing eIF4E, eIF4G, and eIF4A (Gingras et al., 1999; Merrick, 2004). When eIF4B is phosphorylated it makes the helicase activity of eIF4A more efficient (Gingras et al., 1999; Shahbazian et al., 2010a).

7.2 Results

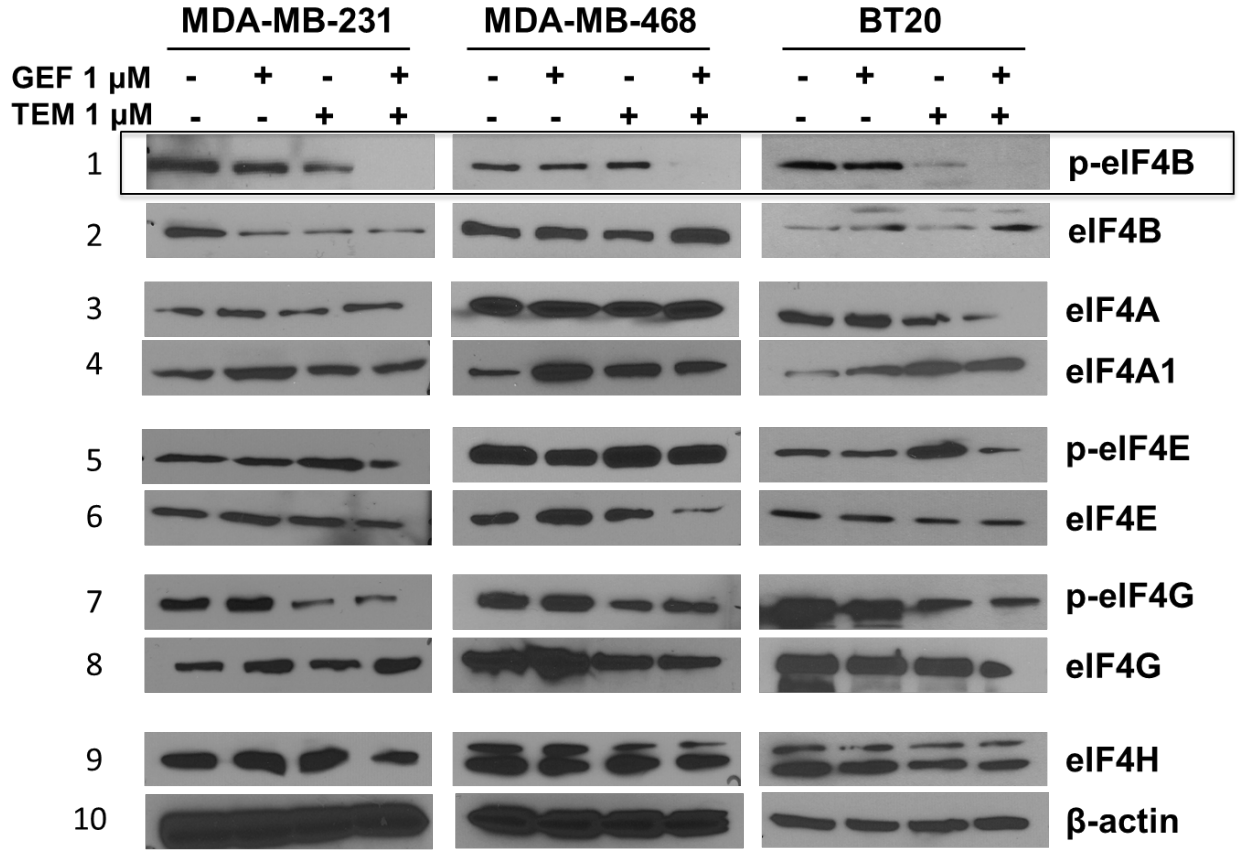
7.2.1 eIF4B phosphorylation is lost with the combination of temsirolimus and gefitinib in TNBC

We analyzed the expression and phosphorylation of a number of translation initiation proteins (Figure 14). Immunoblotting of the eIF4 family showed no change in protein expression across the treatments. However, eIF4B phosphorylation was completely abrogated with the combination gefitinib and temsirolimus while remaining unchanged with single agent treatment consistently in each of the three cell lines (Figure 14, row 1). These data suggest that inhibition of both EGFR and mTOR signaling is required to abrogate eIF4B phosphorylation in the TNBC cell lines we tested.

7.2.2 Decrease in eIF4B expression decreases cell viability in TNBC

We further measured cell viability when eIF4B is knocked down through siRNA constructs. As there currently is no available eIF4B specific inhibitor, using siRNA allowed us to directly target the translation initiation factor and compare knockdown to EGFR and mTOR inhibitor treatment. Cell viability as measured through MTT showed a similar drop in viability with GEF+TEM treatment compared to eIF4B knockdown in MDA-MB-231 cells (Figure 15). There is a significant decrease between the #8 eIF4B knockdown construct and NT, and #8 and a nonsilencing control. It is important to note that #8 provided a more consistent and strong knockdown compared to #7. Our data suggest that the EGFR and mTOR pathways converge on eIF4B and when both pathways are inhibited, eIF4B is also inhibited, stalling translation initiation and regulating the observed synergy.

Figure 14: eIF4B phosphorylation is a fragile point in EGFR and mTOR signaling.



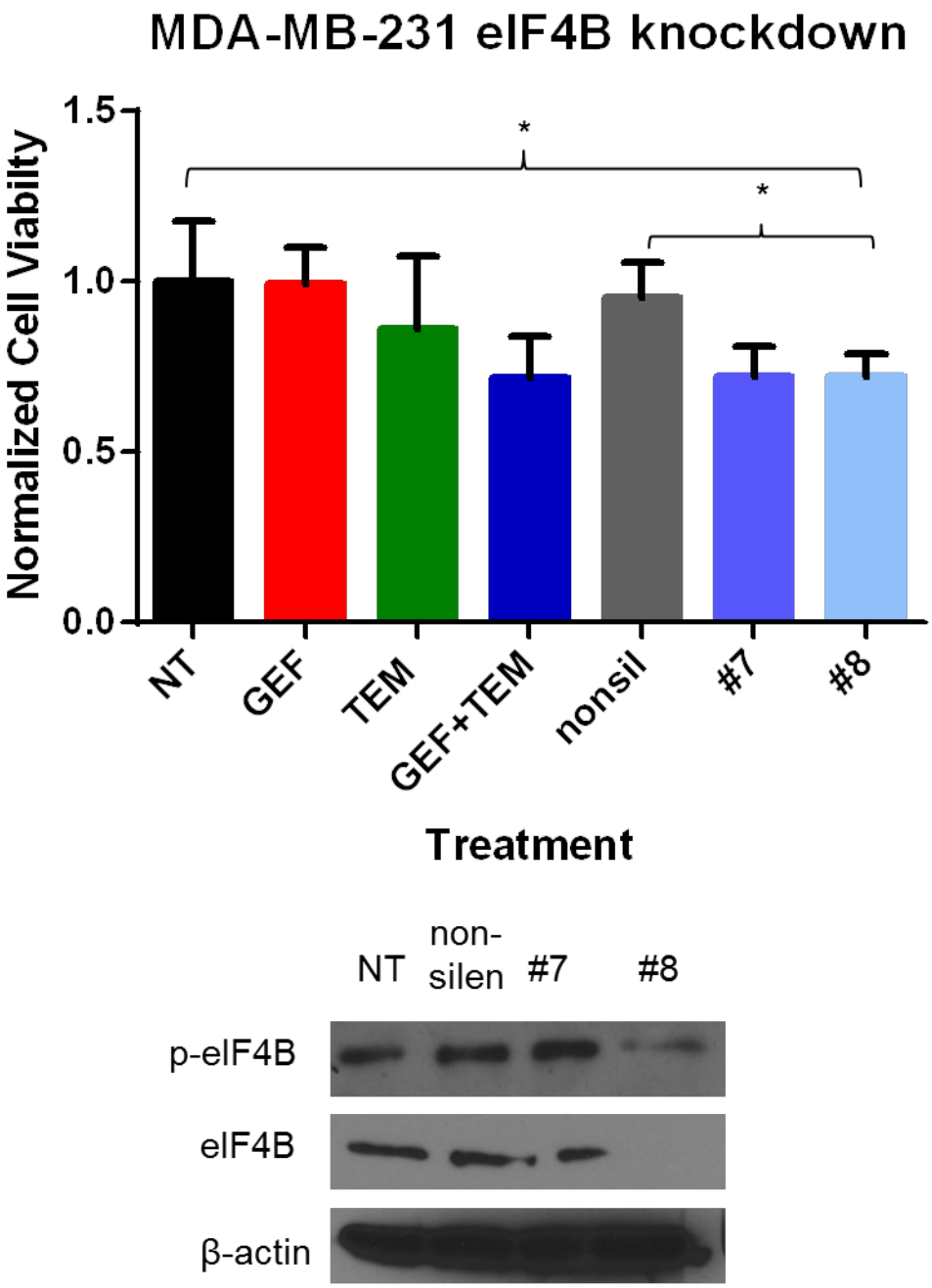
Immunoblot analysis of TNBC cells after treatment with 1 μM gefitinib (GEF) and/or temsirolimus (TEM) for 24 hours. Lysates were collected and blotted for eIF4 family member expression and phosphorylation after electrophoresis on a SDS-PAGE gel. eIF4B phosphorylation is completely abrogated with the combination treatment and only changes minimally when treated with single agent GEF or TEM in all three cell lines. Other family members remain phosphorylated across the treatments.

Taken together our data suggest that the regulation of translation initiation may be a critical component of EGFR and mTOR inhibitor synergy in TNBC.

