THE ROLE OF SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 1 (STAT1) AND 3 (STAT3) IN PRIMARY AND METASTATIC BREAST CANCER

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For my parents who helped me in all things great and small

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ABSTRACT

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Breast cancer is the most frequently diagnosed malignancy and the second most lethal cancer in women. Metastasis in breast cancer is invariably responsible for patient death and is comprised of many steps, of which proliferation in vital organs is responsible for morbidity and mortality due to vital organ failure. Patients with the metastatic disease are limited to chemotherapy, which non-specifically targets proliferating cells. Despite it being initially effective, chemotherapy is associated with high toxicity and many patients develop resistance. Thus, there is an urgent need to characterize the biology of metastatic breast cancer to develop targeted therapies for the late-stage disease.

EGFR is a member of the ErbB family of receptor tyrosine kinases, which have particular relevance in breast tumorigenesis. Clinical studies show that high expression levels of EGFR in the primary mammary tumors correlate with poor prognosis and decreased survival of breast cancer patients due to metastasis. Patient data is supported by experimental and pre-clinical studies, which describe various signaling pathways that mediate the oncogenic effects of EGFR, such as the MAPK, STAT3, and PI3K pathways. Despite these well-documented roles of EGFR, clinical trials evaluating EGFR inhibitors (EGFRi) in metastatic breast cancer have been unanimously unsuccessful in improving patient prognosis, and the mechanisms that contribute to this intrinsic resistance are unknown.

To characterize the signaling events that govern EGFR behavior in metastatic breast cancer resistant to EGFRi, we utilized multiple pre-clinical breast cancer progression series and patient-derived cells that display the intrinsic resistance phenomenon. In these models, EGFR functions as a pro-apoptotic molecule whose ligand-mediated activation results in growth inhibition and/or apoptosis of metastatic breast cancer cells. Here we show that in the later stages of metastasis, increased nuclear translocation of EGFR leads to increased physical access to STAT1 and STAT3 molecules residing in the nucleus. Indeed, an EGFR mutant that is defective in endocytosis is

unable to elicit STAT1/3 phosphorylation. Additionally, specific inhibition of nuclear EGFR function using the EGFR kinase inhibitor gefitinib linked to a nuclear localization signal (NLS-gefitinib) prevents EGF-induced STAT1/3 phosphorylation. Altogether, these findings implicate nuclear localization of EGFR in downstream STAT1/3 signaling in metastatic breast cancer.

Subsequently, we examined the involvement of nuclearly-activated STAT1/3 signaling in the apoptotic function of EGFR. NLS-gefitinib treatment or genetic/pharmacologic inhibition of STAT1/3 efficiently blocks EGF-induced apoptosis in metastatic breast cancer cells resistant to EGFRi. These findings were utilized therapeutically by activating EGFR with EGF treatment while simultaneously blocking the downstream proliferative MAPK:ERK1/2 pathway using the MEK1/2 inhibitor trametinib. EGF + trametinib combination preserved STAT1 signaling while effectively blocking the MAPK pathway, thus potentiating EGF-mediated apoptosis in metastatic breast cancer cells. Importantly, combined administration of trametinib and EGF resulted in STAT1-mediated apoptosis of primary mammary tumor cells, which respond to EGF in a proliferative fashion. These data provide a novel approach of targeting metastatic breast cancer by biasing EGFR signaling towards nuclear activation of STAT1/3 signaling resulting in apoptosis.

Our studies herein also examined the role of STAT3 in primary mammary tumor cells overexpressing EGFR. Depletion of STAT3 expression normalized the transformed phenotype of these cells *in vitro* and resulted in smaller mammary tumors *in vivo*. These results implicate STAT3 in EGFR-driven breast tumorigenesis localized to the mammary tissues. Further, systemic dissemination of breast cancer is associated with activation of the JAK1/2:STAT3 signaling axis. Despite the involvement of STAT3 in EGFR-mediated oncogenesis in the primary tumor setting, targeting JAK1/2:STAT signaling with the JAK1/2 inhibitor ruxolitinib proved ineffective in inhibiting the growth and invasion of metastatic cells derived from these primary tumors. These results are in agreement with the role of STAT1/3 in driving the pro-apoptotic function of EGFR in metastatic breast cancer cells. Altogether, these investigations provide a plausible explanation for the inability of JAK1/2 inhibitors to effectively target metastatic breast cancer in clinical and experimental investigations. Further, these findings indicate that the development of therapeutics or molecular tools that efficiently activate STAT1/3 signaling in metastatic breast cancer may represent an important concept for eradicating tumors resistant to targeted therapies.

CHAPTER 1. INTRODUCTION

(Extracted from the below publications:

- *Ali R, Wendt MK. The paradoxical functions of EGFR during breast cancer progression. Signal transduction and targeted therapy. 2017;2:16042.*
- *Ali R, et al. Targeting FGFR for the Treatment of Breast Cancer. In book: Resistance to Targeted Therapies in Breast Cancer. December 2017)*

1.1 Introduction to Breast Cancer (BC) and Subtypes

Breast cancer (BC) is the most commonly diagnosed and the second most lethal cancer in women (Torre et al., 2015). Metastasis is invariably responsible for patient mortality in BC (Redig & McAllister, 2013). Indeed, BC patients presenting with non-metastatic tumors at the time of diagnosis have 94-98% of cause-specific 5-year survival; however, this percentage drops to less than 40% for patients presenting with disseminated tumors (DeSantis et al., 2016). BC metastasis is comprised of many steps, of which proliferation at vital organs is responsible for morbidity and mortality due to vital organ failure (Steeg, 2016). Treatment of metastatic BC is limited to chemotherapy, which targets hyper-proliferating cells. Despite it being initially effective, chemotherapy is associated with high toxicity and many patients develop resistance (Uhm et al., 2009). These observations underscore the urgent need to identify the oncogenic drivers of metastatic BC and translate this knowledge into targeted strategies for clinical intervention.

BC is a heterogeneous disease that is conventionally classified by pathological features such as tumor grade, size and node status, and by immunohistochemistry for estrogen receptor alpha (ER-α), progesterone receptor (PR), and human epidermal growth factor receptor 2 (Her2) receptor. Since the late eighties, these tools have provided information regarding therapeutic decision-making and patient prognosis. For example, breast cancer patients that express ER-α were reported by Fisher *et al.* to particularly benefit from anti-estrogens (1989). Moreover, Slamon *et al.* showed that Her2 overexpression correlates with aggressive behavior in breast and ovarian cancers (1989). Despite the clinical utility of these classification systems, accumulating evidence has suggested tumors with similar histological characteristics do not necessarily follow the same pathologic progression and display differential responses to similar treatments. Thus, ongoing

research has aimed to understand the heterogeneity of breast cancer subtypes and uncover druggable molecular targets for more accurate subtyping of breast cancers and effective therapeutic choice making.

Molecular subtyping of breast cancer was described using cDNA microarrays that established the underlying diversity in gene expression patterns from various patient-derived breast tumors and cell-lines (Hu et al., 2006; Perou et al., 2000; Sørlie et al., 2001; Sotiriou et al., 2003). These distinctive molecular portraits of breast cancer subtypes were correlated with the traditional histological classifications to create the breast cancer subtypes: luminal A, luminal B, Her2-enriched, basal-like, and claudin-low (Prat et al., 2010; Sørlie et al., 2001). The prognostic value of this subtyping was significantly improved by Parker *et al.* who introduced a 50-gene set that predicts patient outcome and responsiveness to chemotherapy, known as the Prediction Analysis of Microarray 50 (PAM50) (Parker et al., 2009). Essentially, the PAM50 is a gene-list that faithfully differentiates breast cancer subtypes without the need for full genomic analyses (Bastien et al., 2012). Recently, the PAM50 is beginning to be applied clinically as NanoString and Prosigna have developed a clinical diagnostic based around the analysis of the PAM50 leading to a Prosigna score that correlates to tumor subtype and prognosis (Wallden et al., 2015). This and other molecular diagnostics such as MammaPrint® and OncotypeDX® serve to better stratify patients and are beginning to strongly influence treatment decisions. Overall, the classification system of breast cancer continues to evolve to generate new subtypes and refine existing ones (Cancer Genome Atlas Network, 2012; Ciriello et al., 2015; Curtis et al., 2012; Ellis & Perou, 2013).

1.2 Introduction to Resistance to Targeted Therapies in BC

The past three decades have witnessed the emergence of targeted therapeutics in clinical and translational breast cancer research. As the term implies, targeted therapies act by inhibiting very specific characteristics needed for tumor cell growth and survival, in contrast to traditional chemo- and radio- therapies, which less specifically target hyper-proliferating cells. By this mechanism, targeted therapies have a lower incidence of toxic side effects and a larger therapeutic index than chemotherapy. In recent years, clinical application of targeted therapies has essentially tested the theory of oncogene addiction. Fundamentally, oncogene addiction states that despite their diverse array of genetic aberrations, tumor cells depend on one dominant oncogene for

maintenance of malignant potential, metastatic spread, and resistance to cytotoxic stress (reviewed in (Torti & Trusolino, 2011)).

These observations fueled intense investigations to identify and target driver oncogenes in order to halt cancer progression and improve patient prognosis. Indeed, these efforts have resulted in the successful design and formulation of various targeted therapies for the treatment of breast cancer in the form of small-molecule inhibitors and monoclonal antibodies (Masoud & Pagès, 2017). Despite the initial success of many of these agents, breast cancer cells acquire resistance to molecularly-targeted therapies by reactivating the inhibited oncogenic pathway or switching to alternative pathways for survival. Further, there are numerous reports of inherent resistance in breast cancer where targeting oncogenes identified from primary tumor analysis does not yield clinical benefit for that patient in the metastatic setting (Ali & Wendt, 2017; Ellis & Hicklin, 2009). Overall, understanding the molecular plasticity that underlies both acquired and inherent resistance is of tremendous importance to reduce mortality due to metastatic breast cancer.

1.3 Luminal Breast Cancer

The luminal A and B subtypes account for more than 60% of breast cancer cases, and while they differ in their gene expression profiles and prognosis, luminal A and B cells express ER-α and PR. Luminal A is the most common (~40% of all cases) and generally correlates with a lower proliferative index and good overall prognosis. Thus, current guidelines suggest luminal A patients receive endocrine therapy and be spared chemotherapy (Goldhirsch et al., 2011). Indeed, multiple studies have demonstrated that the use of endocrine therapy in luminal A patients correlates with lower recurrence rates and is more beneficial than chemotherapy. Thus, anti-estrogens are likely to remain the first-line treatment option for luminal A breast cancer (Albain et al., 2010; Dowsett et al., 2010; Paik et al., 2004, 2006).

The luminal B subtype accounts for ~20% of all breast cancer subtypes and is characterized by relatively lower ER-α expression, increased proliferation as measured by Ki67 staining, and poorer prognosis as compared to luminal A (Sotiriou & Pusztai, 2009). Unlike the luminal A subtype, luminal B breast cancer has been shown to be more sensitive to chemotherapy than endocrine therapies (Wirapati et al., 2008). This observation prompted investigations to identify molecular pathways for efficient drug development. This biomarker identification remains crucial to uncovering molecular targets for luminal B breast cancer as Ki67 staining and

interpretation, the cornerstone of distinguishing luminal A and B subtypes, is known to be associated with significant variability that may impede the accurate classification of luminal A versus B and thus choice of endocrine versus chemotherapy (Polley et al., 2013). Broader application of the PAM50 will help to alleviate much of this misdiagnosis, but additional targeted therapies for the luminal B subtype are still needed.

1.4 Acquired and Intrinsic Resistance in Luminal Breast Cancer

Extended adjuvant therapy with aromatase inhibitors after ER-α inhibitors prolongs disease-free progression in luminal breast cancer (Goss et al., 2003; Jakesz et al., 2007). However, both inherent and acquired resistance to endocrine therapy has been reported in metastatic luminal breast cancer. An established mechanism of acquired resistance to ER-α antagonists in initially responsive patients is the downregulation of ER-α where these tumors become independent of estrogen signaling for survival (Encarnación et al., 1993). Inherent resistance to endocrine therapy involves loss of PR in metastatic tumors (Branković-Magić, Janković, Nesković-Konstantinović, & Nikolić-Vukosavljević, 2002). Despite these established mechanisms, molecular tools are needed to prospectively predict patient groups that will exhibit resistance. Further, the oncogenic drivers that allow for primary versus metastatic discordance in ER-α expression are yet to be identified for luminal breast cancers.

1.5 Her2-Enriched Breast Cancer

Her2-enriched breast cancer constitutes 15-20% of all breast cancer subtypes, and as the name implies is characterized by high expression levels of Her2. Her2 is a member of the ErbB family of receptor tyrosine kinases (RTKs) and a well-established proto-oncogene. The molecular mechanisms of Her2-mediated oncogenesis are complex and involve receptor oligomerization leading to constitutive receptor activity and activation of downstream signaling cascades to induce cell-proliferation, invasion, and metastasis (reviewed in (Moasser, 2007)). Given these findings, kinase inhibitors and monoclonal antibodies (mAbs) have been formulated to target Her2 expressing tumors. Trastuzumab was the first Her2-targeted mAb to be approved by the Food and Drug Administration (FDA) in combination with chemotherapy as adjuvant therapy for Her2-overexpressing breast cancer patients with nodal involvement (Perez et al., 2014). Trastuzumab

binding to Her2 inhibits intracellular signaling and triggers cellular toxicity (reviewed in (Spector & Blackwell, 2009)). Pertuzumab is also a Her2-targeting mAb that binds a different domain of Her2 (Cho et al., 2003; Franklin et al., 2004; Ng, Lum, Gimenez, Kelsey, & Allison, 2006). Recently, pertuzumab in combination with trastuzumab and chemotherapy was FDA-approved for the treatment of metastatic Her2-overexpressing breast cancer. Trastuzumab has also been chemically linked to emtansine, a powerful chemotherapeutic, to produce an antibody-drug conjugate known as T-DM1 that effectively delivers emtansine specifically to Her2 overexpressing cells (Barok, Joensuu, & Isola, 2014; Lewis Phillips et al., 2008; Phillips et al., 2014). In addition to mAbs, several kinase inhibitors have also been developed for targeting Her2 and other ErbB members. Lapatinib competitively inhibits both Her2 and EGFR and was the first FDA approved kinase inhibitor for Her2-amplified advanced breast cancer used in combination with chemotherapy (Di Leo et al., 2008; Geyer et al., 2006). Other recently developed kinase inhibitors of Her2 and other ErbB family members include the drugs neratinib and afatinib, which covalently inhibit Her2 and EGFR as well as Her4, which is another member of the ErbB family. Neratinib has been shown to significantly increase disease-free survival in Her2-overexpressing breast cancer patients that had previously received trastuzumab-chemotherapy combination or trastuzumab alone (Burstein et al., 2010; Chan et al., 2016). Overall, targeting the ErbB family in Her2-enriched breast cancer has revolutionized the treatment of patients of the Her2-subtype.

1.6 Acquired and Intrinsic Resistance in Her2-enriched Breast Cancer

Despite the success of Her2-targeted therapies, clinical resistance remains a substantial problem. Studies have described mechanisms that alter Her2 isoform or co-receptor expression leading to inhibition of trastuzumab binding as a potential mechanism of resistance (Price-Schiavi et al., 2002; Spector & Blackwell, 2009). Furthermore, resistance to trastuzumab has been demonstrated to result from activation of an interleukin 6 (IL6) signaling loop that essentially results in subtype switching to a triple-negative breast cancer (TNBC) phenotype, which is characterized by the absence of ER-α, PR and Her2 expression (Burnett et al., 2015; Korkaya et al., 2012). Similarly, resistance to lapatinib has recently been linked to general kinome reprogramming, leading to the activation of several alternate growth pathways (Stuhlmiller et al., 2015). In attempts to overcome these mechanisms, a recent clinical trial utilized neratinib as extended adjuvant therapy after completing trastuzumab standard therapy, which demonstrated a

significant increase in disease-free survival (ExteNET Trial) (Chan et al., 2016). These findings suggest that other ErbB family members that are not targeted by trastuzumab may be at play in facilitating resistance. Finally, as is the case with ER-α expression in luminal breast cancer, primary versus metastatic tumor discordance has also been described for Her2 and is an intuitive mechanism of resistance to Her2-targeted therapies (Niikura et al., 2012). Currently, the mechanism responsible for Her2 discordance and the emergence of new oncogenic drivers that accompany this phenomenon are yet to be established.