7.2.3 P70S6K and P90RSK are responsible for phosphorylating eIF4B downstream of EGFR and mTOR signaling

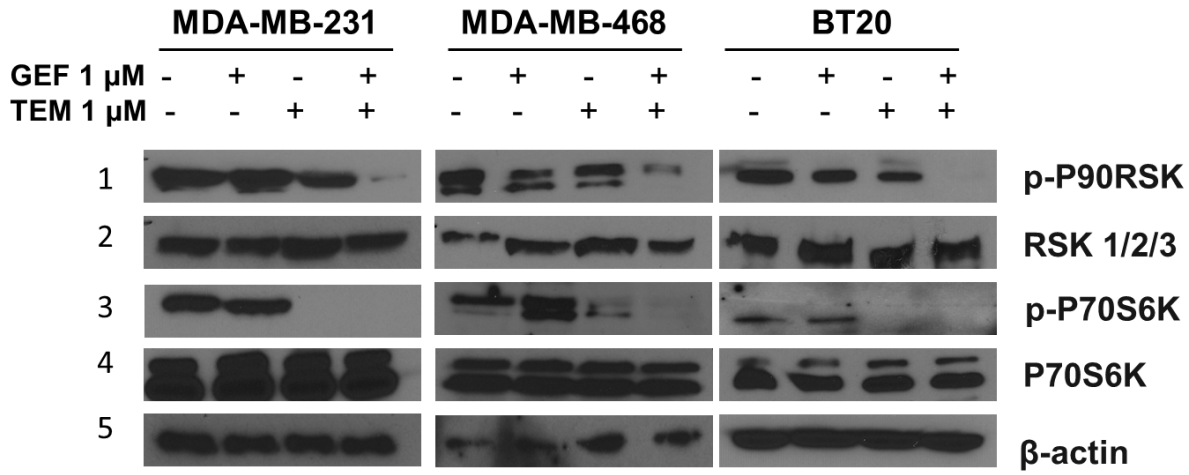
Our data suggest that eIF4B is a point of convergence between the EGFR and mTOR pathways. P90RSK and P70S6K are two kinases that phosphorylate eIF4B on Ser422 (Shahbazian et al., 2006). P90RSK is a downstream effector of EGFR through Ras-MAPK activation and P70S6K is downstream of PI3K/mTOR activation (Raught et al., 2004). Through P90RSK activation, eIF4B is phosphorylated after growth factor signaling from EGFR and a complex signaling network (Yarden and Sliwkowski, 2001). To determine if P90RSK and P70S6K are involved in the activation of eIF4B downstream of EGFR and mTOR in TNBC, we treated the cell lines with gefitinib and/or temsirolimus then measured phosphorylation of P90RSK and P70S6K as surrogates for activation of each protein through immunoblotting. We found that p-P90RSK (S380) was inhibited with the combination of gefitinib and temsirolimus while remaining activated with single agent treatment with gefitinib and temsirolimus (Figure 16). Additionally, P70S6K phosphorylation is abrogated through mTOR inhibition and immunoblotting showed inhibition with single agent treatment of temsirolimus and the combination (Figure 16). Taken together, our results suggest both P90RSK and P70S6K are kinases in the EGFR and mTOR pathways that may be responsible for signaling to translation machinery through eIF4B in TNBC.

Figure 15: eIF4B knockdown has a similar effect on cell viability as gefitinib and mTOR combination.



When eIF4B is knocked down through siRNA (#7, 8) there is a significant decrease in cell viability similar to that of the combination treatment of GEF+TEM (1 μM) in MDA-MB-231 cells. Viability was determined through MTT assay after 72 hour drug treatment and knock down. Immunoblotting of a concurrent 6-well plate shows #7 and #8 successfully knocked down eIF4B while the nonsilencing (non-silen) control had no effect on protein levels.

Figure 16: P70S6K and P90RSK are responsible for phosphorylating eIF4B downstream of mTOR and EGFR signaling.



TNBC cells were treated with 1 μM gefitinib (GEF), 1 μM temsirolimus (TEM) or the combination. Lysates were collected and immunoblotted with the indicated antibodies. p-P90RSK is decreased with the combination while remaining active with single agent treatment. p-P70S6K is inhibited through temsirolimus treatment alone and is a known downstream target of mTOR inhibition. Both kinases are responsible for phosphorylating eIF4B and provide a link between EGFR and mTOR signaling which must be inhibited to get the observed synergy in BT20, MDA-MB-231, and MDA-MB-468 cell lines. P90RSK is activated through EGFR signaling and P70S6K through mTOR.

7.2.4 P90RSK and P70S6K inhibition display similar characteristics to EGFR and mTOR synergy

P90RSK and P70S6K are kinases that directly phosphorylate eIF4B (Shahbazian et al., 2006). Our data suggest that when treated with temsirolimus, P70S6K was dephosphorylated but only the combination of GEF+TEM was able to inhibit phosphorylation of P90RSK. To more specifically test the effect of the dephosphorylation of P70S6K and P90RSK on eIF4B we used two selective inhibitors of P90RSK and P70S6K, BI-D1870 and AT7867, respectively (Table 9). As seen with GEF+TEM combination treatment, when BI-D1870 and AT7867 (BI+AT) were combined in all experimental cell lines, the combination was synergistic (Table 9). Figure 17 further shows a decrease in p-eIF4B only when treated with BI+AT. We tested the growth effects of BI-D1870 and AT7867 on the TNBC cell lines over an 8 day period as described previously. We saw a significant decrease in cell growth with single agent treatment (BI orange and AT green line) but the combination of BI+AT (grey line) decreased growth to the greatest extent (Figure 18). Together, our data demonstrate that dual inhibition of P90RSK and P70S6K displays a similar phenotype as the EGFR and mTOR inhibitor combination, further implicating P90RSK and P70S6K in the pathway conferring synergy in TNBC cell lines.

7.2.5 Inhibiting EGFR and mTOR blocks cap-dependent translation

Eukaryotic cells use two types of translation, cap-dependent, and cap-independent also known as internal ribosome entry site (IRES) translation (Merrick, 2004). Most proteins (95-97%) are translated through the cap-dependent machinery and therefore utilize the eIF proteins to mediate translation, but cap-independent

translation is possible for transcripts that have a special mRNA sequence that can bind the ribosome without the cap (Merrick, 2004; van Gorp et al., 2009; Wendel et al., 2004). We utilized a dual luciferase assay, which is able to measure both cap-dependent and cap-independent translation simultaneously after treatment with gefitinib and temsirolimus. This allowed us to look at the effects of gefitinib and temsirolimus on eIF4B activity through a measurement of its role in translation. Using the reporter plasmid (11510:pFR_HCV_xb) we can measure the expression of firefly luciferase as regulated by cap-dependent translation and the expression of renilla luciferase regulated by cap-independent translation (Petersen et al., 2006). The two types of translation were measured using a dual injector plate reader (Figure 19). Data are presented as normalized cap-dependent/ cap-independent translation. There was a significant decrease ($p<0.05$) in cap-dependent translation after combination treatment of gefitinib and temsirolimus that was not observed with single agent treatment while cap-independent translation remained unchanged by the drug treatments. Our results suggest the combination treatment only decreases cap-dependent translation most likely through the modulation of eIF4B. Treatment has a negligible effect on cap-independent translation, which does not require eIF4B.

7.3 Conclusions

Direct inhibition of P90RSK and P70S6K further implicates eIF4B as an important regulatory point downstream of EGFR and mTOR pathways and upon which they converge to regulate translational control. Since the vast majority of proteins translated within the cell are through a cap-dependent mechanism and therefore require eIF4B, a potential mechanism of action for gefitinib and temsirolimus synergy is through inhibiting

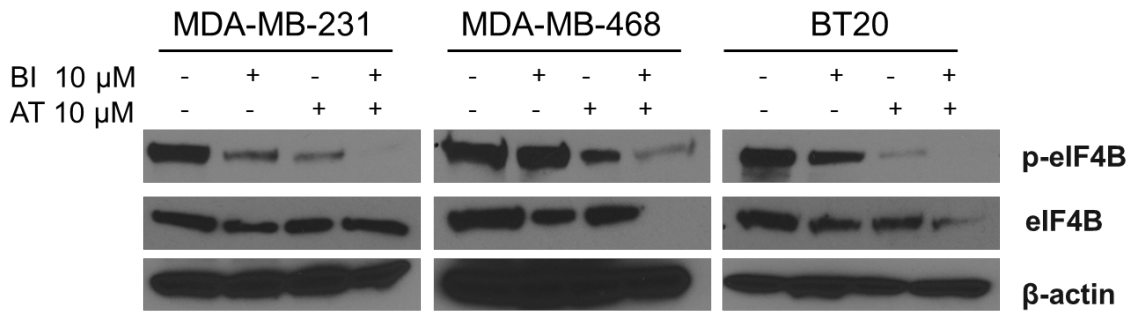
the translation of many proteins in TNBC cell lines (Merrick, 2004). The importance of translation in cancer is beginning to gain notoriety in the field and our data provide further evidence suggesting translational control can play a major role in regulating drug efficacy. EGFR and mTOR are two well-studied signaling proteins and much is known about their pathways. However, our data suggest an important crosstalk between the two signaling cascades that converge on eIF4B to regulate translational control and this process may be exploited in drug development for TNBC.

Table 9: GI_{50} values and synergy of BI-D1870 and AT7867.

	BI-D1870 (RSK inhib)	AT7867 (S6K inhib)	BI+AT	CI value
BT20	7.41 μM	5.93 μM	2.73 μM	0.66
MDA-MB-231	9.57 μM	25.5 μM	3.24 μM	0.32
MDA-MB-468	5.79 μM	8.58 μM	3.54 μM	0.29

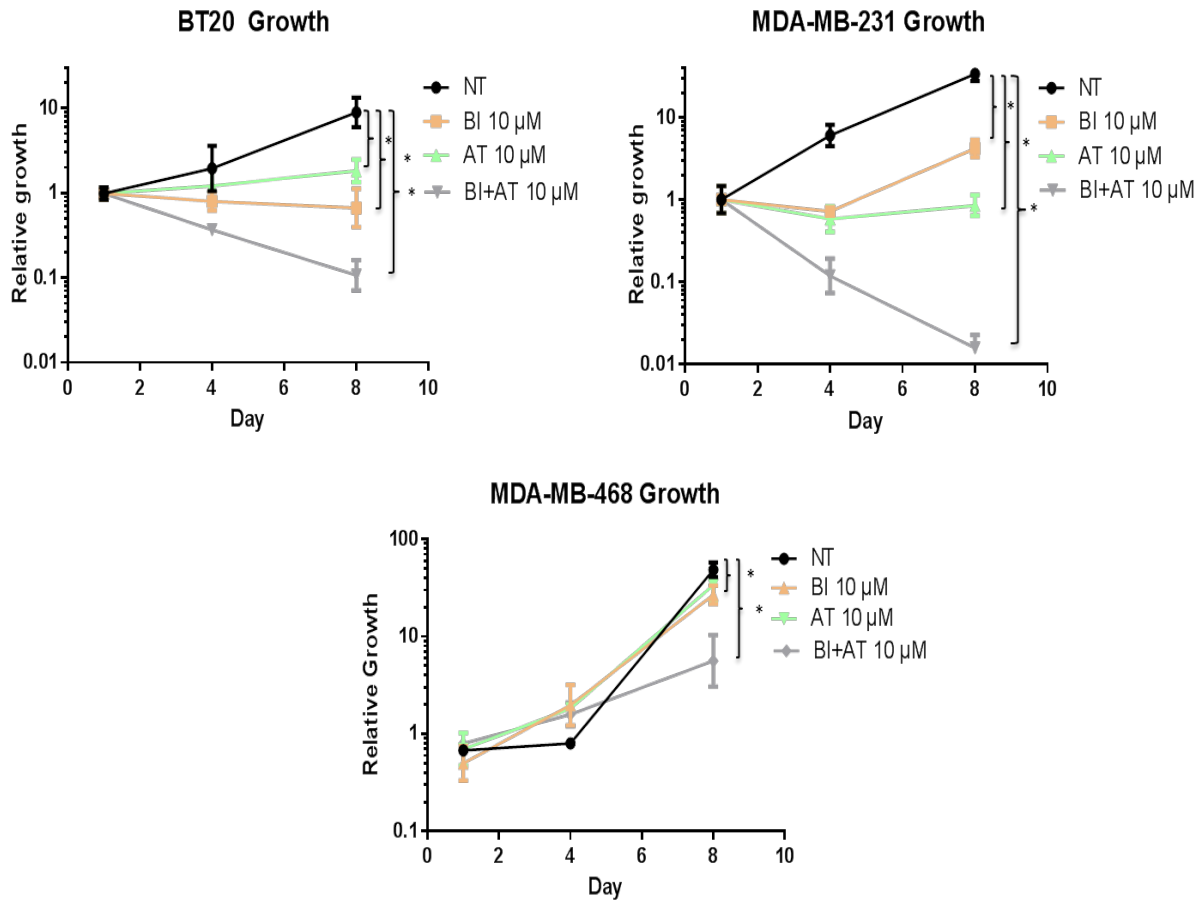
The GI_{50} values for the P90RSK inhibitor BI-D1870 and the P70S6K inhibitor AT7867 are presented. The CI values were also calculated using CalcuSyn software and the combination was found to be synergistic in all three TNBC cell lines. BT20 CI<0.66, MDA-MB-231 CI<0.32, MDA-MB-468 CI<0.29.

Figure 17: BI-D1870 and AT7867 deceases p-eIF4B.



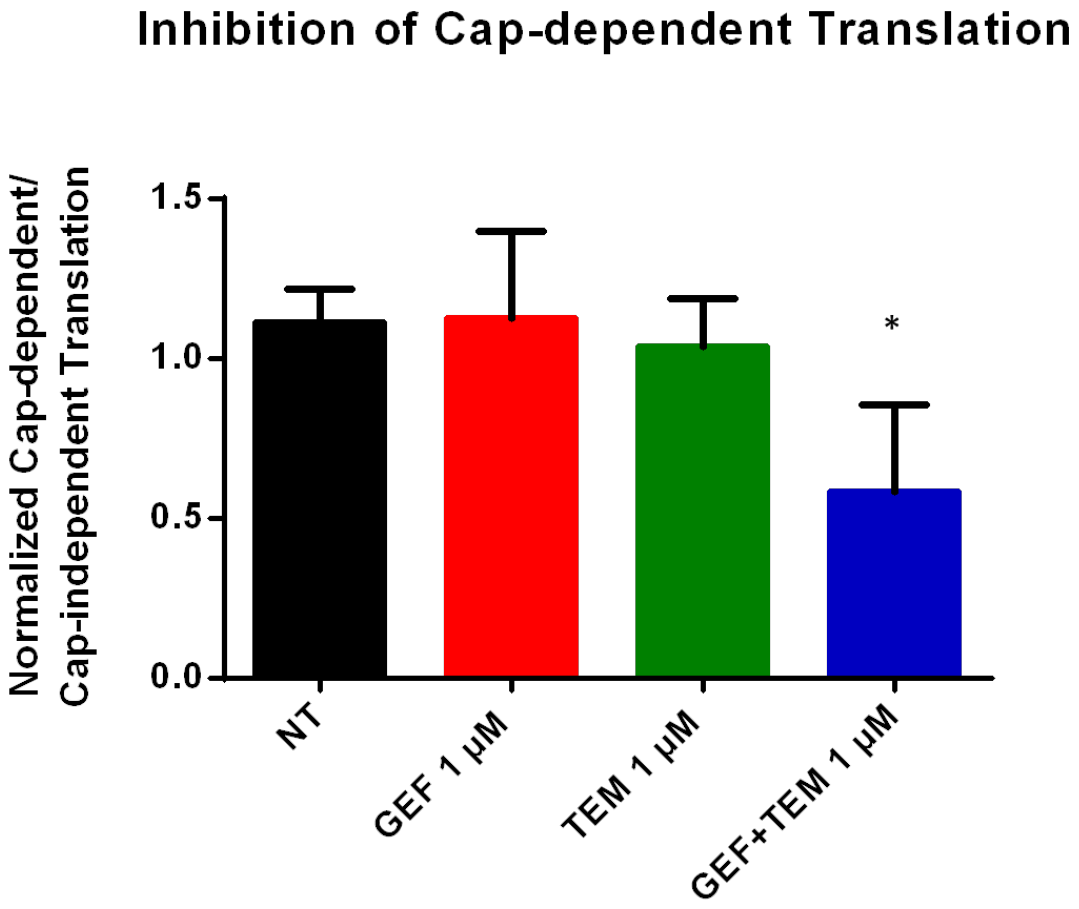
Combination treatment with BI-D1870 and AT7867 decreases p-eIF4B. Single agent inhibition of p-P90RSK and p-P70S6K had minimal effects at decreasing the respective kinases but when combined abrogate signaling.

Figure 18: Growth assays with BI-D1870 and AT7867.



Cell growth assays were done on MDA-MB-468 cells over 8 days in triplicate. Cells were treated every other day with 10 μM BI-1870 (BI) and/ or AT7867 (AT) and counted on days 1, 4, and 8 with a hemacytometer. ANOVAs were performed on GraphPad Prism software with a significant decrease with the combination of BI and AT compared to NT and single treatment p*<0.05. There was also a significant decrease between NT and AT in all cell lines and also in between NT and BI in BT20 and MDA-MB-231 p*<0.05.

Figure 19: Inhibiting EGFR and mTOR signaling blocks cap-dependent translation.



The plasmid 11510:pFR_HCV_xb was transfected into HEK293T cells to measure cap-dependent translation through firefly luciferase and cap-independent translation through renilla luciferase. Luciferase was measured using a dual-luciferase reporter assay system from whole cell lysates. Values within each experiment were normalized to 1.0 as NT (no treatment). Relative light units from the firefly luciferase was plotted over relative light units from the renilla luciferase. Each experiment was performed in triplicate at least two times. There is a significant decrease ($p<0.05$) in translation with GEF+TEM (blue bar) treatment. Treatments were for 24 hours at 1 μM. The decrease in cap-dependent translation is further evidence implicating eIF4B dephosphorylation through EGFR and mTOR inhibition is important in translation regulation. eIF4B provides a point of convergence for the EGFR and mTOR pathways and their role in regulating translation initiation.

CHAPTER 8: STAT3 signaling in EGFR and mTOR synergy in TNBC

8.1 Introduction

STATs are known to play a role in many types of cancer including lung, breast, and prostate (Quesnelle et al., 2007). These transcription factors can be activated by cell surfaces receptors such as EGFR, and by mediary proteins including JAK and mTOR before they translocate to the nucleus and control the transcription of certain genes (Buettner et al., 2002). STAT3 is a known oncogene that has been studied in a variety of cancers including lung, gastric, and breast (Table 5). The association of STAT3 with EGFR and mTOR has been previously explored in gastric cancer and was found to be a negative prognostic marker (Inokuchi M et al., 2011). Elevated expression of all three proteins within the gastric tumor correlated with a higher grade and stage. Important to this work, STAT3 can be phosphorylated on two sites by EGFR (Y705) and mTOR (S727) respectively. Both sites must be phosphorylated for full and robust activation of STAT3. Signaling input from both the membrane bound EGFR and cytoplasmic mTOR protein enable STAT3 to be controlled by many signals leading to the transcription of genes often associated with a proliferative and tumorigenic phenotype.

8.2 Results

8.2.1 STAT3 phosphorylation is lost in response to gefitinib and temsirolimus

Early studies found STAT3 as a potential target of EGFR/mTOR dual inhibition. Specifically, we found a decrease in phosphorylation of STAT3 in all three TNBC cell lines tested when treated with GEF+TEM while single agent treatment had no effect (Figure 20). The phosphorylation of both Y705 and S727 was only decreased with the

combination treatment. The continued phosphorylation of the individual sites with single agent treatment further demonstrates TNBC cell line drug resistance to temsirolimus and gefitinib as the EGFR is still able to signal to Y705 and mTOR to S727, respectively. Only the combination causes a decrease in p-STAT3. These data suggest that, similar to the phosphorylation of eIF4B, STAT3 may be a mediator of gefitinib and temsirolimus synergy in TNBC.

8.2.2 A STAT3 inhibitor decreases cell viability and abrogates EGFR and mTOR fragile point signaling

We sought to further investigate the role of p-STAT3 in conferring the synergy we observe with EGFR and mTOR inhibitors. To determine if an inhibitor of STAT3 mimics gefitinib and temsirolimus dual treatment, we utilized the STAT3 inhibitor (STATTIC). STATTIC is a small molecule inhibitor that inhibits the dimerization domain of STAT3 and its phosphorylation at Y705 abrogating the transcription factor's ability to signal. As seen in Figure 21, STATTIC inhibits STAT3 phosphorylation to a greater extent than the decrease seen in GEF+TEM as expected since it is a direct inhibitor. We then used STATTIC treated cells and measured phosphorylation of other proteins in our predicted EGFR and mTOR synergy pathway. A constitutively active Stat3c plasmid (described below) was also used to overexpress active STAT3 and measure the effect on phosphorylation of the proteins. We observed that STAT3 overexpression increased the phosphorylation. Our data propose that STATTIC is able to abrogate p-P70S6K, p-P90RSK, and p-eIF4B while Stat3c increases phosphorylation in p-P90RSK and p-eIF4B (Figure 21). The data suggest that STAT3 may also have a role in regulating eIF4B and P90RSK and, when STAT3 is dephosphorylated, eIF4B and P90RSK are

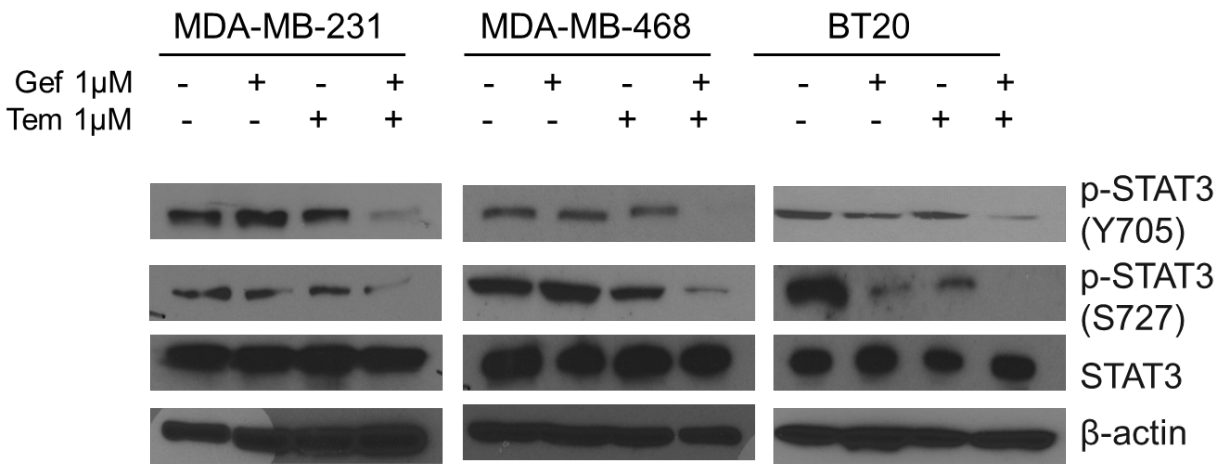
inactivated. While these results are preliminary and much more work would need to be done to validate the role of STAT3 as an upstream signal or transcriptional regulator of P90RSK and P70S6K, our data give exciting evidence for a more intricate crosstalk mechanism within the cell.