1.7 Basal-like Breast Cancer

The basal-like subtype accounts for ~20% of breast cancer and is characterized by increased expression of basal/myoepithelial markers (cytokeratins 5/6, 14, & 17) and epidermal growth factor receptor 1 (EGFR1 or EGFR) (Ciriello et al., 2015; Wallden et al., 2015). While there is yet no unified positive definition of this subtype, the basal-character conversely correlates with the lack of ER-α, PR, and Her2 amplification, and thus the basal-like term is often used interchangeably with TNBC (characteristics of basal-like breast cancer extensively reviewed in (Rakha, Reis-Filho, & Ellis, 2008)). Being a diagnosis of exclusion, TNBC has the worst prognosis of all breast cancer subtypes as it lacks targeted therapies (Sorlie et al., 2003). Indeed, chemotherapy remains the mainstay of treatment for patients with the basal/TNBC subtype as it has been shown to be more sensitive to neoadjuvant chemotherapy compared to the luminal subtypes (Rakha, El-Sayed, Reis-Filho, & Ellis, 2009; Rakha et al., 2008). However, TNBC is characterized by a higher incidence of breast cancer type 1 susceptibility protein (BRCA1) mutations (Haffty et al., 2006). BRCA1 along with Poly ADP-ribose polymerase (PARP) enzymes have critical roles in DNA-damage repair. Thus, TNBC patients with BRCA1 mutations are particularly sensitive to PARP inhibitors. Indeed, PARP inhibitors have recently been approved for BRCA1 mutant ovarian cancer and clinical trials are currently ongoing evaluating PARP inhibition in the context of BRCA1 mutant TNBC (ClinicalTrials.gov Identifier: NCT 02032823) (Faraoni & Graziani, 2018). Another interesting finding is that TNBC is enriched for mutations in the tumor suppressor p53. Given the participation of p53 in cell-cycle arrest and induction of apoptosis in response to DNA damage, p53 mutant TNBC cells proceed in the cell cycle in the presence of DNA damage resulting from chemotherapy. These observations prompted the initiation of trials assessing the efficacy of treating TNBC with cyclin-dependent kinase (CDK)

inhibitors followed by a DNA-damaging chemotherapeutic. Indeed, this sequential combination was shown to induce synthetic lethality in TNBC cells resulting in favorable patient response compared to either drug alone (Jabbour-Leung et al., 2016). Finally, ongoing trials are also currently evaluating immune checkpoint inhibitors in the treatment of metastatic TNBC (NCT02555657). TNBC has been sub-classified into five clinically relevant subtypes: the basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), and the luminal androgen receptor (LAR) subtype (Lehmann et al., 2011). Further molecular characterizations such as these will continue to drive diagnostic criteria for appropriate stratification of patients into groups that will best respond to developing therapies.

1.8 Acquired and Intrinsic Resistance in Basal-like Breast Cancer

While basal-like breast cancer is initially sensitive to chemotherapy, patients often relapse (Uhm et al., 2009). The mechanisms of this acquired resistance are likely to be several-fold, but a major theme is an overexpression or activation of ATP-binding cassette (ABC) transporters capable of efflux of chemotherapies from the cell (O'Reilly et al., 2015). EGFR overexpression is characteristic of basal-like/TNBC and has been intensely investigated as a potential candidate for targeted therapies, given its well-established oncogenic roles in other types of cancer. Yet, clinical trials assessing the effectiveness of EGFR kinase inhibitors and monoclonal antibodies have failed to improve outcomes of EGFR-positive BC patients with metastatic disease. The mechanism of this intrinsic resistance to EGFR inhibitors remains largely unknown; however studies from our lab suggest a loss of EGFR expression and function in the metastatic setting in favor of fibroblast growth factor receptor (FGFR)-driven tumor growth (Wendt, Taylor, Schiemann, Sossey-Alaoui, & Schiemann, 2014; Wendt et al., 2015).

1.9 Intrinsic Resistance to EGFR Inhibitors in Metastatic BC and the EGFR paradox

Epidermal growth factor receptor (EGFR) is the first discovered of the ErbB family of receptor tyrosine kinases which includes a total of four members: ErbB1/EGFR, ErbB2/Her2, ErbB3, and ErbB4 (Cohen, Fava, & Sawyer, 1982). ErbB members form homo- and heterodimeric cell-surface receptors with unique extracellular domains yielding ligand-binding specificity. Downstream signaling from these receptors proceeds via tyrosine-phosphorylation (Yarden &

Sliwkowski, 2001). Since its discovery, EGFR has been characterized as a mediator of a wide variety of signal transduction events that control cell proliferation, migration, and survival. Overexpression of EGFR transforms NIH3T3 fibroblasts in an EGF-dependent manner (Di Fiore et al., 1987). Aberrant EGFR activation in tumor cells can result from increased transcriptional expression and/or gene amplification. Increased EGFR protein and transcript levels correlate with poor prognosis in various epithelial cancers, such as colorectal cancer (CRC) (Spano et al., 2005), non-small cell lung cancer (NSCLC) (Brabender et al., 2001), endometrial cancer (Konecny et al., 2009), and squamous-cell carcinoma of the head and neck (Rubin Grandis, Melhem, Barnes, & Tweardy, 1996). Another mode of EGFR activation in cancer is activating somatic mutations that result in constitutive kinase activity, these are particularly prevalent in NSCLC (reviewed in (Morgensztern, Politi, & Herbst, 2015)). These findings lead to numerous clinical trials to assess the efficacy of EGFR inhibitors in many of these cancers. Gefitinib is a small molecule EGFR kinase inhibitor that received accelerated approval from the FDA in 2003 but was pulled from the market due to lack of efficacy. These findings were the result of not selecting patients whose tumors contain EGFR activating mutations. Since then, it has been recognized that only NSCLC patients with activating mutations in EGFR respond to gefitinib. This has lead to the 2015 approval of gefitinib as a first-line therapy for NSCLC specifically in patients that test positive for activating EGFR mutations. The addiction of these tumors to EGFR signaling is further demonstrated by the emergence of the secondary activating T790M mutation as a major cause of the tumor resistance to gefitinib. This has resulted in the recent formulation and FDA approval of osimertinib, a compound capable of inhibiting T790M mutant EGFR (Jänne et al., 2015). These lessons in NSCLC have served as a critical example of the need for biomarkers to drive the application of kinase inhibitors to EGFR. However, less controlled clinical trials have demonstrated improved patient outcomes with EGFR inhibitors in unselected patients with pancreatic cancer (Moore et al., 2007), head and neck cancer (Vermorken et al., 2008) and colorectal cancer (Van Cutsem et al., 2007). Ultimately, these studies have led to the FDA approval of ligand blocking antibodies (cetuximab and panitumumab) for the treatment of colorectal and head and neck cancer. However, studies are still ongoing to determine other biomarkers that might improve patient selection for these cancers (Sunakawa et al., 2016).

1.10 Targeting EGFR in Metastatic Breast Cancer

Metastasis in breast cancer is invariably responsible for patient death. The triple-negative subtype (TNBC) is characterized by metastatic progression, poor patient prognosis and is identified by the absence of bio-molecules that form the basis for targeted therapies for the other BC subtypes; namely, estrogen receptor alpha, progesterone receptor, and Her2 amplification. Thus, there are currently no FDA approved targeted therapies for TNBC. TNBC is initially highly sensitive to chemotherapy, but many TNBC patients rapidly develop resistance at which point the metastatic disease is highly lethal (Lehmann, Pietenpol, & Tan, 2015). While activating mutations and gene amplification for EGFR are rare in BC, EGFR expression can be enhanced by increased gene copy number due to polysomy, and enhanced expression of EGFR in the primary tumor is associated with increased metastasis and decreased survival of TNBC patients (Park et al., 2014; Uhm et al., 2009). Concomitant with these clinical findings, studies from the Condeelis lab established a paracrine signaling loop in which macrophage-produced EGF supported tumor cell invasion and dissemination from the primary tumor (Tischkowitz et al., 2007; Wyckoff et al., 2004). Experimental findings such as these prompted the initiation of several clinical trials to assess the effectiveness of EGFR inhibition (EGFRi) in metastatic TNBC. The EGFR kinase inhibitor, erlotinib was evaluated in a Phase II trial of unselected patients with advanced BC having had previously received chemotherapy (Patsialou et al., 2009). Additionally, erlotinib was evaluated in combination with the anti-vascular endothelial growth factor (VEGF) antibody bevacizumab (Dickler, Cobleigh, Miller, Klein, & Winer, 2009). Both of these studies determined that erlotinib did not provide clinical benefits to patients and was not correlated with EGFR expression levels. Gefitinib is another EGFR-specific kinase inhibitor that has been evaluated in metastatic BC in multiple trials. A multicenter phase II study examined the outcomes of gefitinib treatment in unselected metastatic BC patients that had previously received standard chemotherapies. In all, 98.3% of these patients were non-responders and as above there was no correlation between EGFR expression and response to gefitinib (von Minckwitz et al., 2005). Similarly, gefitinib as a monotherapy in metastatic estrogen receptor alpha (ER-α) negative BC patients did not provide clinical benefit in another phase II clinical trial (Green et al., 2009). Engebraaten *et al.* tested the efficacy of combining gefitinib with docetaxel in metastatic BC as compared with docetaxel alone. In this study, the combination was associated with lower partial response rate and higher toxicity than chemotherapy alone (2012). In addition to kinase inhibitors,

clinical trials have also evaluated the addition of the ligand blocking monoclonal antibody cetuximab to the DNA-alkylating agent carboplatin (Carey et al., 2012). Similarly, this study found that fewer than 20% of metastatic TNBC patients responded to cetuximab plus carboplatin. In subsequent studies, the combination of cetuximab with antimicrotubule agents or topoisomerase inhibitors did not increase patient overall survival as compared with these chemotherapies alone, leading to premature trial termination (Crozier et al., 2016; Trédan et al., 2015). These findings have been confirmed in more recent trials examining the efficacy of panitumumab, another ligand-blocking anti-EGFR monoclonal antibody, in the treatment of TNBC. As with other EGFRi, panitumumab did not improve progression-free survival over chemotherapy alone when used in metastatic TNBC (Yardley et al., 2016). In contrast to these adjuvant trials in metastatic disease, the use of panitumumab in combination with chemotherapy did appear efficacious as neoadjuvant therapy for operable stage II–III TNBC (Nabholtz et al., 2014). Overall, despite strong pre-clinical data linking high levels of EGFR to increased metastatic progression and decreased patient survival, TNBC in the metastatic setting appears to be unresponsive to EGFRi (Table 1). The mechanisms of inherent resistance of metastatic BC to EGFRi remain to be fully established.

Table 1. A summary of clinical studies investigating EGFRi therapies for the treatment of breast cancer.

EGFRi class	Drug	Targeted BC patient group	Benefit over control	Reference
EGFR kinase inhibitors	Erlotinib	Phase II of locally advanced or metastatic BC as a monotherapy	No	Dickler et al 2009
	Erlotinib	Phase II of metastatic BC in combination with anti-VEGF mAb	No	Dickler et al 2008
	Gefitinib	Phase II of metastatic BC as a monotherapy	No	Minckwitz et al 2015
	Gefitinib	Phase II of metastatic ER-α negative BC as a monotherapy	No	Green et al 2009
	Gefitinib	Phase II of metastatic BC in combination with chemotherapy	No	Engebraaten et al 2012
EGFR ligand-blocking monoclonal antibody (mAb)	Cetuximab	Phase II of metastatic TNBC, in combination with chemotherapy	No	Carey et al 2012
	Cetuximab	Metastatic TNBC, in combination with chemotherapy	No	Trédan et al 2015
	Cetuximab	Phase II of metastatic BC in combination with chemotherapy	No	Crozier et al 2016
	Panitumumab	Phase II of metastatic TNBC, in combination with chemotherapy	No	Yardley et al 2016
	Panitumumab	Neoadjuvant therapy for operable primary TNBC	Yes	Nabholtz et al 2014

1.11 Potential Mechanisms of Intrinsic Resistance to EGFR inhibitors in Metastatic BC

1.11.1 Diminution of EGFR Expression with Metastatic Progression

Our lab recently developed a model in which overexpression of wild-type (WT) EGFR transforms normal murine mammary gland (NMuMG) cells (Balanis et al., 2013; Wendt, Smith, & Schiemann, 2010; Wendt, Taylor, et al., 2014; Wendt et al., 2015). This EGFR-driven tumor model forms well-differentiated *in situ* mammary tumors. Moreover, metastasis of these EGFR-transformed cells can be driven *in vivo* by transforming growth factor beta (TGF-β)-induced

epithelial-mesenchymal transition (EMT), a key step in BC invasion and metastasis (Wendt, Tian, & Schiemann, 2012). Consistent with the clinical trial data discussed above, these EGFR-driven primary tumors are very sensitive to the EGFR kinase inhibitor erlotinib, but metastatic tumors derived from the same cells demonstrated intrinsic resistant to erlotinib (Wendt et al., 2015). Mechanistically, *in vivo* metastatic selection of these EGFR-transformed cells and MDA-MB-231 cells is associated with a dramatic loss of EGFR expression (Wendt et al., 2015).

This discordance in EGFR expression is observed clinically, in human breast tumors (Choong et al., 2007), mouse models of metastatic colorectal cancer (Scartozzi et al., 2011), ovarian cancer (Pradeep et al., 2014), and lung cancer (Zohrabian et al., 2007). The first observation showing metastatic BC cells can have low to undetectable levels of EGFR was reported for the DU4475 (cutaneous metastasis) and AlAb 496 (lung metastasis) cell models in 1982 (Imai, Leung, Friesen, & Shiu, 1982). Since then, isogenic BC cell line series have been shown to demonstrate EGFR down-regulation through metastatic progression, including the MCF10AT BC progression series, and the D2-HAN series (Choong et al., 2007; Morris, Tuck, Wilson, Percy, & Chambers, 1993; Strickland, Dawson, Santner, & Miller, 2000; Wendt, Taylor, Schiemann, & Schiemann, 2011). In patient-derived BC tissues, EGFR is downregulated with metastasis and this correlates with resistance to EGFR inhibitors (Choong et al., 2007; Dittadi et al., 1993). Similarly, EGFR downregulation through promoter hyper-methylation has been linked to intrinsic resistance to anti-EGFR therapy in colorectal carcinoma (Scartozzi et al., 2011). In BC, however, the mechanism(s) of EGFR attenuation that are responsible for intrinsic resistance to EGFRi remain largely unknown.

1.11.2 EGFR Enhanced Nuclear Transport after Metastasis

EGFR is primarily localized to the plasma membrane, but numerous studies have demonstrated nuclear localization of EGFR where it can undergo several poorly understood functions that are both dependent and independent of kinase activity (Brand et al., 2014; Wang et al., 2012, 2010). Transport mechanisms and functions of nuclear EGFR are discussed in depth in (Brand et al., 2014). Importantly, increased nuclear transport of EGFR has been suggested as a potential mechanism of acquired-resistance to EGFR inhibitors. This was shown in studies demonstrating that long-term treatment of an NSCLC cell line with cetuximab generates cell-clones that have enhanced nuclear EGFR staining (Li, Iida, Dunn, Ghia, & Wheeler, 2009).

Similarly, in BC, nuclear EGFR has been attributed to intrinsic resistance to cetuximab and gefitinib using various TNBC cell-lines (Brand et al., 2014; Yu et al., 2013). Retrospective studies using patient-derived samples linking enhanced nuclear EGFR to clinical EGFRi resistance are yet to be performed. These investigations will be essential to confirm the role of nuclear localization of EGFR in resistance to EGFRi therapy. If differential subcellular localization of EGFR is truly at play during inherent resistance to EGFRi, establishing small molecule inhibitors that specifically localize to these compartments will be essential to understanding and targeting this mechanism in metastatic BC (Yu et al., 2013).

1.11.3 The Growth-inhibitory Function of EGFR

The first observation that EGF inhibits cancer-cell growth at concentrations that are stimulatory to other cells was reported for the rat pituitary GH4CI tumor cell-line and the human epidermoid carcinoma A431 cell-line (Barnes, 1982; Gill & Lazar, 1981; Schonbrunn, Krasnoff, Westendorf, & Tashjian, 1980). EGF inhibition of growth has also been demonstrated for human BC cell-lines, where higher concentrations of EGF decreased DNA synthesis in the MCF-7, SK-Br-3, BT-20, BT-474 cells (Imai et al., 1982). MDA-MB-468 is an EGFR amplified BC cell-line derived from a pleural effusion that is also known to display marked EGF-growth inhibition due to induction of apoptosis (Filmus, Trent, Pollak, & Buick, 1987; Prasad & Church, 1991). The A431 and MDA-MB-468 cell-lines have abnormally high levels of EGFR, and therefore the idea has been purported that the receptor must be present above a critical threshold to induced growth inhibition (Filmus et al., 1987; Kawamoto et al., 1984). However, this does not seem to be solely responsible for this phenomenon as EGF-induced inhibition of cell-growth occurs in various non-EGFR amplified cell-lines (Choi et al., 2010; Imai et al., 1982).

Further, EGF treatment stimulates the growth of several BC cell-lines expressing extremely high levels of EGFR (Carpenter & Cohen, 1979; Di Fiore et al., 1987; Wendt et al., 2015). Overall, the strongest body of literature supports that EGF growth-inhibitory action is largely due to induction of apoptosis. The mechanisms of EGF-induced apoptosis are still not fully understood but seem to involve signaling events that take place following receptor internalization potentially resulting in endosomal accumulation (Hyatt & Ceresa, 2008; Rush, Quinalty, Engelman, Sherry, & Ceresa, 2012). Additionally, EGFR-mediated activation of signal transducer and activator of transcription-1 (STAT1) has been shown to induce apoptosis by activation of caspases, induction

of elements of the interferon pathway, or by mediating cell-cycle arrest by activation of p-21 (Andersen et al., 2008; Chin et al., 1996; Grudinkin, Zenin, Kropotov, Dorosh, & Nikolsky, 2007; Hyatt & Ceresa, 2008; Kottke et al., 1999; Kozyulina, Okorokova, Nikolsky, & Grudinkin, 2013; Ohtsubo, Gamou, & Shimizu, 1998).

1.12 The EGFR Paradox during the Metastatic Progression of Breast Cancer

Recently our lab reported findings that demonstrate a switch in EGFR function between primary and metastatic tumors (Wendt et al., 2015). In this study, EGF treatment of EGFR-amplified primary tumor cells resulted in increased proliferation and these cells were particularly sensitive to EGFR inhibition. Conversely, after EMT-driven *in vivo* metastasis, cells derived from pulmonary metastases that are inherently resistant to EGFRi undergo robust growth inhibition in response to EGF (Wendt et al., 2015). The idea that growth factors have context-dependent dual effects on cell-growth has long been proposed (Sporn & Roberts, 1988). Indeed, growth factors such as interleukin 6 (IL-6) and platelet-derived growth factor (PDGF) are known to paradoxically inhibit the growth of some cell models (Kim, Upadhyay, Li, Palmer, & Deuel, 1995; Minami et al., 1996). The most well-established shift in function in BC is that of TGF-β where it functions as a powerful tumor suppressor in primary tumors but actually drives disease progression in the metastatic setting (Tian & Schiemann, 2009). Further understanding of this shift in EGFR signaling will likely serve to explain the failure of EGFRi for the treatment of metastatic BC, but these findings also present the opportunity to exploit the anti-metastatic function of EGFR as a therapeutic approach.

1.13 Signaling Cascades Mediating EGFR Function with Special Focus on STAT1 and STAT3

EGFR activation can be induced by ligand binding or activating mutations that make the receptor constitutively active in the absence of ligand (Purba, Saita, & Maruyama, 2017). While EGFR expression is commonly increased in triple-negative breast tumors, activating mutations of EGFR are uncommon (Jacot et al., 2011; Kim, Jang, Lee, & Bae, 2017; Uramoto, Shimokawa, Nagata, Ono, & Hanagiri, 2010). As mentioned previously, increased levels of EGFR expression in triple-negative breast cancer (TNBC) correlates with poor prognosis (Rosell et al., 2013; Tischkowitz et al., 2007). This overexpression leads to increased sensitivity to ligand binding by

increased receptor sheer number and ultimately results in the activation of survival signaling pathways (Pines, Köstler, & Yarden, 2010). EGFR primarily exists in a monomeric form spanning the cell membrane and is composed of an extracellular ligand-binding domain, followed by a single transmembrane domain, a kinase domain, and finally the C-terminal loop, the last two of which reside intracellularly (Ogiso et al., 2002). In its inactive state, the kinase domain of EGFR is bound to the C-terminal loop, which prevents the catalytic activity of the kinase domain by blocking accessibility to target substrates. Ligand binding results in a structural change in the extracellular domain leading to homo-dimerization with another ligand-bound EGFR through dimerization arms on the extracellular domains (Zhang, Gureasko, Shen, Cole, & Kuriyan, 2006). EGFR is also able to hetero-dimerize with another ErbB family member (Her2, ErbB3, and ErbB4) due to high structural similarities among these receptors. Despite the similarities of the ErbB receptors, they have distinct structural features that contribute to a diverse spectrum of outcomes with respect to gene expression (Citri & Yarden, 2006). Moreover, eleven different ligands have evolved to bind EGFR and other ErbB receptors with differing binding affinities and specificities, thus further diversifying the biologic outcome of EGFR activation (Yarden, 2001). After ligand binding, the conformational change in the extracellular portion of EGFR is relayed to the intracellular domains, leading to dissociation of the kinase domain and C-terminal loop, and the formation of an asymmetric dimer between the two kinase domains of the EGFR homo- or hetero- dimer.

This asymmetric binding is essential for activation as one “activator” kinase from one EGFR molecule upholds the “receiver” kinase of EGFR’s interacting partner in the optimal conformation for catalysis. Subsequently, the “receiver kinase” is activated and phosphorylates a number of tyrosine residues in the C-tail of the former “activator” kinase. Phosphorylation of tyrosine residues generates docking sites for a number of adapter proteins and non-receptor kinases for phosphorylation events that ultimately activate signaling pathways and modulate transcription of target genes (Ogiso et al., 2002; Pines et al., 2010; Zhang et al., 2006).

Multiple signal transduction pathways mediate the biologic functions of EGFR, including the mitogen-activated protein kinase (MAPK) pathway that activates extracellular signal-regulated kinase1/2 (ERK1/2), the phosphoinositide 3-kinase (PI3K) pathway, the phosphoinositide phospholipase C (PLC-γ), and the signal transducer and activator of transcription (STAT) pathway (Ali & Wendt, 2017) (Figure 1).

EGFR signaling through these particular pathways regulates cell proliferation, differentiation, migration, and apoptosis (Wee & Wang, 2017). Of particular relevance to breast oncogenesis is the STAT3 signaling pathway. Indeed, STAT3 signaling has been implicated in driving tumor progression and stem-like character of human breast neoplasms (Banerjee & Resat, 2016; Idowu et al., 2012; Marotta et al., 2011). STAT3 is a member of the STAT family of transcription factors that include STAT1, 2, 3, 4, 5, and 6 (Ihle, 2001). Several signaling systems can activate STAT3, including epidermal growth factor (EGF) (Shao, Cheng, Cook, & Tweardy, 2003), interferons (Ho & Ivashkiv, 2006), and interleukins (ILs) such as interleukin-6 (IL-6).

The STAT3 gene is reported to transcribe to a number of mRNA products. The "full-length" STAT3-alpha (STAT3α or simply STAT3) translates to a protein product that contains a DNA binding domain, a SRC Homology 2 (SH2) domain that contains a tyrosine as the 705 amino acid, and a trans-activation domain that contains a serine as the 727 amino acid. The SH2 domain and the amino acids tyrosine-705 and serine-727 are crucial for STAT3 activation and regulation of activity (Goldberg, Abutbul-Amitai, Paret, & Nevo-Caspi, 2017). For example, in the canonical pathway of STAT3 activation downstream of EGFR, STAT3 is recruited (through its SH2 domain) to the phosphorylated tyrosine residues at the EGFR cytoplasmic tail after EGFR-ligand binding. After recruitment, STAT3 is phosphorylated on tyrosine-705 by the activated kinase domain of EGFR (Srivastava & DiGiovanni, 2016). This phosphorylation event can also be mediated by non-receptor tyrosine kinases such as the SRC family of tyrosine kinases which has 9 members: Src, Yes, Fyn, Fgr, Lck, Hck, Blk, Lyn and Frk (Annerén, Cowan, & Melton, 2004).

Phosphorylation of STAT3 at tyrosine-705 triggers homo-dimerization with a second p-STAT3-Y705 molecule or hetero-dimerization with another STAT3 molecule via SH2 domain interactions. Following dimerization, STAT3 homo- or hetero- dimer complexes translocate into the nucleus to regulate gene transcription. STAT3 can also be further phosphorylated at the serine-727 residue by serine kinases such as mTOR and the MAP kinases (Chung, Uchida, Grammer, & Blenis, 1997; Yokogami, Wakisaka, Avruch, & Reeves, 2000). Following these phosphorylation events, STAT3 dimers recruit co-activator or co-repressor proteins through the trans-activation domain and translocate to the nucleus. These complexes bind sequence-specific DNA regions termed STAT-inducible elements (SIEs) and regulate transcription of target genes (Chung et al., 1997; Wen, Zhong, & Darnell, 1995; Yokogami et al., 2000; Zhang, Blenis, Li, Schindler, & Chen-Kiang, 1995). Serine-727 phosphorylation has been reported to be essential for 'full activation' of

STAT3 as a transcription factor, since phosphorylation at this site can facilitate oligomerization of multiple STAT3 dimers at promoters of target genes. Such structures are claimed to provide optimal control over gene transcription due to the fact that target genes of STAT3 commonly have multiple SIE elements in their promoter regions (Lin et al., 2014). A number of target genes have been reported to mediate the oncogenic functions of STAT3 activation, including the cell-cycle gene cyclin-D1, inflammatory cytokines such as interleukin-10 (IL-10), pro-survival and anti-apoptotic genes such as C-myc, Bcl-xL, Mcl-1, and Bcl, vascular endothelial growth factor (VEGF), and transforming growth factor β (TGF-β) (Fagard, Metelev, Souissi, & Baran-Marszak, 2013).

The STAT3 gene can transcribe to a shorter variant, termed STAT3-beta (STAT3-β), by alternative splicing. STAT3-β mRNA translates to a protein product that lacks the trans-activation domain of STAT3-α containing the serine-727 phosphorylation site. STAT-β, however, retains the DNA binding domain and the SH2 domain containing tyrosine-705. Thus, STAT3-β is capable of dimerization through tyrosine-705 mediated phosphorylation but lacks gene-regulation activity due to missing tarns-activation domain and serine-727 (Maritano et al., 2004). Due to these unique properties as compared to STAT3-α, the beta STAT3 isoform was initially described to have anti-tumorigenic functions by playing a dominant-negative effect (Caldenhoven et al., 1996). However, a recent study proposed this isoform to be a biologically active molecule (Zammarchi et al., 2011). Overall, much less is known regarding the biological functions of STAT3-β compared to the oncogenic full-length isoform and the involvement of STAT3-β signaling downstream of EGFR remains unknown.

Signal transducer and activator of transcription 1 (STAT1) is another member of the STAT family of transcription factors. Similar to STAT3, the 'full-length' STAT1 is composed of a DNA-binding domain, an SH2-domain containing a tyrosine as amino acid 701, and a transactivation domain containing a serine as the amino acid 727. STAT1 gene can also be alternatively spliced to a shorter isoform that lacks the trans-activation domain containing the Serine-727 amino acid (termed STAT1-β) (Baran-Marszak et al., 2004). In addition to its structural resemblance to STAT3, STAT1 follows a similar mode of activation in terms of recruitment to activated receptor tyrosine kinases (RTKs) through SH2 domains, dimerization, and translocation to the nucleus to regulate gene expression (Zhang & Liu, 2017). Despite their significant similarities, STAT1 and STAT3 mediate very different and commonly opposing functions in carcinogenesis. Indeed, while

STAT3 induces the transcription of genes associated with oncogenesis, STAT1 activation precipitates anti-proliferative and apoptotic effects (Avalle, Pensa, Regis, Novelli, & Poli, 2012). This is consistent with the well-established function of STAT1 as the canonical mediator of interferon signaling and apoptosis in virally infected cells (Ogiso et al., 2002). While EGFR is an established proto-oncogene whose activation is linked to transformation, increased proliferation, and metastasis, EGFR activation results in growth arrest and/or apoptosis in certain cancer cell-lines. As discussed previously, the vast majority of these studies implicate STAT1 in these anti-tumorigenic effects via induction of cell-cycle arrest and/or apoptosis (Ali & Wendt, 2017). However, very little is known regarding the mechanism by which EGFR signals to STAT1 and the therapeutic utility of EGFR:STAT1 signaling induction. Elucidation of this mechanism could provide novel avenues for targeting tumors that are driven by EGFR signaling.

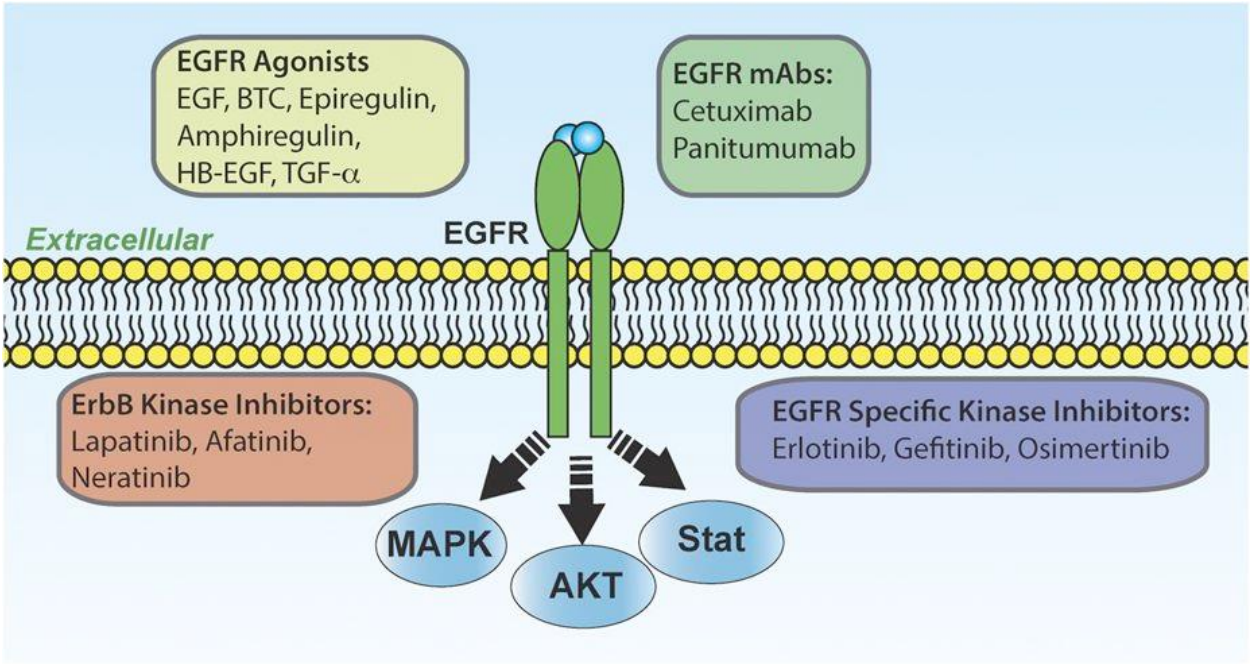

Figure 1. EGFR ligands, oncogenic signaling, and clinical inhibitors. A schematic representation of the activators, inhibitors, and outcomes of EGFR signaling. EGFR is part of the four-membered ErbB superfamily (ErbB1–4). These receptors form several different homo- and heterodimers (here we only depict the EGFR homodimer). EGFR is capable of binding several different extracellular ligands that agonize the receptor leading to activation of several downstream signaling events including, but not limited to, those listed. Several therapeutics have been developed to antagonize EGFR including monoclonal antibodies (mAbs) that block ligand binding as well as several different kinase inhibitors. In addition to EGFR, some of these kinase inhibitors also target other ErbB receptors, supporting their use in Her2-amplified BC. All of the listed therapies are FDA approved for various cancers with the exception of Neratinib (Ali & Wendt, 2017).