8.2.3 Constitutively active STAT3 blocks gefitinib and temsirolimus synergy in TNBC

Our results suggest STAT3 may play a role in regulating EGFR and mTOR inhibitor synergy in TNBC cell lines. To further study the importance of STAT3 we utilized a constitutively active STAT3 plasmid (Stat3c) transfected into TNBC cells and then treated with gefitinib and temsirolimus to assess synergy. If STAT3 plays a vital role in regulating EGFR and mTOR inhibitor synergy through its decreased phosphorylation, a constitutively active STAT3 would negate the synergy. Gefitinib and temsirolimus would be unable to modulate STAT3 activity and turn off the downstream signaling pathways. Therefore, we performed MTT viability assays to determine if the GEF+TEM combination had the same synergistic effect when STAT3 remained phosphorylated through transfection of Stat3c.

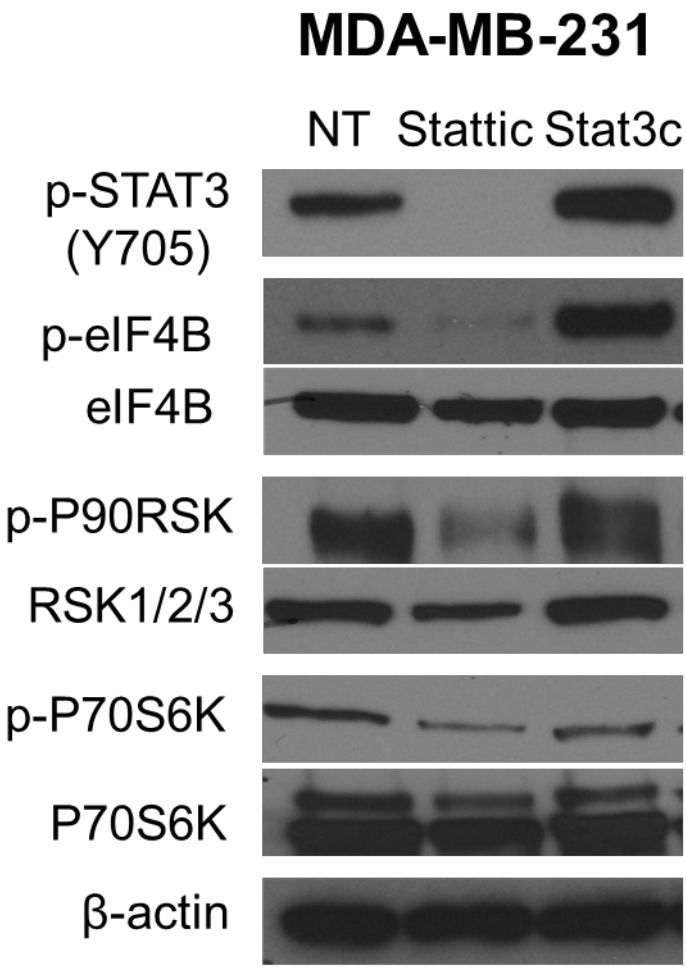
Transfection efficiency as observed through the GFP tag on Stat3c is only about 30% in MDA-MB-231 and MDA-MB-468 cells and lower in BT20 (Figure 22 white cells in Stat3c row compared to cells in Brightfield row, estimated transfection efficiency based on visualized GFP positive cells). Immunoblotting for p-STAT3 performed alongside the MTT viability study, shows after 72 hours, there is a marked increase in p-STAT3 (Y705) suggesting that the cells transfected with Stat3c do contain higher levels of constitutively active STAT3. To determine if gefitinib and temsirolimus treatment

Figure 20: Gefitinib and temsirolimus combination decreases p-STAT3.



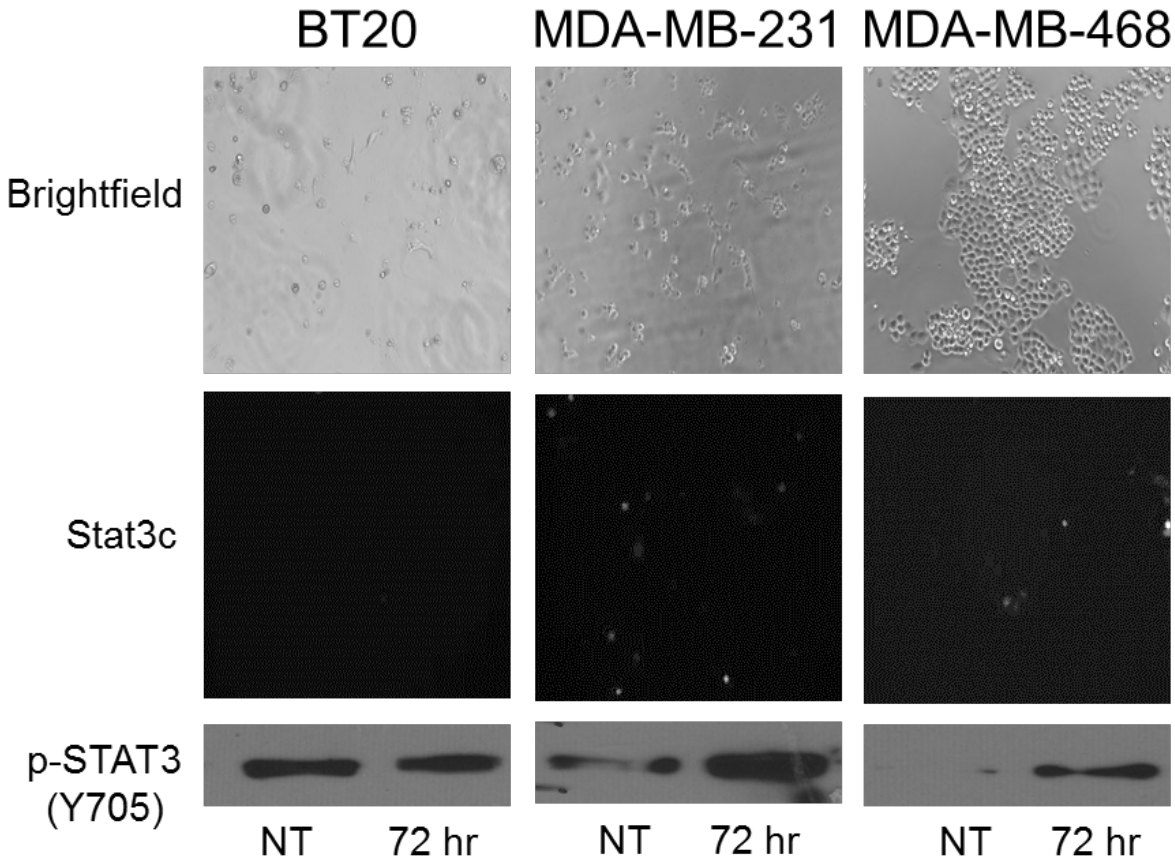
TNBC cells were treated with 1 μM gefitinib (GEF), 1 μM temsirolimus (TEM) or the combination. Lysates were collected and immunoblotted with the indicated antibodies. p-STAT3 (Y705) and (S727) are decreased with the combination while remaining active with single agent treatment. Both sites must be phosphorylated for full activation of STAT3.

Figure 21: STAT3 inhibition through STATTIC decreases EGFR and mTOR regulated proteins.



STAT3 inhibition through STATTIC treatment decreases EGFR and mTOR related proteins including p-eIF4B, p-P90RSK and p-P70S6K in a similar manner to EGFR and mTOR dual inhibition through GEF+TEM. Stat3c transfected cells have increased phosphorylation of proteins.

Figure 22: Constitutively active STAT3 by Stat3c plasmid negates gefitinib and temsirolimus synergy in TNBC cell lines.



Stat3c constitutively active plasmid was transfected into TNBC cells over 72 hours. Immunoblotting was done to measure protein phosphorylation while fluorescent images were also taken measuring transfection efficiency through the GFP tag on Stat3c. MDA-MB-231 and MDA-MB-468 cells had the best transfection as measured though GFP and immunoblotting. MTT cell viability assays were also done to determine gefitinib and temsirolimus combination effect on the cells lines with Stat3c. CI values were calculated using CalcuSyn software and the CI>1.0. BT20 CI<2.23, MDA-MB-231<1.07, MDA-MB-468 <1.48. GFP positive green cells were changed to grey scale for better visualization when printed to appear white on a black background instead of green.

remained synergistic in cells transfected with Stat3c, synergy studies were performed as described above in section 3.4 Using CalcuSyn to calculate the CI values in the presence of Stat3c, we found that all three cell lines no longer exhibited synergy in response to gefitinib and temsirolimus (CI=2.23 for BT20, CI=1.07 for MDA-MB-231, and CI=1.48 for MDA-MB-468). These results support a role for STAT3 in regulating the synergy observed when TNBC cells are treated with EGFR and mTOR inhibitors.

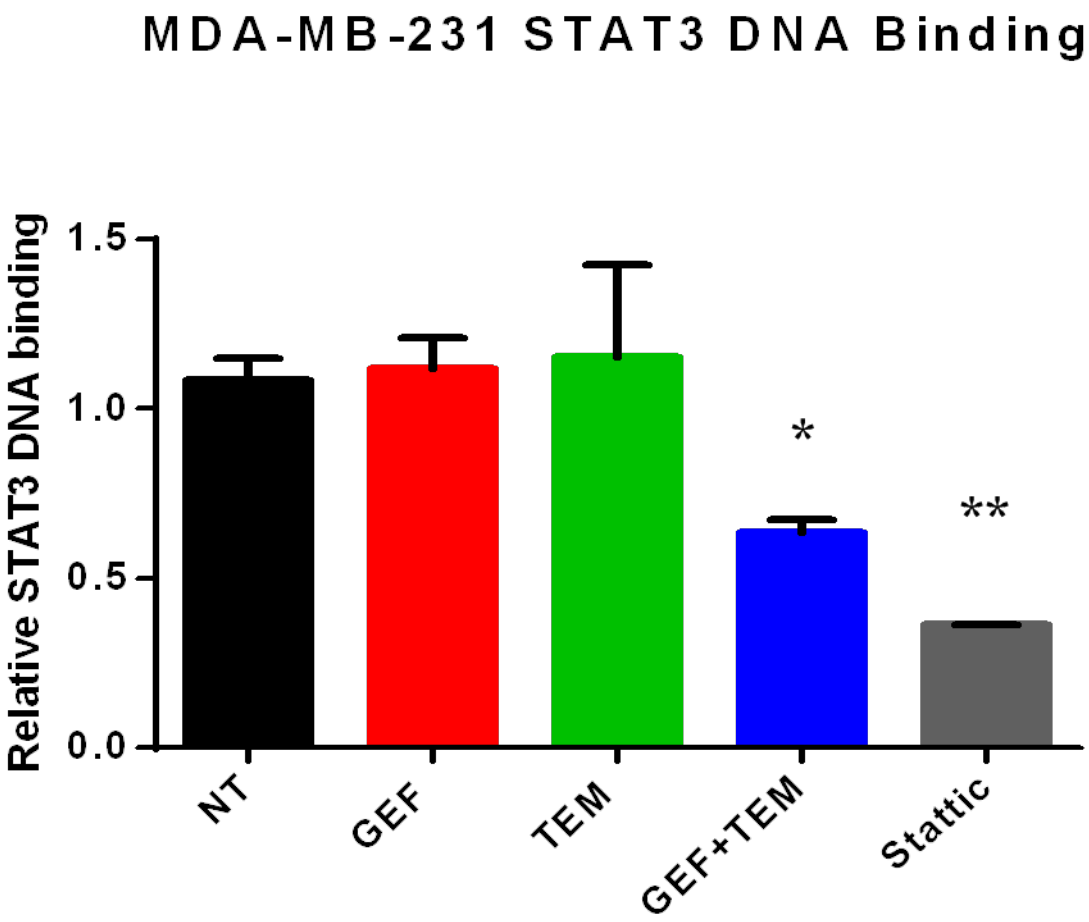
8.2.4 EGFR and mTOR inhibitors block STAT3 DNA binding