CHAPTER 2. THE ROLE OF STAT1 IN METASTATIC BREAST CANCER (BC)

(As published in:
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2.1 Abstract

Inhibition of epidermal growth factor receptor (EGFR) signaling by small-molecule kinase inhibitors and monoclonal antibodies has proven effective in the treatment of multiple cancers. In contrast, metastatic breast cancer (BC) derived from EGFR-expressing mammary tumors is inherently resistant to EGFR-targeted therapies. Mechanisms that contribute to this inherent resistance remain poorly defined. Here, we show that in contrast to primary tumors, ligand-mediated activation of EGFR in metastatic BC is dominated by STAT1 signaling. This change in downstream signaling leads to apoptosis and growth inhibition in response to epidermal growth factor (EGF) in metastatic BC cells. Mechanistically, these changes in downstream signaling result from an increase in the internalized pool of EGFR in metastatic cells, increasing physical access to the nuclear pool of STAT1. Along these lines, an EGFR mutant that is defective in endocytosis is unable to elicit STAT1 phosphorylation and apoptosis. Additionally, inhibition of endosomal signaling using an EGFR inhibitor linked to a nuclear localization signal specifically prevents EGF-induced STAT1 phosphorylation and cell death, without affecting EGFR:ERK1/2 signaling. Pharmacologic blockade of ERK1/2 signaling through the use of the allosteric MEK1/2 inhibitor, trametinib, dramatically biases downstream EGFR signaling toward a STAT1-dominated event, resulting in enhanced EGF-induced apoptosis in metastatic BC cells. Importantly, combined administration of trametinib and EGF also facilitated an apoptotic switch in EGFR-transformed primary tumor cells, but not normal mammary epithelial cells. These studies reveal a fundamental distinction for EGFR function in metastatic BC. Furthermore, the data demonstrate that pharmacological biasing of EGFR signaling toward STAT1 activation is capable of revealing the apoptotic function of this critical pathway.

2.2 Introduction

Breast cancer metastasis is a multi-step process that culminates in vital organ invasion and proliferation by cancer cells. These later events of metastasis are responsible for patient morbidity and mortality in breast cancer (Steeg, 2016). Developing targeted therapies for metastatic breast cancer faces many challenges. Paramount to these challenges is the high degree of molecular changes that characterize metastatic lesions compared to primary tumors, which constantly brings into question the utility of primary tumor analysis to guide metastatic therapy (Cejalvo et al., 2017; Vignot, Besse, André, Spano, & Soria, 2012). Thus, understanding signaling events specific to metastatic breast tumors is essential to identify potential therapeutic targets and biomarkers for late-stage disease.

Similar to more established breast cancer-associated genes, such as estrogen receptor (ER) and human epidermal growth factor receptor 2 (Her2), primary versus metastatic tumor discrepancies have also been described for epidermal growth factor receptor (EGFR)-expressing mammary tumors (Ali & Wendt, 2017; Cejalvo et al., 2017; Choong et al., 2007; Niikura et al., 2012). Breast cancer cells predominantly respond to EGFR agonists in a proliferative fashion supporting its role as an oncogene. Indeed, studies from our group and others have linked activation of EGFR to mammary epithelial cell transformation, increased proliferation, and several early steps of metastasis (Wendt et al., 2010; Wyckoff et al., 2004). Various signaling pathways facilitate these oncogenic roles of EGFR, including the p38 mitogen-activated protein kinase, extracellular signal-regulated kinases 1 and 2 (ERK1/2), signal transducer and activator of transcription 3 (STAT3), and phosphoinositide 3-kinase (PI3K). These experimental findings are supported by clinical studies that report high expression of EGFR in primary mammary tumors is predictive of reduced patient survival (Park et al., 2014; Tischkowitz et al., 2007). However, subsets of cancer cells, including those originating from the breast, respond to epidermal growth factor (EGF) via cell cycle arrest and induction of apoptosis (Choi et al., 2010; Jackson & Ceresa, 2016; Lim, Jeon, Koh, & Wu, 2015; Wendt et al., 2015). These observations are corroborated by the antitumor response of *in vivo* administered EGF (Lim et al., 2015). Many studies describe the growth-inhibitory functions of EGFR to be mediated by STAT1, which is an established tumor suppressor and mediator of apoptosis downstream of interferon signaling (Andersen et al., 2008; Chin, Kitagawa, Kuida, Flavell, & Fu, 1997; Grudinkin et al., 2007). We have recently shown that EGFR function changes from oncogenic in primary tumors to growth-inhibitory and apoptotic in

metastatic tumors (Ali & Wendt, 2017; Balanis et al., 2013; Wendt et al., 2010, 2015) (Figure 2A and B). The importance of this paradoxical function of EGFR is substantiated by the failure of EGFR inhibition (EGFRi) to improve the clinical outcomes of metastatic breast cancer patients (Ali & Wendt, 2017; Carey et al., 2012; Crozier et al., 2016; Dickler et al., 2009, 2008; Smith et al., 2007; Sunakawa et al., 2016; Trédan et al., 2015; von Minckwitz et al., 2005; Yardley et al., 2016) (Figure 2C). Inhibition of specific pathways downstream of EGFR is also being pursued for clinical applications. In particular, the compound trametinib is an allosteric inhibitor of MEK1/2, the kinases directly upstream of ERK1/2 (Gilmartin et al., 2011). As opposed to direct inhibition of growth factor receptors, targeting of downstream pathways requires consideration that the cellular effects of inhibition may also arise via differential activation of alternate signaling pathways downstream of a common driver receptor.

In the current study, we demonstrate the apoptotic function of EGFR in metastatic breast cancer is dependent on STAT1 and we address the hypothesis that pharmacologic inhibition of MEK1/2 with trametinib will bias EGFR signaling toward a STAT1-dominated, apoptotic signaling pathway. These findings identify unique molecular signaling events that specifically manifest in metastatic BC, and identify a pharmacological approach to enhance STAT1-induced apoptosis and limit primary and metastatic tumor growth.

2.3 EGF-mediated STAT1 Phosphorylation Increases with Metastasis

Our previous studies demonstrate that EGFR overexpression is capable of transforming normal murine mammary gland (NME) cells (Balanis et al., 2013; Balanis, Yoshigi, Wendt, Schiemann, & Carlin, 2011; Wendt et al., 2010, 2015). Transient induction of epithelial–mesenchymal-transition (EMT) via treatment with TGF-β facilitates the metastasis and inherent resistance of these cells to EGFRi (Figure 2A and B) (Wendt et al., 2015). To investigate differential downstream signals generated by EGFR in these isogenic cells of increasing metastatic capacity, we examined the phosphorylation of STAT1 and ERK1/2 in response to exogenous EGF stimulation.

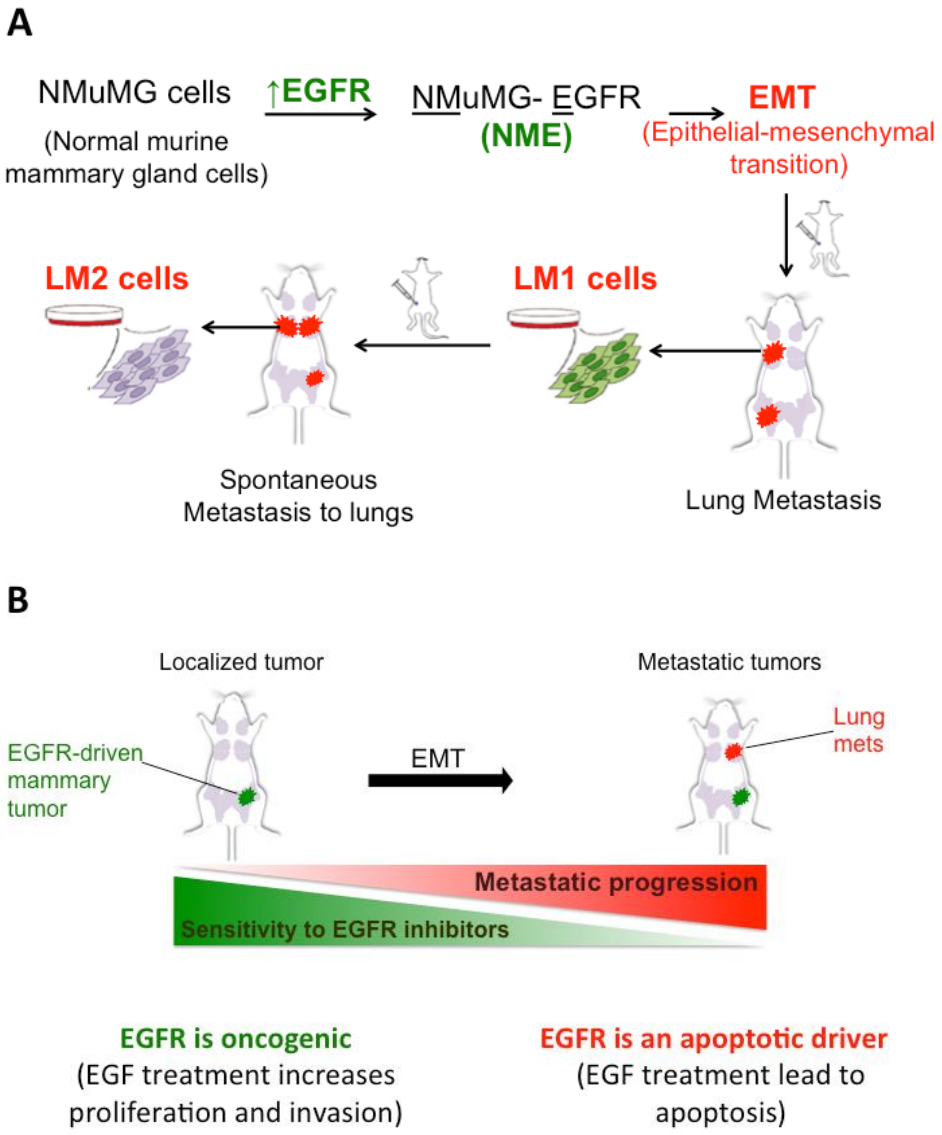
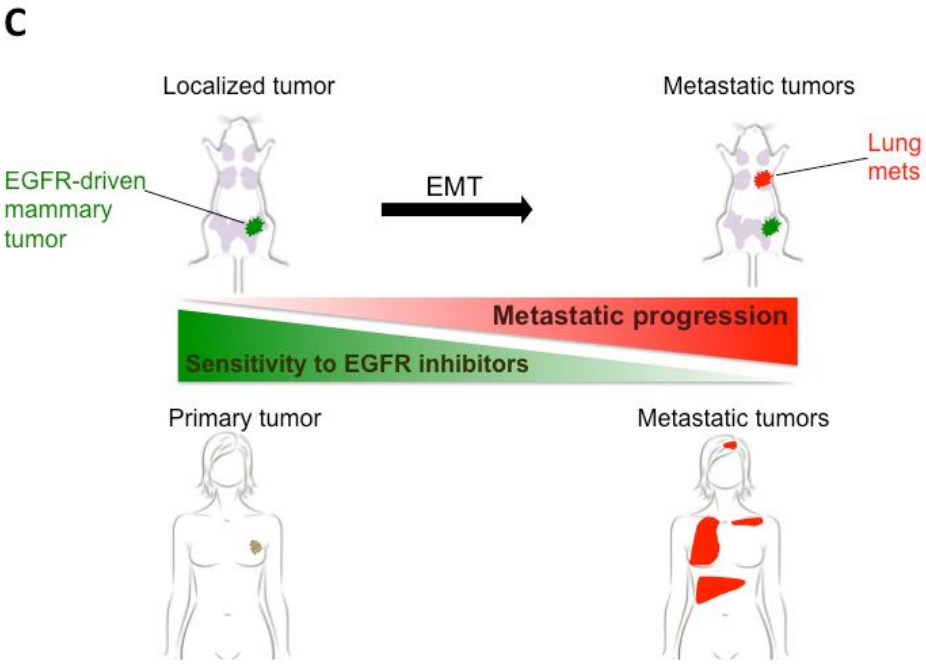


Figure 2. Generation of a breast carcinoma progression series representative of the clinical resistance of metastatic breast cancer to EGFR inhibitors. **A.** Schematic representation of EGFR-transformed EMT-driven lung-metastatic breast cancer progression series (referred to as NME series). In this model, overexpression of human wild-type (WT) EGFR transforms the otherwise non-transformed normal murine mammary gland (NMuMG) cells. Indeed, engraftment of EGFR-overexpressing NMuMG cells (referred to here as NME cells) onto the mammary fat-pad of Nu/Nu mice leads to the development of mammary tumors. Induction of epithelial-mesenchymal transition (EMT) in NME cells by treatment with transforming growth factor-β (TGF-β) greatly enhances their invasive capacity and lung metastasis. **B.** EMT-driven metastasis of NME cells is associated with inherent resistance to EGFR inhibitors and a change in the function of EGFR in response to EGF stimulation, from oncogenic in the primary mammary tumors to apoptotic in metastatic NME-LM1 and NME-LM2 cells (schematic is drawn to summarize results from (Wendt et al., 2015)). **C.** NME breast carcinoma progression series generated in our laboratory is comparable to the inherent resistance phenomenon of metastatic breast cancer to EGFR inhibitors observed in the clinic (schematic is drawn to summarize results from (Wendt et al., 2015)).

Figure 2 continued



As shown in Figure 3B and C, EGF treatment resulted in enhanced phosphorylation of STAT1 in lung-metastatic, LM1 and LM2, cells as compared to the non-metastatic NME cells. In contrast, phosphorylation of ERK1/2 in response to EGF was similar in all cell types, therefore the ratio of ERK1/2 to STAT1 phosphorylation is dramatically altered in metastatic cells (Figure 3 B and C). Importantly, the enhanced STAT1 signaling in the LM cells occurs despite EGFR returning to levels similar to endogenous in non-transformed NMuMG cells (Figure 3B). Unlike EGF, interleukin 6 (IL6)-induced STAT3 activation was similar across all cells of the NME series (data are not shown). Taken together, these findings suggest that through metastasis there is not a general propensity to increase STAT activation, but there is a specific increase in the EGFR:STAT1 signaling axis.

To expand these observations, we derived additional metastatic lines from different anatomical locations. Metastases from two different lymph nodes were subcultured and termed NME-Lym1 and NME-Lym2 cell lines (Figure 3D). Consistent with our previously reported observations in lung metastases, these lymph node metastases display increased resistance to the EGFR inhibitor erlotinib as compared to primary tumor NME cells when cultured under three-dimensional (3D) organotypic conditions (Figure 4). Furthermore, the downstream EGFR signaling in these lymph node metastases also became dominated by STAT1 phosphorylation

(Figure 3E). The diminution of EGFR through metastasis is further supported by previous reports from our lab and others showing EGFR downregulation in the RAS-transformed MCF10A breast carcinoma progression series (Choong et al., 2007; Strickland et al., 2000; Wendt et al., 2015). To examine EGF-induced downstream signaling in this additional isogenic model of metastatic progression, we engineered the metastatic Ca1a cells to re-express EGFR using stable or doxycycline-inducible expression systems (Figure 3F and 5). EGF stimulation of Ca1a cells stably or transiently replenished with EGFR expression led to robust phosphorylation of EGFR and STAT1 and induction of apoptosis (Figure 3F and 5). Overall, these data indicate that enhanced STAT1 signaling downstream of EGFR activation correlates with EGF-mediated growth inhibition in metastatic BC. Figure 6 is a schematic representing the overall conclusions of the above findings.