After STAT3 is phosphorylated it can homo- or heterodimerize, then translocate to the nucleus to bind STAT3 DNA-binding elements and control gene expression (Furqan et al., 2013). We have previously shown that GEF+TEM combination is able to decrease STAT3 phosphorylation on both Y705 and S727 (Figure 20). In order to measure STAT3 activity we utilized an ELISA (Figure 23). The ELISA is able to qualitatively measure sample binding to a STAT3 response element oligo allowing us to determine the effect of drug treatment on STAT3 activity. We found that MDA-MB-231 cells when treated with single agent gefitinib and temsirolimus (red and green bars) had minimal effect on DNA binding ability. However, the combination (GEF+TEM, blue bar) showed a significant decrease in STAT3 activity ($p^*<0.05$). STATTIC treatment also had a significant decrease in DNA binding. Taken together, our results suggest that EGFR and mTOR dual inhibition decreases STAT3 phosphorylation on both Y705 and S727 to modulate DNA binding. This decrease in STAT3 DNA binding is another mechanism of action that may be responsible for the synergistic effects of gefitinib and temsirolimus in TNBC cell lines.

8.3 Conclusions

Immunoblot studies showed a decrease in STAT3 phosphorylation on both Y705 and S727 in response to GEF+TEM combination, while single agent treatment had no effect on phosphorylation. Further studies demonstrated that inhibition of STAT3 through a small molecule inhibitor, STATTIC, decreased cell viability and also inhibited the phosphorylation of interested proteins in the EGFR/mTOR pathway that are important in regulating synergy; P90RSK, P70S6K, and eIF4B. Through constitutive activation of STAT3 via plasmid transfection, we were able to negate the previously observed synergy of GEF+TEM in all three TNBC cell lines suggesting that the decreased phosphorylation of STAT3 is an important mediator of the EGFR and mTOR inhibitor synergy. DNA binding ability of STAT3 was also significantly decreased when treated with the drug combination. Our results provide evidence that STAT3 is an important mediator of EGFR/mTOR inhibitor synergy in TNBC.

Figure 23: Gefitinib and temsirolimus in combination significantly decrease STAT3 DNA binding.



STAT3 DNA binding was measured through an ELISA. There is a significant decrease in DNA binding when treated with the GEF+TEM combination and STATTIC that is absent in single agent treatment. p*<0.05 **<0.01

CHAPTER 9: Conclusions

While survival rates for breast cancer patients are rising, those with TNBC continue to have no suitable and successful drug therapy other than standard cytotoxic chemotherapy. These patients do not respond to common hormone and HER2 driven therapies since they do not express the cellular targets. TNBC patients have limited non-surgical treatment options. Adjuvant cytotoxic chemotherapy is often given but high resistance rates are common (Chacon and Costanzo, 2010; Santana-Davila R, 2010). Research has been done in TNBC to find an effective targeted therapy that improves the response rates while avoiding resistance and treatment limiting side effects. This work focused on the overexpression of EGFR as TNBC often expresses high levels of the receptor. Inhibitors targeting the EGFR are approved for different types of cancers and, therefore, the receptor has potential to be exploited as a target in TNBC (Hawk, 2010; Santana-Davila R, 2010). Our research found that the mTOR pathway remained activated in EGFR inhibitor resistant TNBC cell lines. Further evidence suggests that mTOR and EGFR inhibitor combination has a synergistic effect at decreasing TNBC cell viability and significantly decreases growth and colony formation. Our data confirm the findings of others studying colon, prostate, and breast cancers that suggest the use of mTOR inhibitors in combination with EGFR inhibitors to be of potential benefit (Bianco et al., 2008; Liu et al., 2011). Everolimus was shown to sensitize GEO colon, PC3 prostate, and MDA-MB-468 TNBC cells to gefitinib and cetuximab and decrease their growth in a dose-dependent manner. Using mouse xenografts the study showed a decrease in colon cancer tumor burden by 90% when the drugs were used in combination (Bianco et al., 2008). While EGFR inhibitors as monotherapy have not

been effective due to intrinsic resistance, our results and those of others suggest that combinations of EGFR and mTOR inhibitors are synergistic and their combined potential as a targeted therapy in TNBC needs to be further studied (Liu et al., 2011; O'Regan and Hawk, 2011).

Similar to published studies, our work found that dual treatment with gefitinib and temsirolimus had a synergistic effect on decreasing TNBC cell viability, growth, and clonogenic survival. While the combination of EGFR and mTOR inhibitors has been previously observed in TNBC, the mechanism of synergy is not understood. Our studies found an EGFR and mTOR crosstalk involving eIF4B and STAT3 that provides evidence for regulating the observed synergy.

Inhibiting both EGFR and mTOR signaling was required to abrogate eIF4B phosphorylation and subsequent cap-dependent translation in TNBC cell lines. Cancer cells have an increased metabolic and proliferative rate requiring a high demand for available protein. Aberrant translation has been implemented in cancer progression for a number of years as patients with ribosomal disorders have an increased cancer risk (Loreni et al., 2013; Montanaro et al., 2012). eIF4E is often considered an oncogene as it was shown to induce transformation in cells (Lazaris-Karatzas et al., 1990). eIF4E is a member of initiation factors that have altered expression in different types of cancer (Silvera et al., 2010). Current research has also explored the potential benefit of targeting the eIF4F complex in BRAF inhibitor resistant melanoma, colon, and thyroid cancer cell lines. The authors, published in the most current issue of Nature, reported that patient tumors resistant to inhibitors had increased activation of eIF4F resulting in higher cap-dependent translation (Boussemart et al., 2014). In the same issue, another

group discovered the oncogene ability of eIF4A in T-ALL and an inhibitor of eIF4A was able to decrease ALL cell growth (Wolfe et al., 2014). It was previously discovered that the MAPK and mTOR/PI3K pathways converge on eIF4B but to our knowledge this is the first evidence of EGFR and mTOR inhibitor synergy being regulated by eIF4B in TNBC (Shahbazian et al., 2006). Taken together, these results have identified a new fragile point, eIF4B phosphorylation, and cap-dependent translation as a mediator of EGFR and mTOR crosstalk in TNBC.

Our results suggest that the common EGFR and mTOR signaling proteins, MAPK and AKT, are not responsible for regulating the synergy. Inhibiting EGFR and mTOR through gefitinib and temsirolimus had no effect on MAPK and AKT phosphorylation. This result corresponds with other studies in colon and lung cancer that found AKT and MAPK maintain activity after EGFR and mTOR inhibitor treatment (Bianco et al., 2008; La Monica et al., 2009). While MAPK and AKT are often considered the main signaling proteins for the EGFR and mTOR pathways, respectively, our data suggest a more prominent role for P70S6K and P90RSK in TNBC cell lines resistant to EGFR inhibitors. P90RSK is downstream of MAPK and known to be up regulated in breast and prostate cancer. It can activate P70S6K through direct phosphorylation or through mTOR activation (Clark et al., 2005; Hennessy et al., 2005; Romeo and Roux, 2011). Both P90RSK and P70S6K are involved in protein translation through their ability to directly phosphorylate eIF4B. P70S6K is also known to phosphorylate another key translation factor, 4E-BP1. When 4E-BP1 is phosphorylated it releases eIF4E, the rate-limiting step in translation initiation, allowing it to form the eIF4F complex initiating translation. Our data suggest that P70S6K regulation through

EGFR and mTOR inhibition acts independently of 4E-BP1 as immunoblotting indicates no change in 4E-BP1 phosphorylation after treatment (data not shown). Instead the gefitinib and temsirolimus combination inhibits eIF4B phosphorylation through P70S6K and P90RSK inhibition and decreases cap-dependent translation leading to the synergy we observe in TNBC cell lines.

STAT3 is a transcription factor that is implicated in the oncogenesis of many types of cancer, including breast. It can be activated through a variety of signaling proteins including EGFR and mTOR, which bind to individual phosphorylation sites (Y705 and S727, respectively), both of which are required for full activation of STAT3. Our data suggest STAT3 phosphorylation selectively decreases with GEF+TEM combination while remaining unchanged with single agent treatment. Further evidence using STATTIC, a STAT3 inhibitor, and Stat3c constitutively active plasmid found that specific STAT3 activation is able to negate the synergistic effect we observe with GEF+TEM as measured through cell viability MTT assays. Our data suggest that, similar to eIF4B regulation, STAT3 is regulated downstream of EGFR and mTOR signaling and may be responsible for regulating TNBC cell synergy.