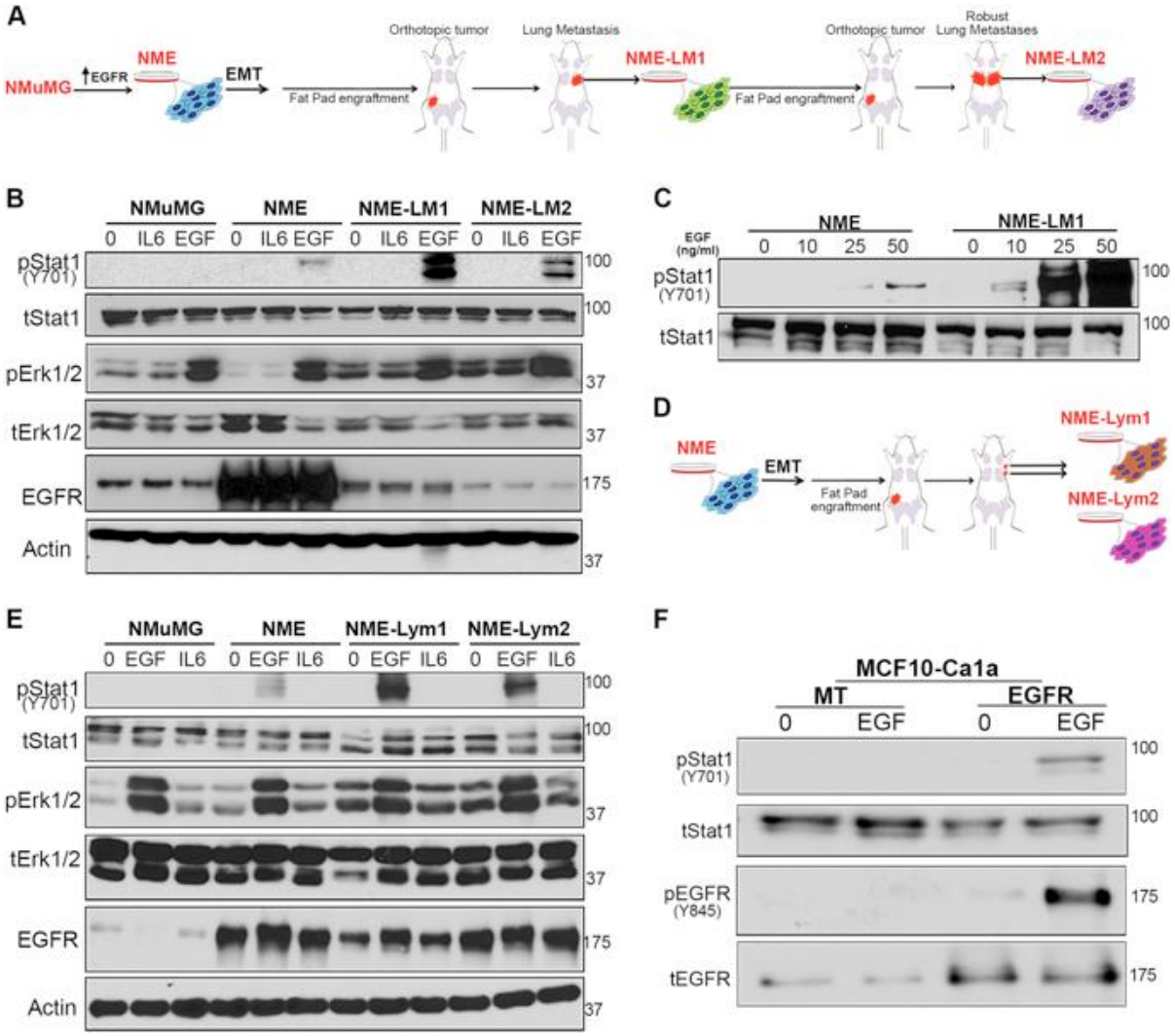


Figure 3. The STAT1:ERK1/2 signaling ratio downstream of EGFR is altered in metastatic cells compared to primary breast cancer cells. **A.** Schematic representation of the EGFR-transformed lung-metastatic breast cancer progression series as described in Figure 2A. **B.** Cells described in panel A were analyzed by immunoblot for phosphorylation of STAT1 and ERK1/2 in response to a 30-minute EGF stimulation (50 ng/ml). IL6 and BSA (0) served as protein stimulation controls, and analysis of total levels of EGFR, STAT1, ERK1/2, and Actin served as loading controls. C. Non-metastatic (NME) and lung metastatic (NME-LM1) cells as described in panel A were stimulated for 30 minutes with the indicated concentrations of EGF and analyzed for phosphorylation of STAT1. Analysis of total STAT1 served as a loading control. **D.** Schematic representation of the EGFR-transformed lymph node metastatic breast cancer progression series. Metastatic cells were isolated from two separate lymph nodes and termed NME-Lym1 and NME-Lym2. **E.** Cells described in panel D were analyzed for phosphorylation of STAT1 and ERK1/2 in response to a 30-minute EGF stimulation (50 ng/ml). IL6 and BSA served as stimulation controls, and analysis of total levels of EGFR, STAT1, ERK1/2, and Actin served as loading controls. **F.** EGFR was ectopically expressed in metastatic MCF10-Ca1a cells. These cells were stimulated with EGF (50 ng/ml) for 30 minutes and analyzed for phosphorylation of STAT1 and EGFR. Analysis of total levels of EGFR and STAT1 served as loading controls. All immunoblots shown are representative of at least three independent experiments.

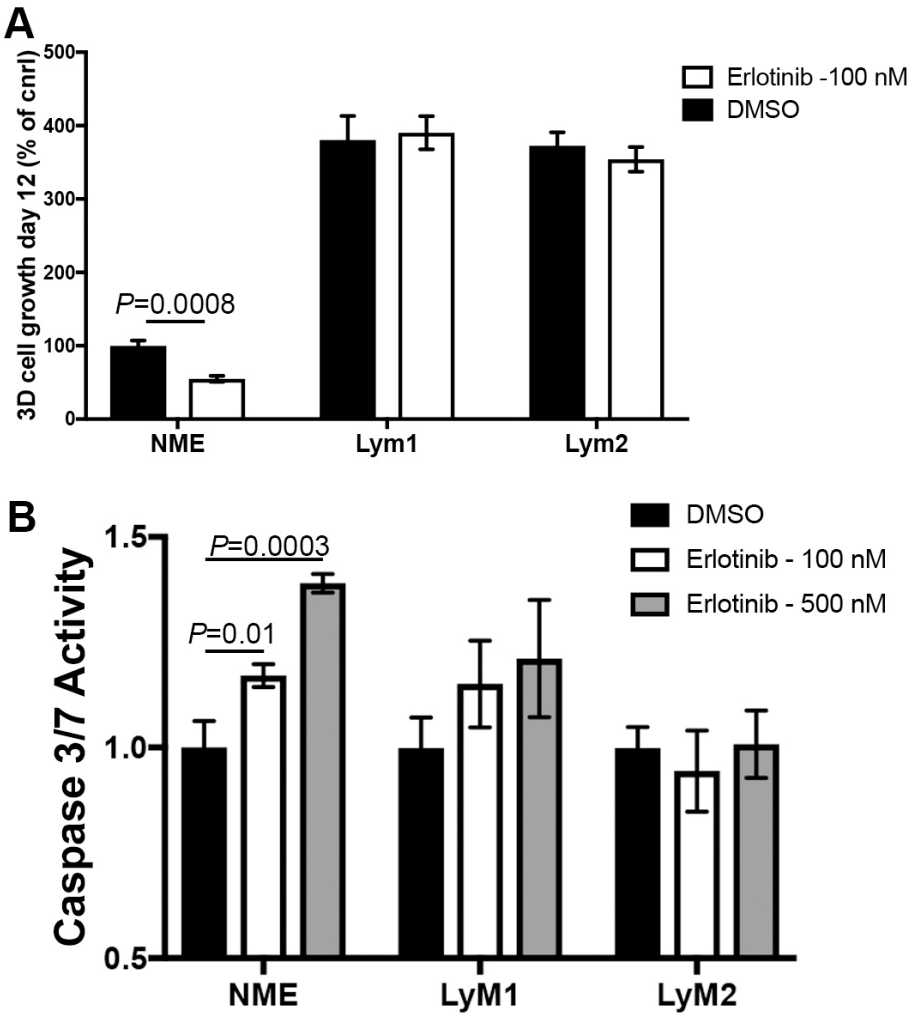


Figure 4. EGFR-transformed lymph node metastases are resistant to inhibition of EGFR kinase activity. **A.** EGFR transformed mammary epithelial cells (NME) and their isogeneic lymph node-derived metastatic counterparts (Lym1 and Lym2) expressing firefly luciferase were cultured under 3D conditions in the presence or absence of the EGFR inhibitor erlotinib for 12 days at which point cellular viability was quantified by bioluminescence. **B.** The cells described in panel A were cultured on 2D tissue plastic and treated for 24 hours with the indicated concentrations of erlotinib and subsequently analyzed for caspase 3/7 activity. Data are the mean ± SE of three separate experiments completed in triplicate, resulting in the indicated P-values.

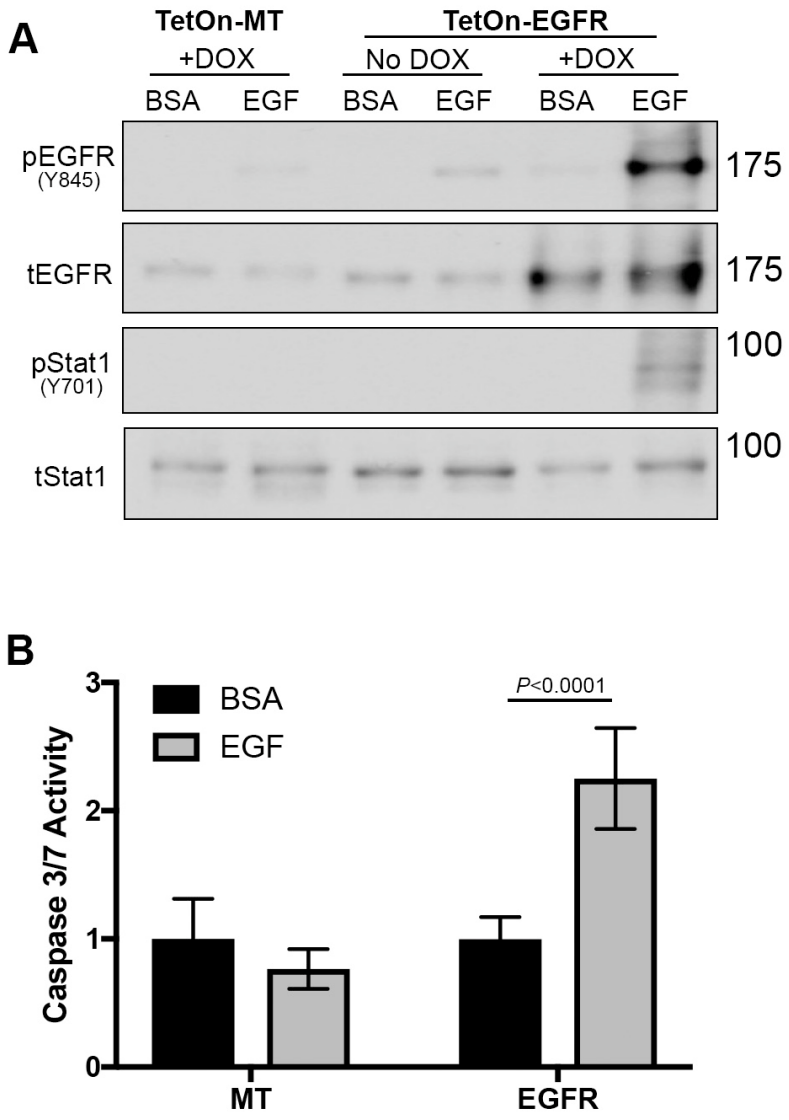


Figure 5. Induction of EGFR in metastatic breast cancer cells is sufficient for ligand-induced STAT1 phosphorylation and apoptosis. **A.** Metastatic MCF10-Ca1a cells were constructed to express EGFR under the control of a tetracycline-induced promoter. Following a 24-hour induction with doxycycline (DOX: 1 μg/ml) these cells were stimulated with EGF (50 ng/ml) for 30 minutes and assayed for phosphorylation of EGFR and STAT1. Expression of total EGFR and STAT1 served as loading controls. **B.** MCF10-Ca1a cells were constructed to stably express EGFR. These cells were stimulated with EGF (50 ng/ml) for 24 hours and assayed for caspase 3/7 activity.

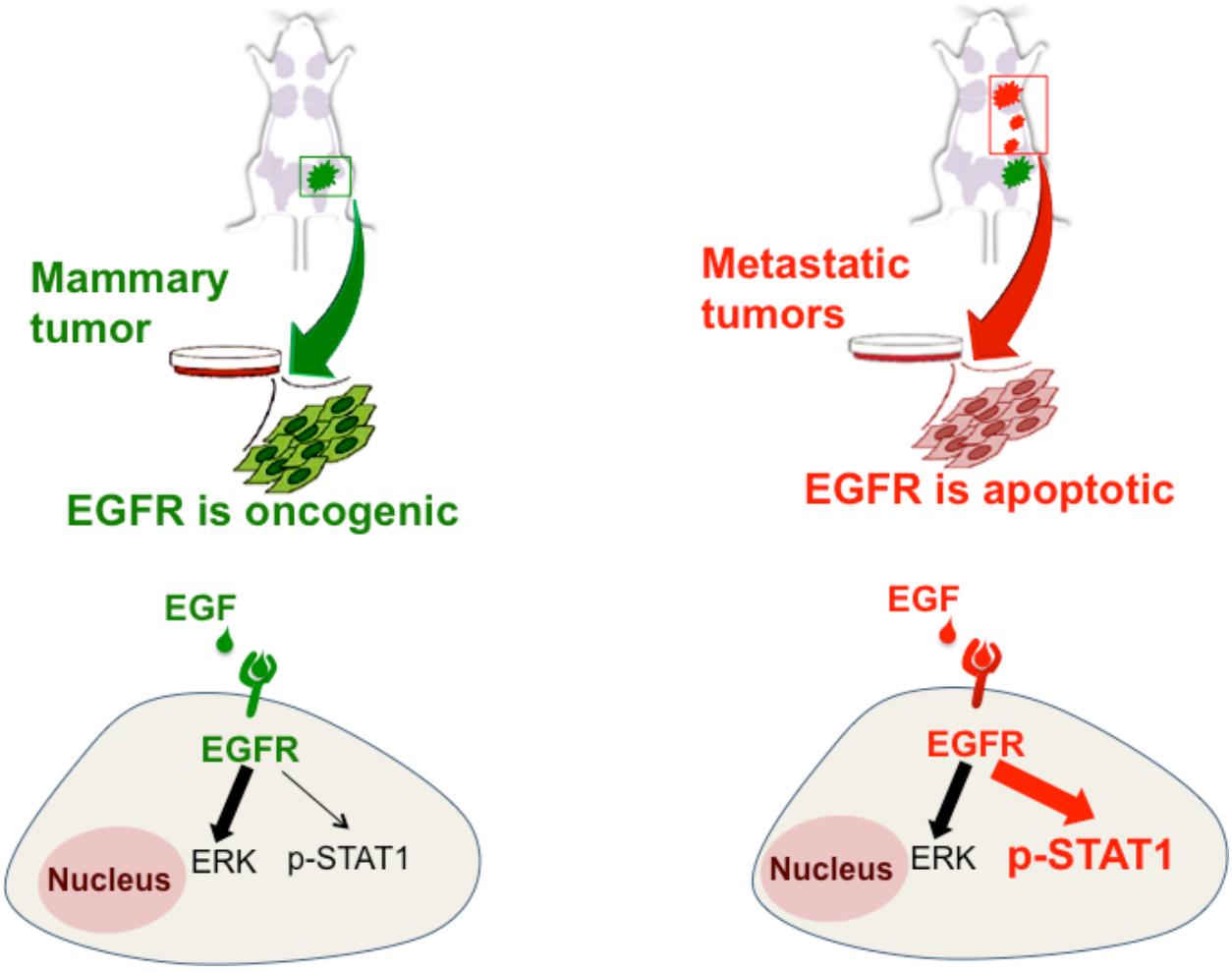


Figure 6. Increased STAT1 phosphorylation is responsible for the altered STAT1:ERK1/2 signaling ratio downstream of EGFR in metastatic cells compared to primary breast cancer cells. Schematic showing part 1 of the final model: In response to ligand stimulation, there is increased STAT1 phosphorylation in metastatic breast cancer cells compared to primary mammary tumor ones. ERK1/2 is phosphorylated to a similar extent downstream of EGFR in primary and metastatic breast cancer cells.

2.4 STAT1 Is Required for EGF-mediated Apoptosis in Metastatic BC

Previous studies indicate that STAT1 activation by EGF or other cytokines inhibits proliferation and induces apoptosis (Andersen et al., 2008; Chin et al., 1997; Grudinkin et al., 2007). Therefore, we sought to define the functional role of STAT1 downstream of EGFR activation in metastatic breast cancer cells. Indeed, EGF stimulation of both cell-lines derived from lymph node metastases, Lym1 and Lym2, resulted in cell rounding and enhanced caspase 3/7 activity (Figure 7A and B). Depletion of STAT1 expression in the metastatic Lym1 cells using two

different shRNA sequences prevented the ability of EGF to induce apoptosis (Figure 7C and D). Identical results were observed when STAT1 was depleted in the Lym2 cell-line (data not shown). In these analyses, we also pharmacologically blocked ERK1/2 signaling through the addition of trametinib. While the addition of trametinib alone had no effect on apoptosis, it did potentiate the ability of EGF to induce apoptosis in these cells (Figure 7D). Importantly, this effect was similarly dependent on STAT1 expression (Figure 7D). These data clearly implicate the functional involvement of STAT1 in EGF-induced apoptosis in metastatic breast cancer. Figure 8 summarizes the overall conclusions of the above findings.

2.5 Nuclear STAT1 Is Accessed by EGFR Through Endocytosis

Our recent studies demonstrate an enhanced localization of EGFR in the nucleus of metastatic breast cancer cells as compared to primary tumor cells (Bartolowits et al., 2017) (Figure 9). Given the role of STAT1 in facilitating EGF-induced apoptosis in metastatic cells, we next sought to investigate the subcellular localization of STAT1 under non-stimulated and EGF-stimulated conditions. Surprisingly, STAT1 was already localized to the nucleus in NME cells even before ligand stimulation (Figure 10A and 11). Unfortunately, immunostaining these cells with a phospho-specific STAT1 antibody is not possible due to cross-reactivity with phospho-EGFR epitopes. However, our whole-cell and nuclear-fractioned immunoblot analyses indicate that prior to EGF stimulation, STAT1 is not phosphorylated (Figure 10B and 11). Therefore, these data are consistent with previous reports that indicate a pool of STAT1 can exist in the nucleus in an unphosphorylated state (Cheon & Stark, 2009).

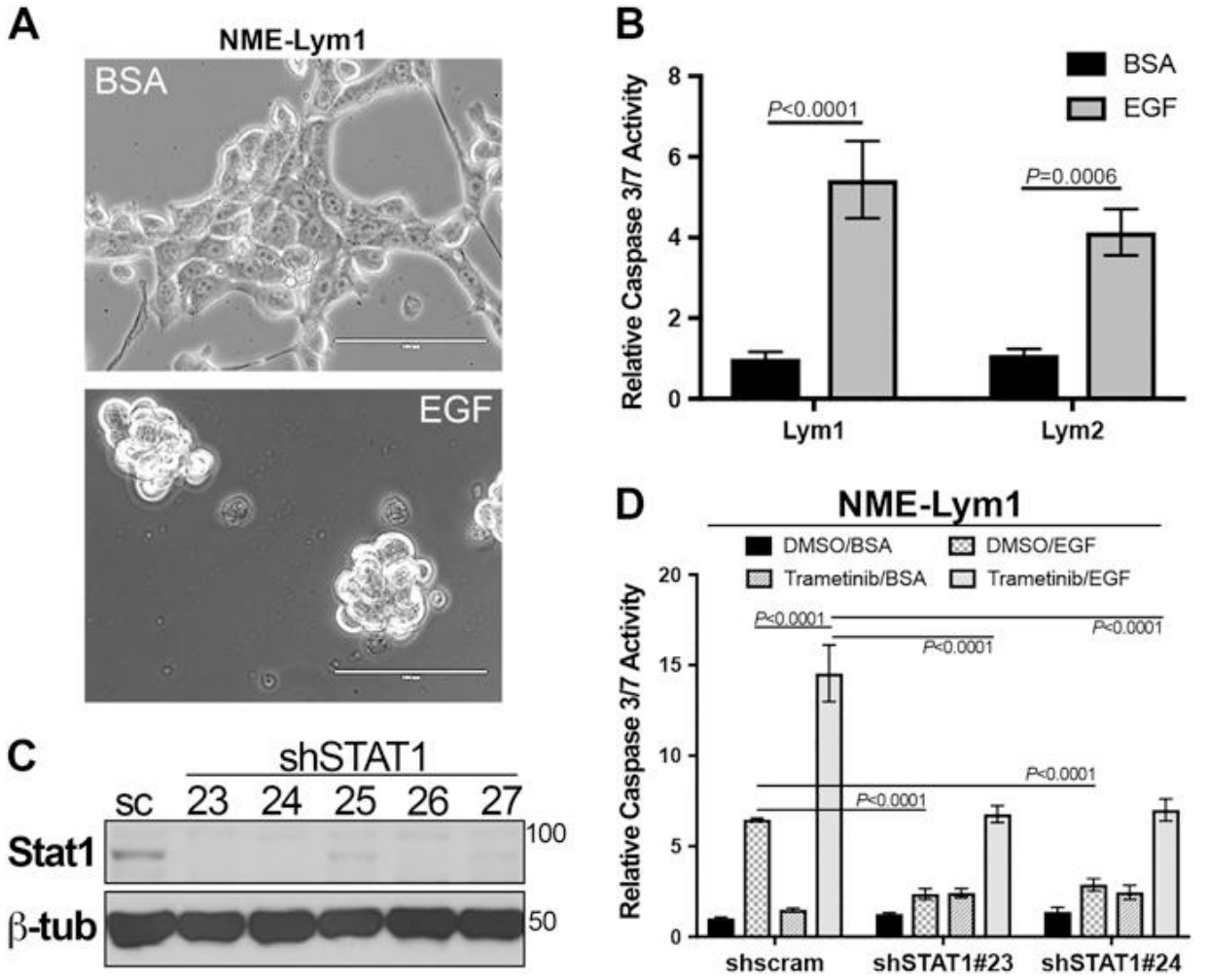


Figure 7. STAT1 is required for EGF-induced apoptosis in metastatic breast cancer cells. **A.** Lymph node metastases (NME-Lym1 and NME-Lym2) were stimulated with EGF (100 ng/ml) for 36 hours and imaged via phase-contrast microscopy. **B.** Following EGF stimulation as described in panel A, cells were assayed for caspase 3/7 activity. **C.** NME-Lym1 cells were constructed to stably express a scrambled control (sc) shRNA or various shRNAs (23–27) targeting STAT1. These cells were analyzed for STAT1 expression by immunoblot. Expression of β-tubulin (β-tub) served as a loading control. **D.** Control (shscram) and STAT1 depleted (shSTAT1#23 and shSTAT1#24) NME-Lym1 cells were treated with EGF as described above and caspase 3/7 activity was assessed. Separate groups of cells were treated with the MEK1/2 inhibitor trametinib (100 nM) in the presence or absence of exogenous EGF (100 ng/ml) and these cells were similarly assayed for caspase 3/7 activity. Data in panels B and D are the mean ± SE of three independent experiments completed in triplicate resulting in the indicated P-values.

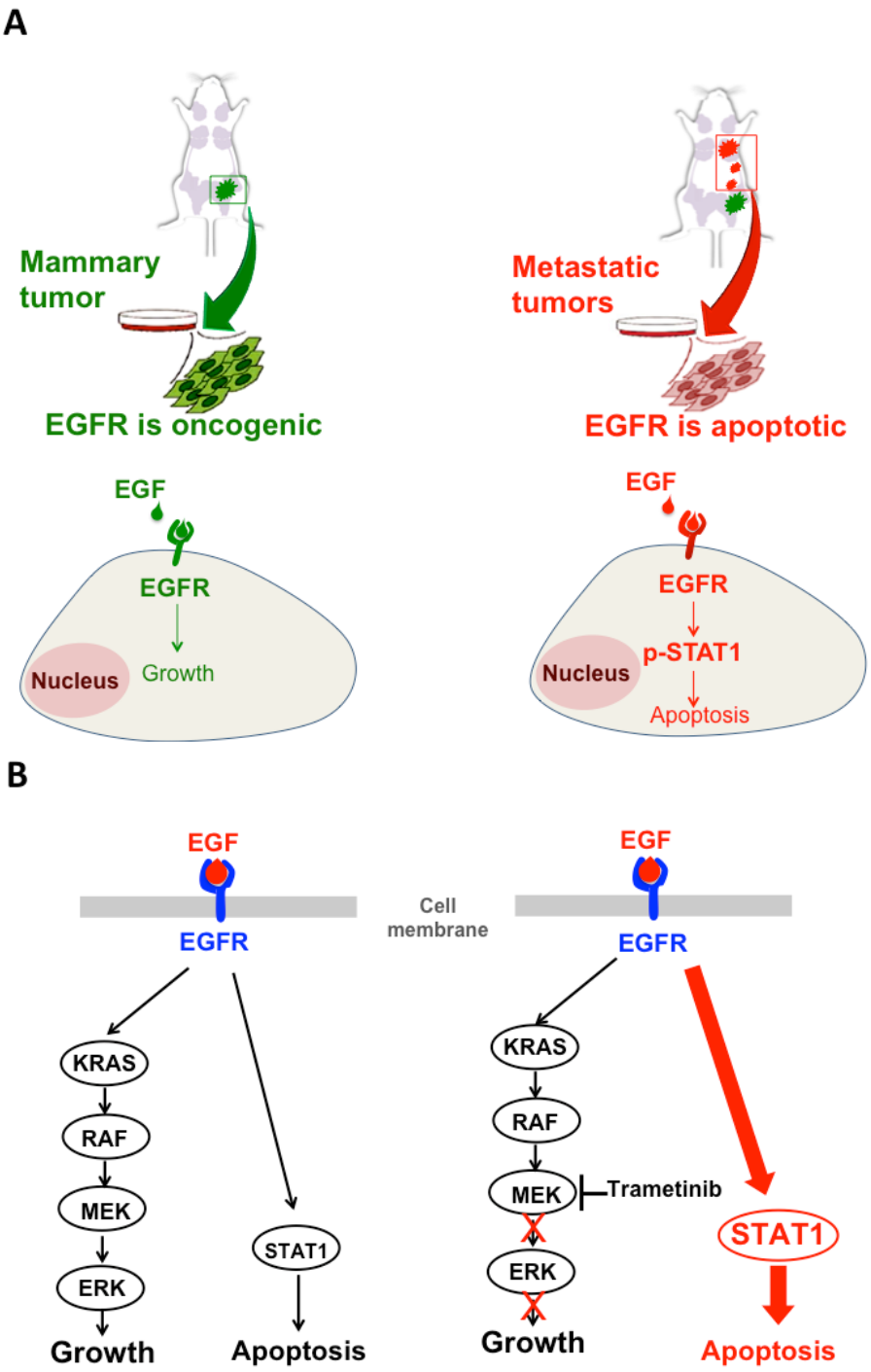


Figure 8. STAT1 is required for apoptosis of metastatic breast cancer cells when treated with EGF or EGF and trametinib combination. **A.** Schematic showing the role of STAT1 in EGF-induced apoptosis. In primary mammary tumors, EGFR activation is associated with increased growth and metastasis of these tumors. Conversely, in metastatic breast cancer cells, EGFR activation is associated with apoptosis and this effect is dependent on STAT1. **B.** Schematic showing the potentiation of EGF-induced apoptosis by combining EGF treatment with trametinib, an inhibitor of the mitogen-activated protein kinase (MAPK) pathway.

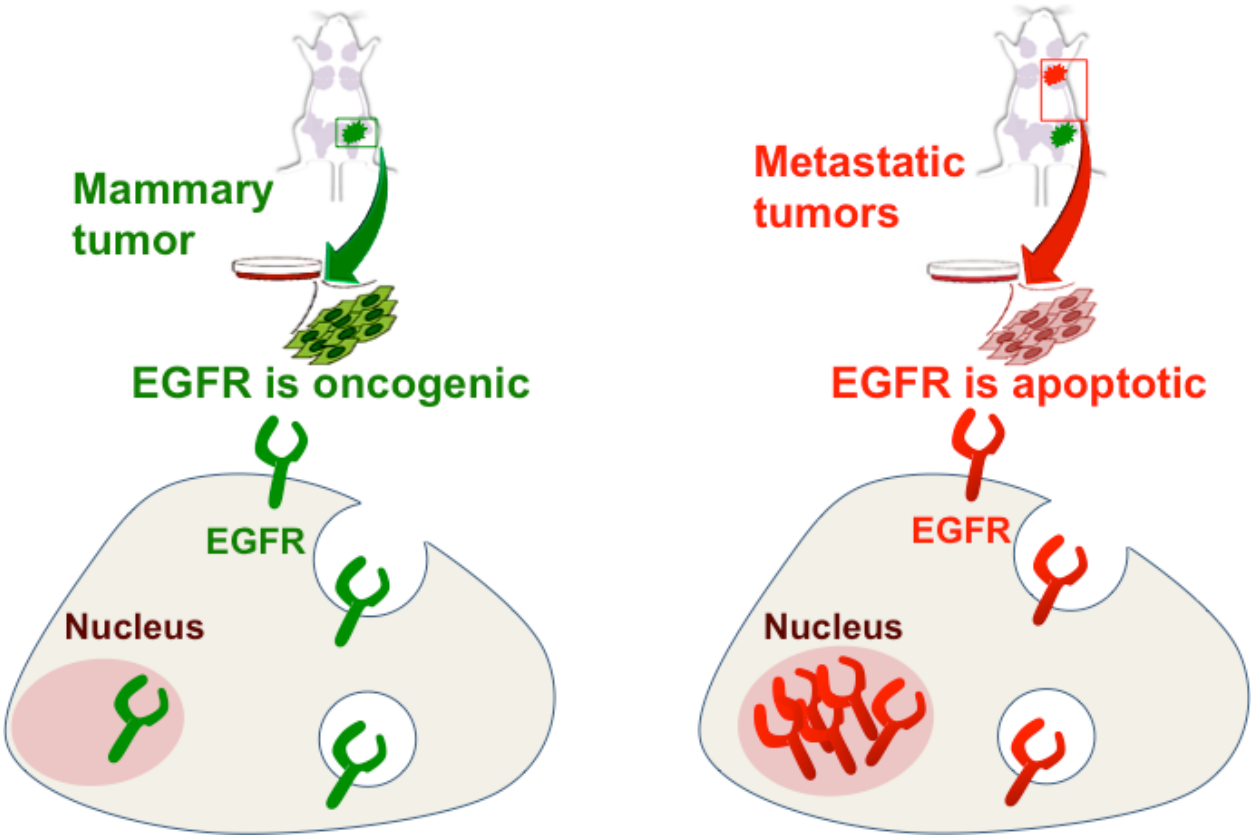


Figure 9. Nuclear localization of EGFR is enhanced in metastatic breast cancer cells as compared to primary tumors. Metastatic progression of breast cancer is associated with increased nuclear translocation of EGFR (schematic is drawn to summarize the results of (Bartolowits et al., 2017)).