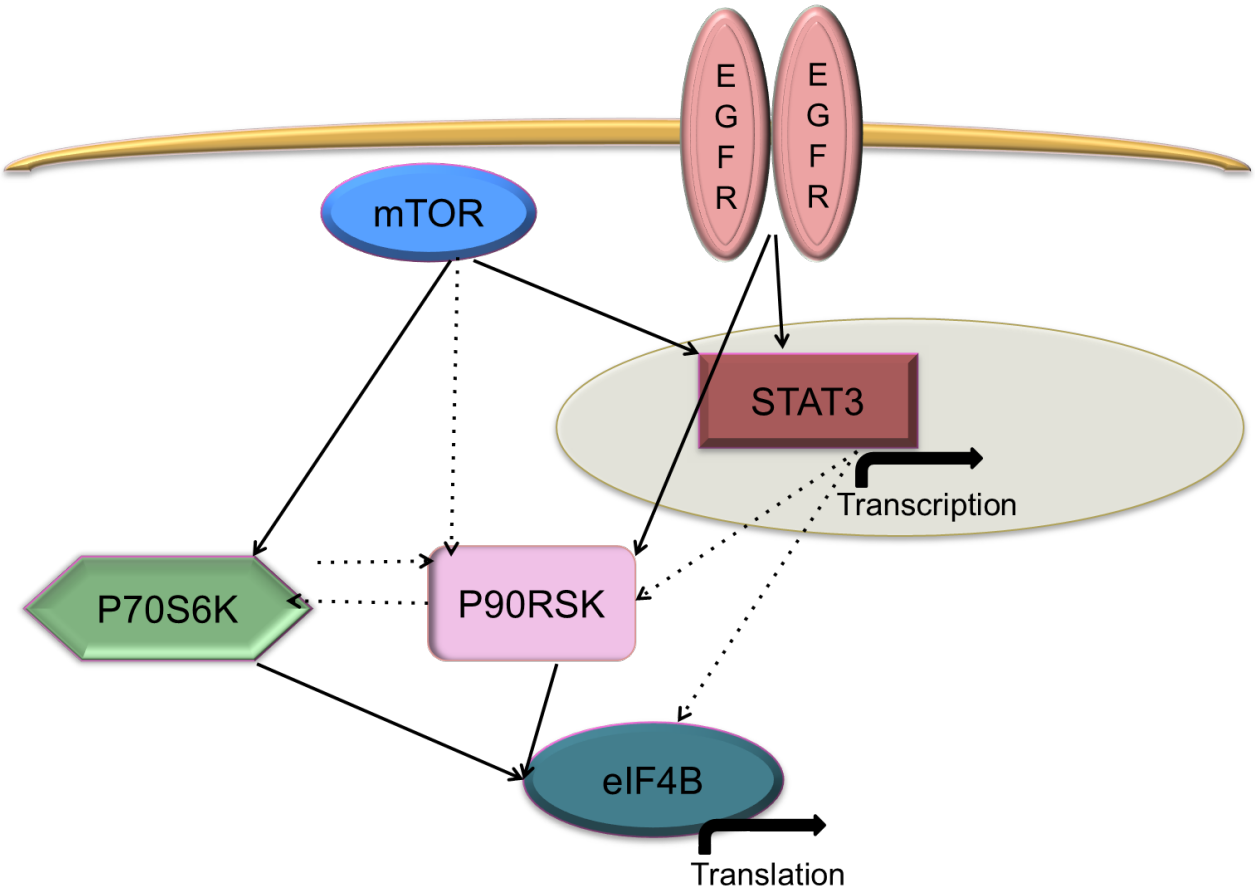
Our studies identified EGFR and mTOR inhibitors as a potentially effective treatment for TNBC and the drug combination needs to be further explored. In addition, these studies suggest P90RSK and P70S6K must be abrogated to mediate the synergistic effects of gefitinib and temsirolimus combination. The effect translation has on cancer cells in regard to the mTOR and EGFR pathways is largely unexplored in TNBC and further implicates eIF4B as a protein of interest in understanding the gefitinib

and temsirolimus synergy. We further present evidence suggesting that STAT3 may also play a role in regulating synergy through DNA binding control.

TNBC has limited therapeutic options and this project sought to find a drug combination to circumvent EGFR resistance using a phospho-proteomic approach. While EGFR and mTOR inhibitors are approved in the clinic for various types of cancer. Confirming the results of others, we suggest a potential use for EGFR/ mTOR inhibitor combination in TNBC that needs to be further explored. The mechanism of action for their observed synergy was previously unknown but we suggest it is through translational control by eIF4B, and transcriptional regulation by STAT3. TNBC is a highly malignant disease and through the data presented in this dissertation, another therapeutic option may become available for patients in the future.

APPENDIX

Supplemental Figure 1: Proposed pathway diagram.



Proposed pathway involving EGFR and mTOR proteins that are involved in regulating synergy to gefitinib and temsirolimus combination in TNBC cell lines. Only when used in combination do the drugs inhibit eIF4B to reduce translation and STAT3 to control transcription. Dashed lines between mTOR, P90RSK, and P70S6K indicate the ability of these proteins to activate each other. Additionally, there may be an extra level of control involving STAT3 regulation of eIF4B and P90RSK (dashed lines) that also contribute to the observed synergy and decreases in growth and survival.

Supplemental Table 1: Proteins identified by phospho-mass spectrometry as not significantly changed after gefitinib treatment.

Gene description	Accession Number
182 kDa tankyrase-1-binding protein	TB182_HUMAN
3-hydroxyacyl-CoA dehydratase 3	HACD3_HUMAN
3-phosphoinositide-dependent protein kinase 1	PDPK1_HUMAN (+1)
40S ribosomal protein S3 OS=Homo sapiens	RS3_HUMAN
60S acidic ribosomal protein P0	RLA0_HUMAN (+1)
60S acidic ribosomal protein P1	RLA1_HUMAN (+1)
6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2	F262_HUMAN
Acetyl-coenzyme A synthetase, cytoplasmic	ACSA_HUMAN
Adenylyl cyclase-associated protein 1	CAP1_HUMAN
Alpha- and gamma-adaptin-binding protein p34	AAGAB_HUMAN
Alpha-taxilin OS=Homo sapiens	TXLNA_HUMAN
AMP deaminase 2 OS=Homo sapiens	AMPD2_HUMAN
Antigen KI-67 OS=Homo sapiens	KI67_HUMAN
AP2-associated protein kinase 1	AAK1_HUMAN
AP-3 complex subunit delta-1	AP3D1_HUMAN
Arfaptin-1 OS=Homo sapiens	ARFP1_HUMAN
Astrocytic phosphoprotein PEA-15	PEA15_HUMAN
Ataxin-2-like protein	ATX2L_HUMAN
ATP-binding cassette sub-family F member 1	ABCF1_HUMAN
AT-rich interactive domain-containing protein 1A	ARI1A_HUMAN
BAG family molecular chaperone regulator 3	BAG3_HUMAN
Bcl-2-associated transcription factor 1	BCLF1_HUMAN
Beta-2-syntrophin	SNTB2_HUMAN
Bifunctional aminoacyl-tRNA synthetase	SYEP_HUMAN
Brefeldin A-inhibited guanine nucleotide-exchange protein 2	BIG2_HUMAN
Bystin	BYST_HUMAN
CAD protein	PYR1_HUMAN
Calnexin	CALX_HUMAN
cAMP-dependent protein kinase type I-alpha regulatory subunit	KAP0_HUMAN
cAMP-dependent protein kinase type II-alpha regulatory subunit	KAP2_HUMAN
Catenin alpha-1	CTNA1_HUMAN
Catenin delta-1	CTND1_HUMAN
Cation-independent mannose-6-phosphate receptor	MPRI_HUMAN

CCAAT/enhancer-binding protein beta	CEBPB_HUMAN
Cleavage and polyadenylation specificity factor subunit 2	CPSF2_HUMAN
Cleavage stimulation factor subunit 3	CSTF3_HUMAN
Coatomer subunit beta'	COPB2_HUMAN
Cofilin-1	COF1_HUMAN
Coiled-coil domain-containing protein 6	CCDC6_HUMAN
Coiled-coil domain-containing protein 86	CCD86_HUMAN
CTTNBP2 N-terminal-like protein	CT2NL_HUMAN
Cyclin-dependent kinase 1	CDK1_HUMAN
Cyclin-dependent kinase 12	CDK12_HUMAN
Death-associated protein 1	DAP1_HUMAN
Death-inducer obliterator 1	DIDO1_HUMAN
Density-regulated protein	DENR_HUMAN
Destrin	DEST_HUMAN
DNA repair protein complementing XP-C cells	XPC_HUMAN
DNA repair protein XRCC1	XRCC1_HUMAN
DNA replication licensing factor MCM2	MCM2_HUMAN
DNA topoisomerase 2-beta	TOP2B_HUMAN
DOCK3_HUMAN-R	DOCK3_HUMAN-R
Double-strand break repair protein MRE11A	MRE11_HUMAN
Drebrin-like protein	DBNL_HUMAN
Dual specificity testis-specific protein kinase 1	TESK1_HUMAN
Dynein heavy chain 14, axonemal	DYH14_HUMAN
E3 SUMO-protein ligase RanBP2	RBP2_HUMAN
E3 ubiquitin-protein ligase BRE1A	BRE1A_HUMAN
E3 ubiquitin-protein ligase CHIP	CHIP_HUMAN
E3 ubiquitin-protein ligase HUWE1	HUWE1_HUMAN
E3 ubiquitin-protein ligase TRIM33	TRI33_HUMAN
E3 ubiquitin-protein ligase ZFP91	ZFP91_HUMAN
Echinoderm microtubule-associated protein-like 4	EMAL4_HUMAN
EFCB6_HUMAN-R	EFCB6_HUMAN-R
Elongation factor 1-beta	EF1B_HUMAN
Elongation factor 1-delta	EF1D_HUMAN
Enhancer of mRNA-decapping protein 4	EDC4_HUMAN
Epidermal growth factor receptor kinase substrate 8-like protein 2	ES8L2_HUMAN
Epidermal growth factor receptor	EGFR_HUMAN
Epiplakin	EPIPL_HUMAN
Eukaryotic translation initiation factor 2A	EIF2A_HUMAN
Eukaryotic translation initiation factor 3 subunit B	EIF3B_HUMAN
Eukaryotic translation initiation factor 3 subunit C	EIF3C_HUMAN

Eukaryotic translation initiation factor 3 subunit D	EIF3D_HUMAN
Eukaryotic translation initiation factor 3 subunit G	EIF3G_HUMAN
Eukaryotic translation initiation factor 3 subunit J	EIF3J_HUMAN
Eukaryotic translation initiation factor 4 gamma 1	IF4G1_HUMAN
Eukaryotic translation initiation factor 4E-binding protein 1	4EBP1_HUMAN
Eukaryotic translation initiation factor 5	IF5_HUMAN
Eukaryotic translation initiation factor 5B	IF2P_HUMAN
FACT complex subunit SSRP1	SSRP1_HUMAN
Fatty acid synthase	FAS_HUMAN
Ferritin heavy chain	FRIH_HUMAN
FH1/FH2 domain-containing protein 1	FHOD1_HUMAN
FIL1L_HUMAN-R	FIL1L_HUMAN-R
Filensin	BFSP1_HUMAN
FK506-binding protein 15	FKB15_HUMAN
General transcription factor II-I	GTF2I_HUMAN
Glucocorticoid receptor DNA-binding factor 1	GRLF1_HUMAN
Glucosamine--fructose-6-phosphate aminotransferase [isomerizing] 1	GFPT1_HUMAN
Glycogen [starch] synthase, muscle	GYS1_HUMAN
Glycogen synthase kinase-3 alpha	GSK3A_HUMAN (+1)
Golgi reassembly-stacking protein 2	GORS2_HUMAN
Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1	GBF1_HUMAN
H/ACA ribonucleoprotein complex subunit 4	DKC1_HUMAN
Heat shock protein beta-1	HSPB1_HUMAN
Heat shock protein HSP 90-alpha	HS90A_HUMAN
Heat shock protein HSP 90-beta	HS90B_HUMAN (+1)
Hepatoma-derived growth factor	HDGF_HUMAN
Heterogeneous nuclear ribonucleoprotein H	HNRH1_HUMAN (+1)
Heterogeneous nuclear ribonucleoprotein K	HNRPK_HUMAN
Heterogeneous nuclear ribonucleoprotein U	HNRPU_HUMAN
Heterogeneous nuclear ribonucleoprotein U-like protein 1	HNRL1_HUMAN
Heterogeneous nuclear ribonucleoprotein U-like protein 2	HNRL2_HUMAN
Heterogeneous nuclear ribonucleoproteins A2/B1	ROA2_HUMAN
High mobility group protein HMG-I/HMG-Y	HMGA1_HUMAN
Histone deacetylase 2	HDAC2_HUMAN
Histone demethylase UTY	UTY_HUMAN
Histone H1.2	H12_HUMAN
Histone H1.5	H15_HUMAN