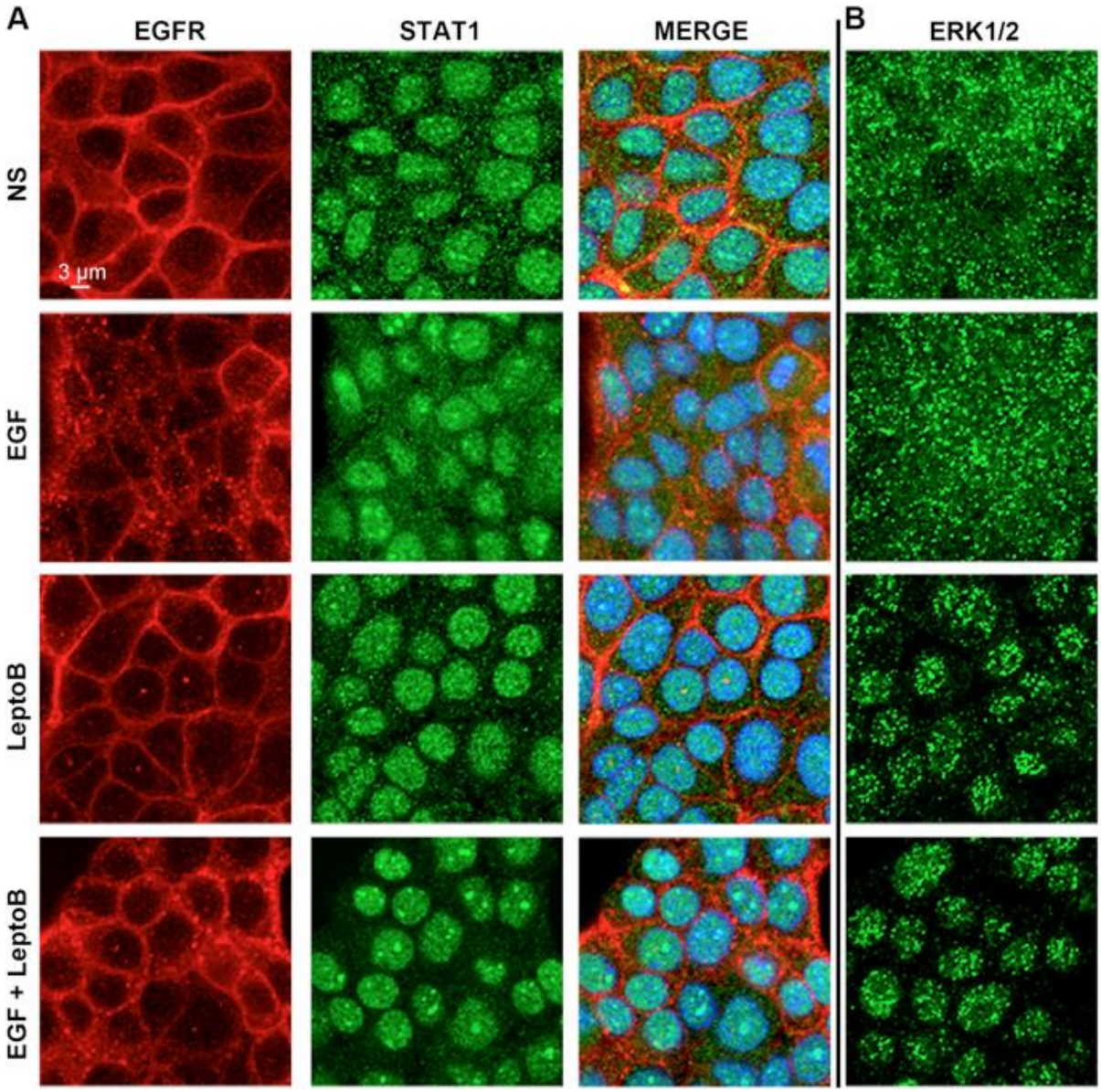


Figure 10. STAT1 is localized in the nucleus prior to phosphorylation. **A.** NME cells were stimulated with EGF for 30 minutes in the presence or absence of the nuclear export inhibitor leptomycin B (LeptoB). These cells were subsequently analyzed by dual immunofluorescence and imaged via confocal microscopy for the localization of EGFR and STAT1. These cells were counterstained with DAPI to visualize the nuclei. **B.** Separate sets of cells were stimulated as in panel **A** and analyzed for localization of ERK1/2. Data in panels **A** and **B** are representative images from at least 10 fields of view over two independent experiments.

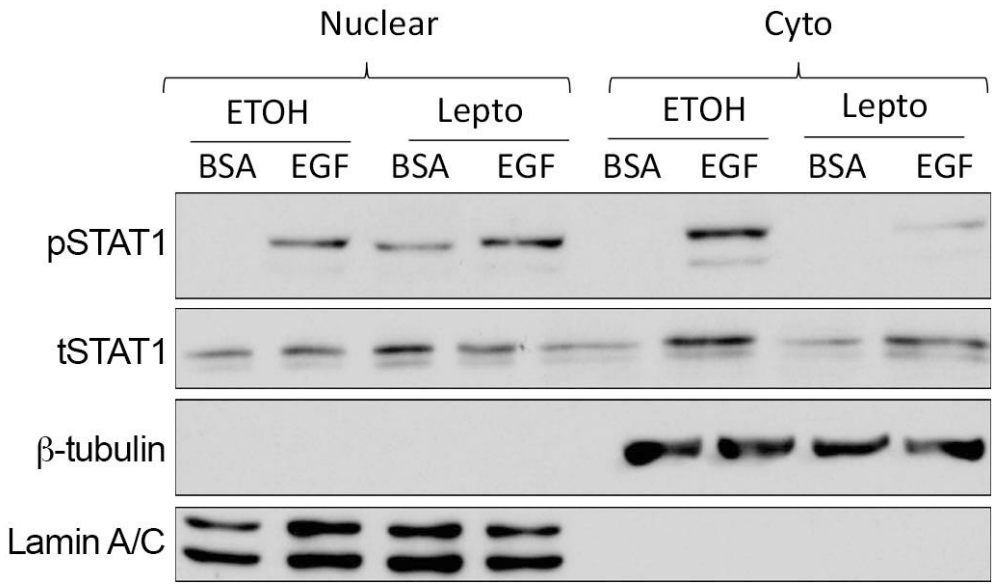


Figure 11. STAT1 is constitutively localized to the nucleus. NME cells were stimulated with EGF for 30 minutes in the presence or absence of the nuclear export inhibitor leptomycin B (Lepto). These cells were split into nuclear and cytoplasmic fractions and each fraction was assayed for the presence of total and phosphorylated STAT1. Lamin A/C and β-tubulin served as loading controls for the nuclear and cytoplasmic fractions, respectively. Immunoblots are representative of three independent experiments.

In contrast, ERK1/2 is primarily localized in the cytoplasm and only moves into the nucleus upon EGF stimulation, an event that is stabilized upon the addition of leptomycin B to prevent nuclear export (Figure 10B). These data suggest that EGFR must gain access to the nucleus to phosphorylate STAT1. Indeed, EGFR internalization in endocytic vesicles can clearly be visualized upon EGF stimulation (Figure 10A). Moreover, a closer examination of EGFR localization using super-resolution microscopy revealed that in certain areas of the cell the plasma membrane is in direct physical contact with the nucleus. Therefore, we hypothesized that upon ligand-induced endocytosis from the plasma membrane, a subpopulation of EGFR molecules has immediate access to the nuclear compartment (Figure 12A). To this end, we utilized an EGFR construct that contains alanine substitutions in the juxtamembrane di-lysine motif (679–680-LL converted to AA). This construct is established to be signaling proficient from the plasma membrane, but deficient in endocytosis upon ligand engagement (Kil & Carlin, 2000). Accordingly, the EGFR-AA construct was not able to induce phosphorylation of STAT1 in

response to EGF (Figure 12B). Taken together, these data consistently indicate that a subset of EGF receptors is able to translocate to the nucleus via endocytosis to phosphorylate STAT1.

2.6 Pharmacological Biasing of EGFR Signaling Promotes EGF-induced Apoptosis in Metastatic Breast Cancer cells

To specifically target the function of the subpopulation of EGF receptors that reach the nuclear compartment, we utilized our recently reported novel EGFR inhibitor (Bartolowits et al., 2017) (Figure 13). This chemical construct contains the EGFR tyrosine kinase inhibitor gefitinib linked to a peptoid moiety encoding the SV40 nuclear localization sequence (NLS-gefitinib). This approach leads to robust accumulation of gefitinib in the nucleus (Bartolowits et al., 2017). Consistent with the notion that only endocytosed EGFR molecules have access to the nuclear pool of STAT1, pretreatment of NME-LM1 cells with this chimeric NLS-gefitinib molecule led to a potent blockade of EGF-induced STAT1 phosphorylation without affecting phosphorylation of ERK1/2 (Figure 14A). In contrast, pretreatment with trametinib drastically alters the STAT1:ERK1/2 activation ratio by completely preventing downstream phosphorylation of ERK1/2, while leading to a slight increase in EGF-induced phosphorylation of STAT1 (Figure 14A).

To quantify the biological implications of these events, we again analyzed caspase 3/7 activation following EGF treatment in the presence or absence of these inhibitors (Figure 14B). Consistent with our findings in Figure 7 implicating the role of STAT1 in facilitating EGF-induced apoptosis, the addition of gefitinib-NLS blocked EGF-induced activation of caspase 3/7 (Figure 14B). Consistent with the induction of apoptosis, cotreatment of these lung metastases with trametinib led to a robust increase in EGF-induced caspase 3/7 activity and Annexin V staining (Figure 14B and 15). Also, NLS-gefitinib was capable of preventing EGF-mediated growth inhibition in the NME-LM1 cells, and NLS-gefitinib increased cell viability under nonstimulated conditions (Figure 14C). In contrast, the addition of EGF augmented the growth-suppressive effects of trametinib in a shorter-term assay (Figure 14D). Consistent with our shRNA depletion studies, the ability of EGF to induce apoptosis is likely dependent on STAT1 since the addition of IL-6, a specific activator of STAT3, did not alter trametinib-induced growth inhibition (Figure 14D). All of these events could be replicated using the NME-LM2 metastatic variant (Figure 16). The only noted difference in the LM2 cells was that no caspase 3/7 activity could be quantified

with EGF alone, but again the addition of EGF and trametinib led to significantly increased apoptosis as compared to trametinib alone (Figure 16A). As cells escape primary mammary tumors, a particularly aggressive subpopulation is able to survive the non-adherent conditions in the blood/lymphatic circulation, and these cells are ultimately responsible for colonizing vital organs. To recapitulate these events in an *in vitro* assay, we generated spheroids of metastatic cells in non-adherent conditions and then transferred these spheroids onto a bed of reconstituted basement membrane (Figure 14E). Using this approach, the metastatic NME-LM1 tumorspheres form highly invasive, multicellular branches over a period of 3 days (Figure 14E). The addition of physiological amounts of EGF or nanomolar concentrations of trametinib partially blocked these events, but a combination of the growth factor and trametinib completely prevented the invasive growth of these highly metastatic cells. Therefore, biasing EGFR signaling toward STAT1 using downstream inhibitors can enhance the apoptotic potential of this pathway. Figure 17 is a schematic representation of the above findings.

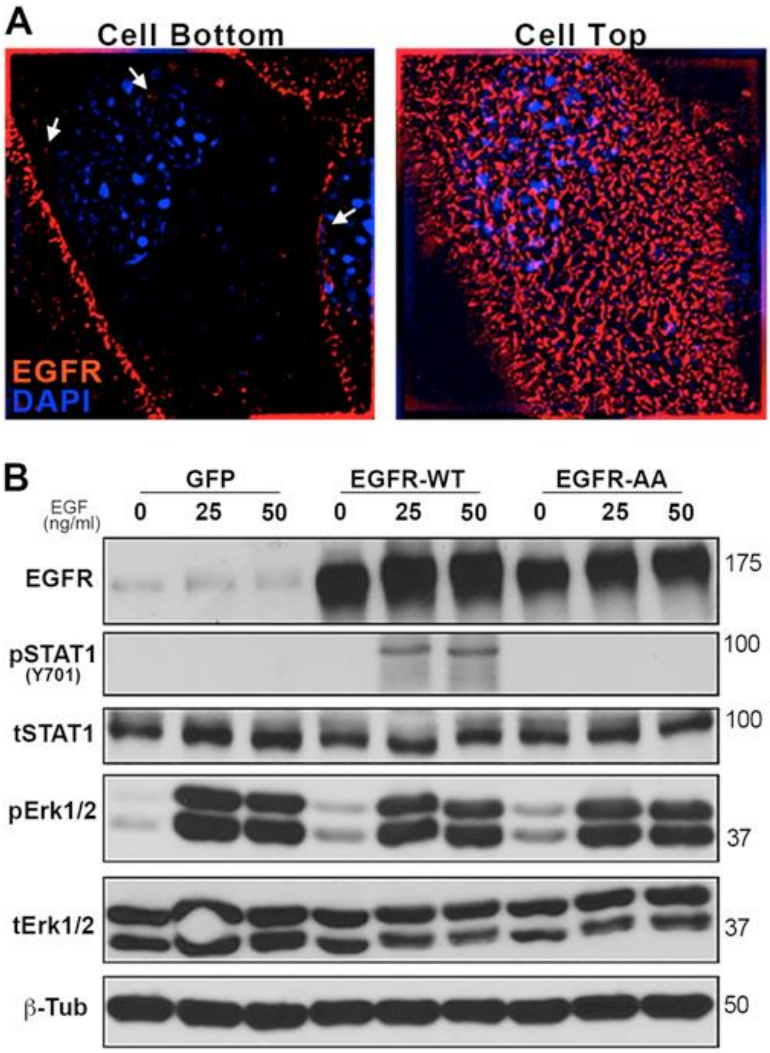


Figure 12. Endocytosis of EGFR is required for phosphorylation of nuclear STAT1. **A.** NME cells were analyzed by immunofluorescence and imaged via super-resolution microscopy for localization of EGFR (100× objective). These cells were counterstained with DAPI to visualize DNA. Images of the same cell are shown for sections taken at the interface of the cell with the coverslip (cell bottom) and top of the cell (cell top). Arrows indicate areas where EGF receptors appear to be in direct contact with the nucleus. Data are representative images from at least 10 fields of view over two independent experiments. **B.** NMuMG cells expressing wild type EGFR (EGFR-WT), the endocytosis-deficient 679–680-AA mutant form of EGFR (EGFR-AA), or GFP as a control, were stimulated with EGF (50 ng/ml) for 30 minutes and analyzed for phosphorylation of STAT1 and ERK1/2. Analysis of total levels of EGFR, STAT1, ERK1/2, and β-tubulin (β-Tub) served as loading controls. Immunoblots are representative of at least three independent experiments.