HIV Tat-specific factor 1	HTSF1_HUMAN
Hsc70-interacting protein	F10A1_HUMAN
IFRD2_HUMAN-R	IFRD2_HUMAN-R
Interferon regulatory factor 2-binding protein 2	I2BP2_HUMAN
Interferon regulatory factor 2-binding protein-like	I2BPL_HUMAN
KDM6A_HUMAN-R	KDM6A_HUMAN-R
Kelch domain-containing protein 4	KLDC4_HUMAN
Keratin, type I cytoskeletal 18	K1C18_HUMAN
Keratin, type I cytoskeletal 19	K1C19_HUMAN
Keratin, type II cytoskeletal 5	K2C5_HUMAN
Keratin, type II cytoskeletal 7	K2C7_HUMAN
Keratin, type II cytoskeletal 8	K2C8_HUMAN
Kinectin	KTN1_HUMAN
Lamina-associated polypeptide 2, isoform alpha	LAP2A_HUMAN (+1)
La-related protein 1	LARP1_HUMAN
La-related protein 4	LARP4_HUMAN
Large proline-rich protein BAG6	BAG6_HUMAN
LIM and SH3 domain protein 1	LASP1_HUMAN
LIM domain and actin-binding protein 1	LIMA1_HUMAN
Lupus La protein	LA_HUMAN
Lysine-specific histone demethylase 1A	KDM1A_HUMAN
Mediator of DNA damage checkpoint protein 1	MDC1_HUMAN
Membrane-associated progesterone receptor component 1	PGRC1_HUMAN
Membrane-associated progesterone receptor component 2	PGRC2_HUMAN
Methylosome subunit pICln	ICLN_HUMAN
Microcephalin	MCPH1_HUMAN
Microfibrillar-associated protein 1	MFAP1_HUMAN
Misshapen-like kinase 1	MINK1_HUMAN
Mitochondrial import receptor subunit TOM22 homolog	TOM22_HUMAN
Mitogen-activated protein kinase 1	MK01_HUMAN
Mitotic checkpoint protein BUB3	BUB3_HUMAN
Monocarboxylate transporter 1	MOT1_HUMAN
Myb-binding protein 1A	MBB1A_HUMAN
Myc box-dependent-interacting protein 1	BIN1_HUMAN
Myelin expression factor 2	MYEF2_HUMAN
Myosin-9	MYH9_HUMAN
Nardilysin	NRDC_HUMAN
Negative elongation factor B	NELFB_HUMAN
Neuroblast differentiation-associated protein AHNAK	AHNK_HUMAN
Niban-like protein 1	NIBL1_HUMAN

Nuclear autoantigen Sp-100	SP100_HUMAN
Nuclear cap-binding protein subunit 1	NCBP1_HUMAN
Nuclear pore complex protein Nup98-Nup96	NUP98_HUMAN
Nuclear ubiquitous casein and cyclin-dependent kinases substrate	NUCKS_HUMAN
Nuclear-interacting partner of ALK	NIPA_HUMAN
Nuclease-sensitive element-binding protein 1	YBOX1_HUMAN
Nucleolar and coiled-body phosphoprotein 1	NOLC1_HUMAN
Nucleolar complex protein 2 homolog	NOC2L_HUMAN
Nucleolar protein 58	NOP58_HUMAN
Nucleolar RNA helicase 2	DDX21_HUMAN
Nucleolin	NUCL_HUMAN
Nucleophosmin	NPM_HUMAN
Nucleosome assembly protein 1-like 4	NP1L4_HUMAN
Osteoclast-stimulating factor 1	OSTF1_HUMAN
Oxysterol-binding protein 1	OSBP1_HUMAN
Oxysterol-binding protein-related protein 11	OSB11_HUMAN
Paralemmin-3	PALM3_HUMAN
Partitioning defective 3 homolog	PARD3_HUMAN
Paxillin	PAXI_HUMAN
Periodic tryptophan protein 1 homolog	PWP1_HUMAN
PERQ amino acid-rich with GYF domain-containing protein 2	PERQ2_HUMAN
PHD and RING finger domain-containing protein 1	PHRF1_HUMAN
Phosphatidylinositol 4-kinase type 2-alpha	P4K2A_HUMAN
Pinin	PININ_HUMAN
Plakophilin-3	PKP3_HUMAN
Pleckstrin homology-like domain family B member 2	PHLB2_HUMAN
Plectin	PLEC_HUMAN
Prelamin-A/C	LMNA_HUMAN
Probable E3 ubiquitin-protein ligase TRIP12	TRIPC_HUMAN
Proline-, glutamic acid- and leucine-rich protein 1	PELP1_HUMAN
Proline-rich AKT1 substrate 1	AKTS1_HUMAN
Proline-rich protein 15	PRR15_HUMAN
Protein arginine N-methyltransferase 3	ANM3_HUMAN
Protein capicua homolog	CIC_HUMAN
Protein FAM83H	FA83H_HUMAN
Protein kinase C and casein kinase substrate in neurons protein 2	PACN2_HUMAN
Protein KRI1 homolog	KRI1_HUMAN
Protein PRRC2B	PRC2B_HUMAN
Protein PRRC2C	PRC2C_HUMAN

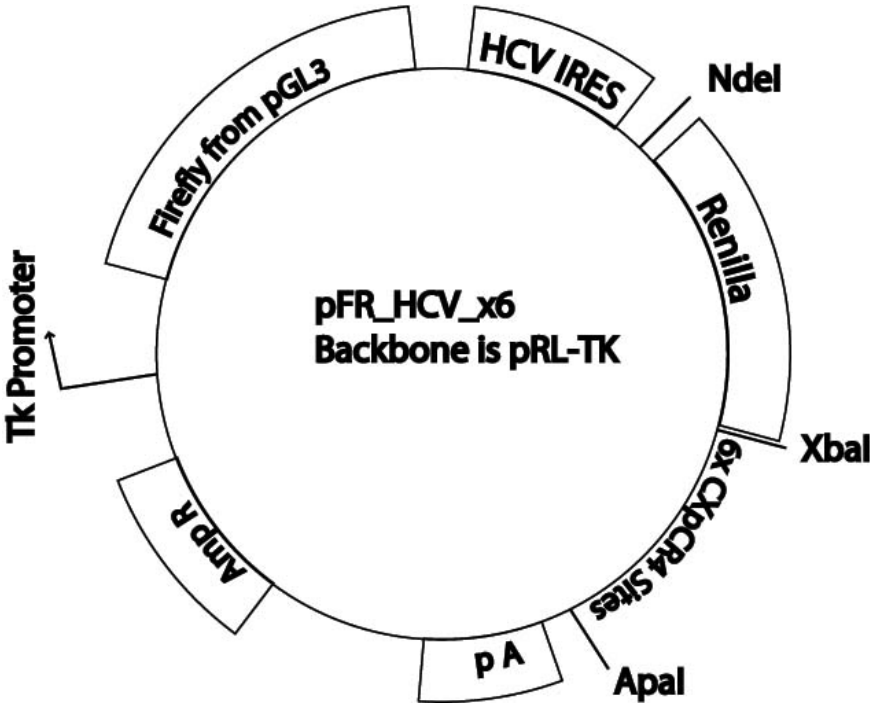
Protein scribble homolog	SCRIB_HUMAN
Protein strawberry notch homolog 1	SBNO1_HUMAN
Protein transport protein Sec61 subunit beta	SC61B_HUMAN
Putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX16	DHX16_HUMAN
Putative protein phosphatase inhibitor 2-like protein 1	IPP2L_HUMAN
Putative protein phosphatase inhibitor 2-like protein 3	IPP2M_HUMAN (+1)
Rab11 family-interacting protein 1	RFIP1_HUMAN
Ral GTPase-activating protein subunit alpha-1	RGPA1_HUMAN
Ras GTPase-activating protein-binding protein 1	G3BP1_HUMAN
Ras-related protein R-Ras2	RRAS2_HUMAN
Regulation of nuclear pre-mRNA domain-containing protein 1B	RPR1B_HUMAN
Regulator of microtubule dynamics protein 3	RMD3_HUMAN
Reticulon-3	RTN3_HUMAN
Reticulon-4	RTN4_HUMAN
Retinoic acid-induced protein 3	RAI3_HUMAN
Rho GTPase-activating protein 10	RHG10_HUMAN
Rho GTPase-activating protein 12	RHG12_HUMAN
Rho GTPase-activating protein 17	RHG17_HUMAN
Rho guanine nucleotide exchange factor 5	ARHG5_HUMAN
Ribosomal L1 domain-containing protein 1	RL1D1_HUMAN
RING finger protein 113A	R113A_HUMAN
RNA-binding protein 39	RBM39_HUMAN
RNA-binding protein NOB1	NOB1_HUMAN
RNA-binding protein Raly	RALY_HUMAN
rRNA/tRNA 2'-O-methyltransferase fibrillarin-like protein 1	FBLL1_HUMAN
Septin-2	SEPT2_HUMAN
Septin-9	SEPT9_HUMAN
Serine/arginine repetitive matrix protein 1	SRRM1_HUMAN
Serine/arginine repetitive matrix protein 2	SRRM2_HUMAN
Serine/threonine-protein kinase B-raf	BRAF_HUMAN
Serine/threonine-protein kinase PAK 4	PAK4_HUMAN
Serine/threonine-protein kinase PRP4 homolog	PRP4B_HUMAN
Serine/threonine-protein kinase SRPK1	SRPK1_HUMAN
Serine/threonine-protein kinase TAO3	TAOK3_HUMAN
Signal recognition particle receptor subunit alpha	SRPR_HUMAN
Sister chromatid cohesion protein PDS5 homolog B	PDS5B_HUMAN
Small acidic protein	SMAP_HUMAN
Sodium/hydrogen exchanger 1	SL9A1_HUMAN
Spectrin beta chain, brain 1	SPTB2_HUMAN

Splicing factor 1	SF01_HUMAN
Splicing factor 3B subunit 2	SF3B2_HUMAN
Splicing factor, arginine/serine-rich 19	SFR19_HUMAN
Src substrate cortactin	SRC8_HUMAN
Stathmin	STMN1_HUMAN
Striatin	STRN_HUMAN
Structural maintenance of chromosomes protein 4	SMC4_HUMAN
Sulfotransferase family cytosolic 2B member 1	ST2B1_HUMAN
Supervillin	SVIL_HUMAN
Survival motor neuron protein	SMN_HUMAN
SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5	SMCA5_HUMAN
Synapse-associated protein 1	SYAP1_HUMAN
Synembryn-A	RIC8A_HUMAN
Target of Myb protein 1	TOM1_HUMAN
TBC1 domain family member 9B	TBC9B_HUMAN
Telomeric repeat-binding factor 2-interacting protein 1	TE2IP_HUMAN
Tensin-3	TENS3_HUMAN
Thioredoxin-related transmembrane protein 1	TMX1_HUMAN
Thyroid hormone receptor-associated protein 3	TR150_HUMAN
Tight junction protein ZO-1	ZO1_HUMAN
Tight junction protein ZO-2	ZO2_HUMAN
Tight junction protein ZO-3	ZO3_HUMAN
Tight junction-associated protein 1	TJAP1_HUMAN
Torsin-1A-interacting protein 1	TOIP1_HUMAN
TRAF-type zinc finger domain-containing protein 1	TRAD1_HUMAN
Transcription intermediary factor 1-beta	TIF1B_HUMAN
Transcriptional activator protein Pur-beta	PURB_HUMAN
Transmembrane and coiled-coil domains protein 1	TMCC1_HUMAN
Transmembrane protein 40	TMM40_HUMAN
Treacle protein	TCOF_HUMAN
Triosephosphate isomerase	TPIS_HUMAN
Tripartite motif-containing protein 16	TRI16_HUMAN
tRNA (cytosine-5-)-methyltransferase NSUN2	NSUN2_HUMAN
Tumor protein D54	TPD54_HUMAN
Tumor suppressor p53-binding protein 1	TP53B_HUMAN
U3 small nucleolar RNA-associated protein 14 homolog A	UT14A_HUMAN
U3 small nucleolar RNA-associated protein 18 homolog	UTP18_HUMAN
Ubiquitin carboxyl-terminal hydrolase 10	UBP10_HUMAN
Ubiquitin-associated protein 2-like	UBP2L_HUMAN

Uncharacterized protein C19orf21	CS021_HUMAN
UPF0414 transmembrane protein C20orf30	CT030_HUMAN
UV excision repair protein RAD23 homolog B	RD23B_HUMAN
Vinexin	VINEX_HUMAN
WD repeat-containing protein 44	WDR44_HUMAN
WW domain-containing adapter protein with coiled-coil	WAC_HUMAN
Yorkie homolog	YAP1_HUMAN
Zinc finger and BTB domain-containing protein 7A	ZBT7A_HUMAN
Zinc finger CCCH-type antiviral protein 1	ZCCHV_HUMAN
Zinc finger FYVE domain-containing protein 19	ZFY19_HUMAN
Zinc finger protein 185	ZN185_HUMAN
Zyxin	ZYX_HUMAN

Proteins identified by phospho-proteomic analysis whose phosphorylation levels do not significantly change after gefitinib treatment. Phosphopeptides were matched to proteomic databases for protein identification using Scaffold software. The amount of phosphorylation was compared between the DMSO vehicle control and gefitinib treated cells.

Supplemental Figure 2: 11510:pFR_HCV_xb Dual reporter luciferase plasmid map.



The dual-reporter luciferase plasmid selectively measures cap-dependent translation through Firefly luciferase and cap-independent translation through Renilla luciferase.

Supplemental Table 2: Mutational status of common genes.

	BT20	MDA-MB-231	MDA-MB-468
p53	mut	mut	mut
BRCA	wt	wt	wt
PTEN	wt	wt	loss
PIK3CA	mut	wt	wt
Kras	wt	mut	wt
Hras	wt	wt	wt
Braf	wt	mut	wt
p16	del	del	wt
p14ARF	del	del	wt
rb1	wt	wt	del
chek2	wt	wt	wt
myc	wt	wt	wt

Common genetic mutations and losses are described in the table arranged by TNBC cell type. mut indicates a mutation, del a deletion, and wt indicates the gene is wild type.

Supplemental Methods:

Phospho- Proteomics Analysis

BT20 cells were washed with ice cold HANK's solution then proteins precipitated with 100% EtOH before cell proteins were scraped from plates and transferred to microcentrifuge tubes. Proteins were solubilized in 0.2 ml of Tris, 10 mM pH=7.5, LiF, 1 mM, Na_3VO_4, 0.1 mM, EDTA (Ethylenediaminetetraacetic acid) 1 mM and LiDS (Lithium Dodecyl Sulfate) 0.5%. Non-soluble material was removed by filtration through Spin Columns (Pierce, Rockford, IL, USA) and protein content determined by BCA protein assay. Samples were reduced with 10 mM dithiothreitol, alkylated with 30 mM iodoacetamide and 900 µg protein digested with 1:100 TPCK-treated trypsin (Sigma) after dilution to reduce LiDS concentration to 0.1% and addition of 10% acetonitrile. Samples were filtered through 0.45 µm 13 mm GHP filters (Pall, Port Washington, NY, USA) then phosphopeptides selected by incubation with 6 mg/sample TiO_2 beads (GL Sciences, Torrance, CA, USA, 5 µm).

The selectivity of the 6 mg of 5 µM TiO_2 procedure for phosphopeptides was 95%. Cell digests were incubated with TiO_2 in 2% TFA (Trifluoroacetic acid) saturated with glutamic acid in 60% acetonitrile. The beads were washed three times with 1% TFA in 60% acetonitrile before eluting phosphopeptides with NH_4OH in 50% acetonitrile. TiO_2 elutes were neutralized with formic acid, dried under vacuum and stored at -80°C until analysis. Eluted peptides solubilized in 0.1% formic acid were then analyzed by LC-MS/MS (Liquid chromatography–mass spectrometry) without further purification.

Mass Spectrometry: All analyses were performed on a Thermo LTQ equipped with ETD (electron-disassociation transfer) (ThermoFisher Scientific, Watham, MA, USA). Samples were loaded on a peptide Captrap (Michrom, Auburn, CA, USA) trapping column and peptide separations achieved using a linear gradient of 5% to 35% acetonitrile to elute from a Majic 0.1 mm x 150 mm AQ C18 column (Michrom). LC-MS/MS was run in a neutral loss mode so that high abundance precursor neutral losses of 24.25, 32.66, or 49.00 m/z found in an MS2 spectrum were selected for MS3 analysis.

Database Searching: Tandem mass spectra were extracted by Proteome Discoverer (ThermoFisher Scientific) version 1.4.0.288. Charge state deconvolution and deisotoping were not performed. All MS/MS data were analyzed using Mascot (Matrix Science, London, UK; version 2.4.0) and X! Tandem (The GPM, thegpm.org; version CYCLONE (2010.12.01.1)). Mascot and X!Tandem were each set up to search the uniprot_sprot_20110405 database (selected for Homo sapiens, 20305 entries) assuming the digestion enzyme trypsin. Spectra were searched with a fragment ion mass tolerance of 0.70 Da and a parent ion tolerance of 3.5 Da. The iodoacetamide derivative of cysteine was specified as a fixed modification. Oxidation of methionine, acetylation of the N-terminus and phosphorylation of serine, threonine and tyrosine were specified as variable modifications.

Criteria for Protein Identification: Scaffold (version 4.0.5, Proteome Software Inc., Portland, OR, USA) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Peptide Prophet algorithm.(Keller et al., 2002)

Protein identifications were accepted if they contained at least one identified phosphopeptide and had a Protein Prophet probability greater than 80%.(Nesvizhskii et al., 2003) Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. All subsequent analysis of protein sets included all proteins or peptides that met the criteria for identification without weighting for the level of confidence in the identification.

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ABSTRACT

COMBATING RESISTANCE TO EPIDERMAL GROWTH FACTOR RECEPTOR INHIBITORS IN TRIPLE NEGATIVE BREAST CANCER

by

JULIE M MADDEN

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Advisor: Dr. Julie L Boerner

Co-Advisor: Dr. Raymond R Mattingly

Major: Cancer Biology

Degree: Doctor of Philosophy

Triple negative breast cancer (TNBC) patients suffer from a highly malignant and aggressive cancer that lacks an effective targeted therapeutic. Although many TNBCs, both in vitro and in vivo, have increased expression of epidermal growth factor receptor (EGFR), EGFR targeted inhibitors, such as gefitinib (GEF), have yet to demonstrate efficacy. Using mass spectrometry to identify pathways that remain activated in the presence of GEF, we found that components of the mTOR signaling pathway remain phosphorylated. While inhibiting mTOR with temsirolimus (TEM) decreased mTOR signaling, EGFR signaling pathways remained activated and the TNBC cell lines continued to proliferate. However, dual treatment with TEM and GEF synergistically decreased cell viability in TNBC cells. Interestingly, abrogation of both EGFR and mTOR signaling did not alter the phosphorylation of key growth signaling molecules including MAPK and AKT. Instead, our data have identified the translational control pathway, specifically, eIF4B as a potentially key regulatory point in EGFR and mTOR inhibitor synergy. Further, we have also identified the transcription factor, STAT3 as

another regulatory point in the EGFR and mTOR inhibitor synergy. Therefore, in this study we hypothesized that mTOR inhibition can sensitize TNBC cells to EGFR TKIs through the inhibition of eIF4B and STAT3 phosphorylation.

eIF4B enhances the helicase activity of eIF4A during translation initiation. As expected, knockdown of eIF4B expression decreased cell viability comparable to the decrease observed with the combination treatment. Importantly, we have identified p70S6K and p90RSK as kinases directly responsible for eIF4B phosphorylation, such that both molecules need to be inactivated in order for eIF4B phosphorylation to be abrogated. This inactivation correlated with a loss of cell growth and viability and a decrease in clonogenic cell survival, potentially through alterations in the cell cycle. Furthermore, cap-dependent translation was inhibited to a greater extent in the combination treatment than GEF or TEM alone. Taken together these data suggest that EGFR and mTOR inhibitor combination abrogates cell growth, viability, and survival via disruption of translational control mechanisms through eIF4B.

STAT3 is a widely considered oncogenic transcription factor that has been implicated in a variety of cancer types. We found a decrease in phospho-STAT3 with the GEF+TEM combination. Further DNA binding ELISAs found STAT3 activity was also significantly decreased with the combination. Overexpression of a constitutively active STAT3 plasmid found that STAT3 activation negates the GEF+TEM synergetic effect on cell viability. Together, these studies suggest a role for STAT3 in EGFR and mTOR inhibitor synergy.

Taken together these data suggest that in the presence of activated MAPK and AKT, EGFR and mTOR inhibitors abrogate growth, viability, and survival via disruption

of eIF4B and STAT3 phosphorylation leading to decreased translation and transcription factor DNA binding, respectively, in TNBC cell lines. The effect translation has on cancer cells in regard to the mTOR and EGFR pathways is largely unexplored in TNBC and further implicates eIF4B as a protein of interest in understanding the gefitinib and temsirolimus synergy. TNBC patients currently have limited treatment options and our data suggest that including an mTOR inhibitor along with an EGFR inhibitor in TNBC with increased EGFR expression should be further explored.

AUTOBIOGRAPHICAL STATEMENT

Education

Wayne State University Detroit, MI
Doctor of Philosophy: Cancer Biology

Hope College Holland, MI
2010 *Bachelor of Science*, Biology

Publications

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Abstracts

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