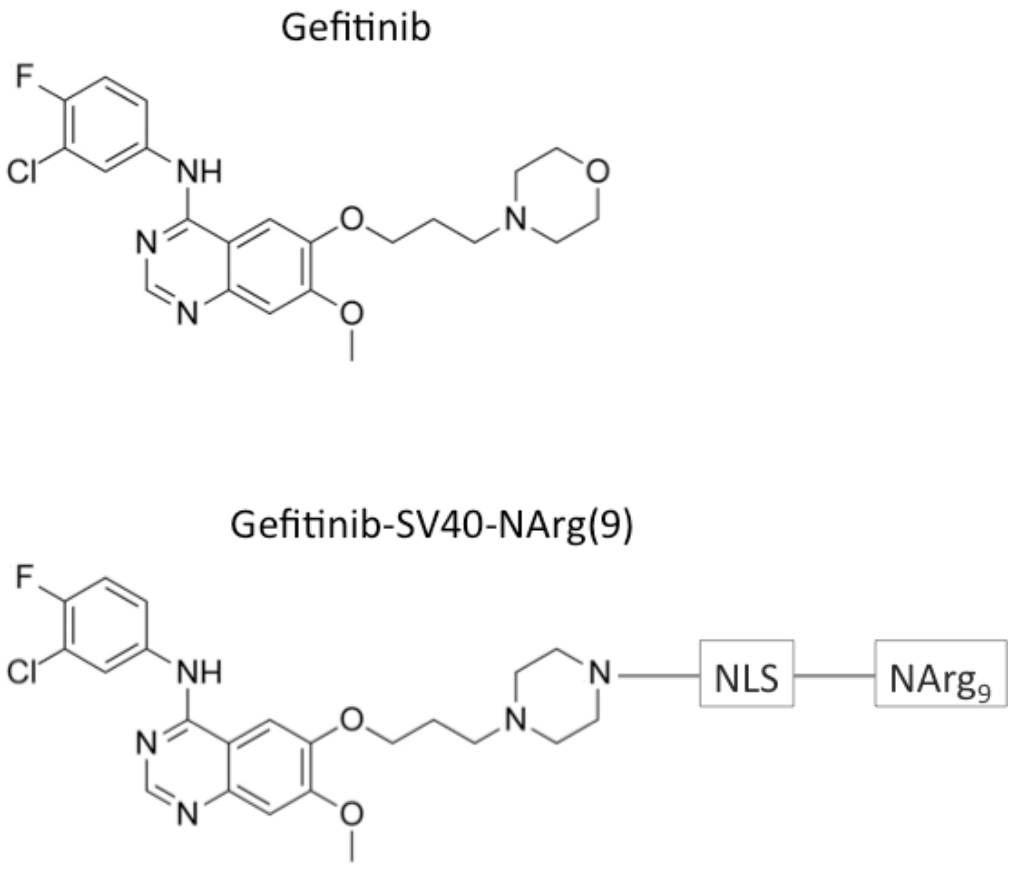


Figure 13. Comparison between the structures of the EGFR inhibitor gefitinib and its derivative NLS-gefitinib. **Top.** Chemical structure of unmodified gefitinib. **Bottom.** Schematic showing the basic structure of NLS-gefitinib in which gefitinib is chemically linked to a nuclear localization signal (NLS) and a nine-membered poly-arginine peptoid sequence (schematic is drawn to illustrate the compound from (Bartolowits et al., 2017)).

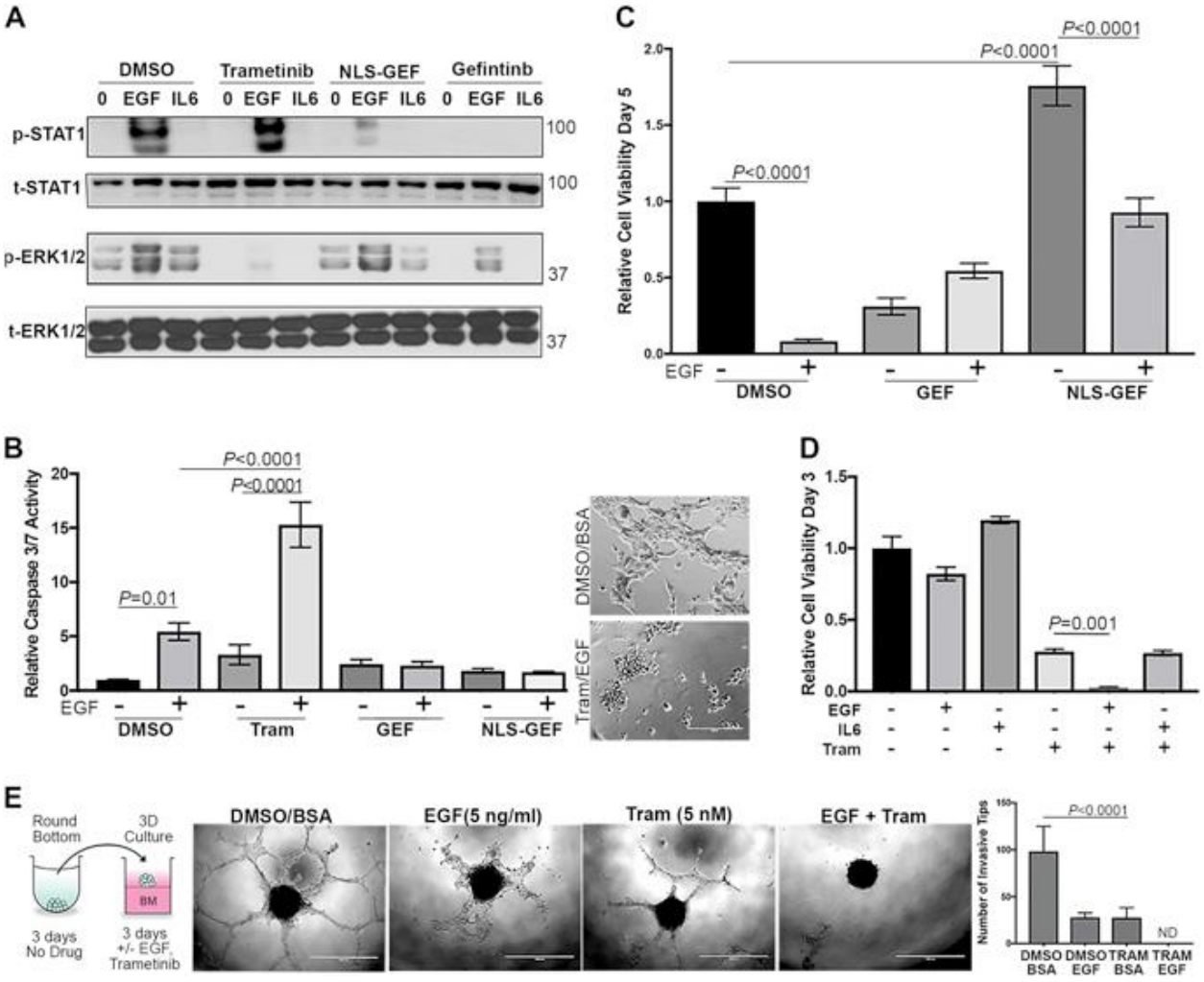


Figure 14. Inhibition of ERK1/2 signaling augments EGF-induced apoptosis in metastatic breast cancer cells. **A.** Lung metastatic (NME-LM1) cells were pretreated with trametinib, gefitinib, or a nuclear localization sequence-gefitinib conjugate (NLS-GEF) and then stimulated with EGF (50 ng/ml) for 30 minutes. These cells were subsequently analyzed for phosphorylation of STAT1 and ERK1/2. IL6 and BSA (0) served as protein stimulation controls and total levels of STAT1 and ERK1/2 were assessed as loading controls. **B.** NME-LM1 cells were stimulated with EGF (100 ng/ml) in the presence or absence of trametinib (Tram), gefitinib (GEF), or the nuclear localization sequence-gefitinib conjugate (NLS-GEF). Following 24 hours of treatment, these cells were assayed for caspase 3/7 activity. (Inset) Representative images of cells under control and Tram/EGF stimulation are shown. **C.** As in panel B, NME-LM1 cells were stimulated with EGF (50 ng/ml) in the presence of the indicated inhibitors for 5 days at which point changes in cellular viability were quantified. **D.** NME-LM1 cells were stimulated as indicated for 3 days at which point cell viability was quantified. **E.** NME-LM1 tumorspheres were formed in round-bottom wells and subsequently transferred to a hydrogel layer of basement membrane in the presence or absence of EGF and trametinib. Tips of invading cellular branches were quantified. Data in panels B-E are the mean ± SE of three independent experiments completed in triplicate

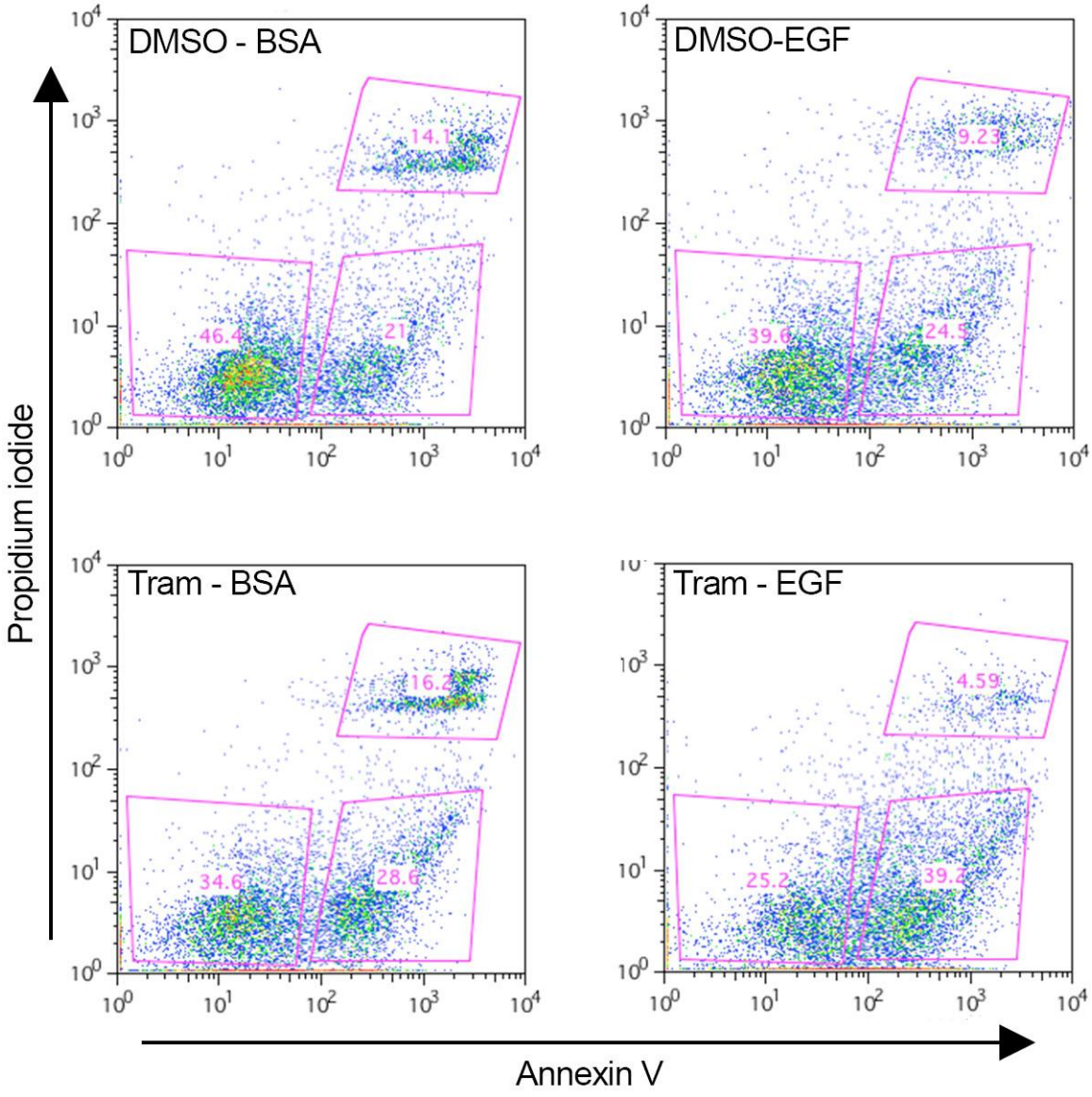


Figure 15. Combined treatment with Trametinib enhances EGF-induced apoptosis. NME-LM1 cells were stimulated with EGF (100 ng/ml) in the presence or absence of trametinib (Tram). Following 24 hours of treatment, these cells were trypsinized and stained with Annexin V antibodies and propidium iodide. Stained cells were analyzed by flow cytometry. The percentage of the total cell population in each gate is indicated.

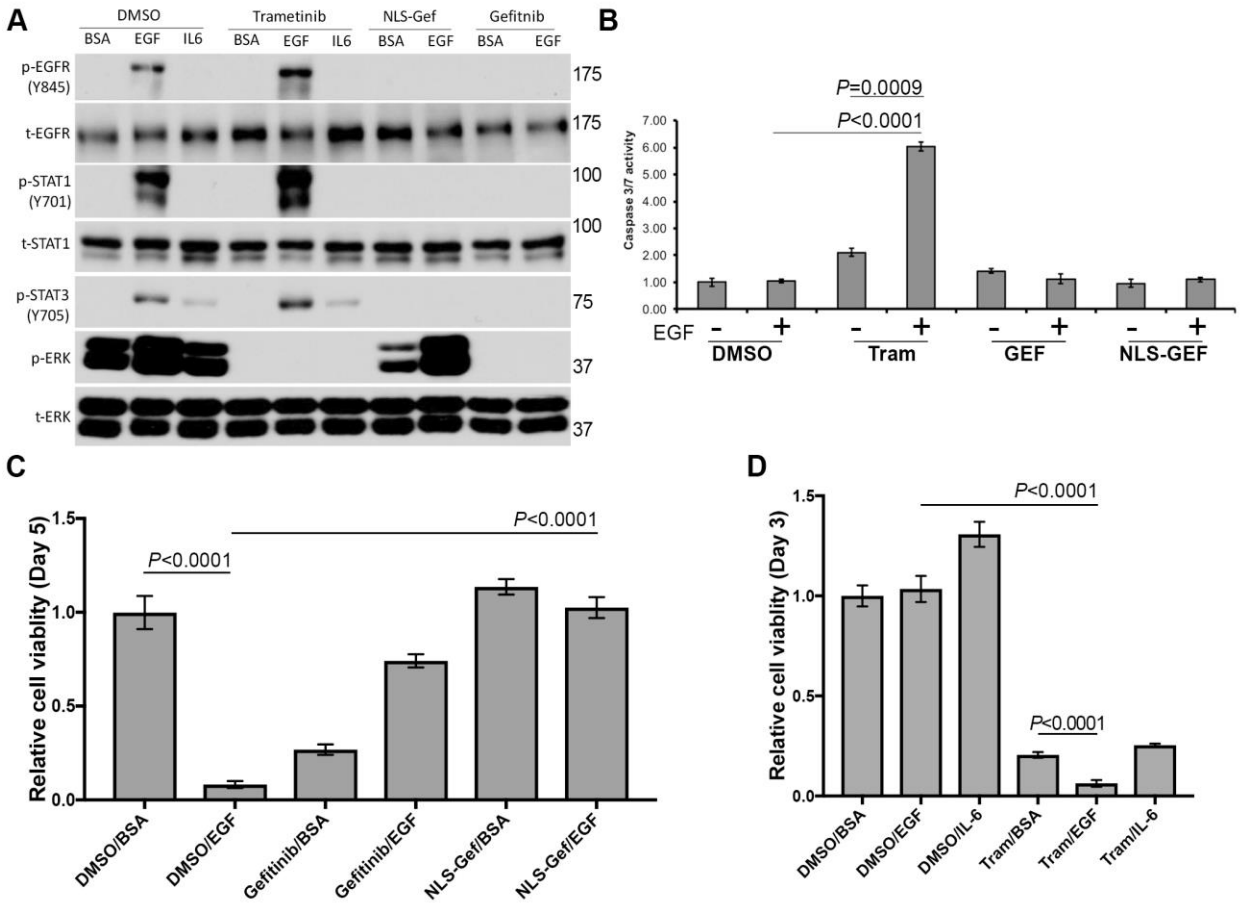


Figure 16. Inhibition of ERK1/2 signaling causes EGF-induced apoptosis in lung metastatic breast cancer cells. **A.** Lung metastatic (NME-LM2) cells were pretreated with trametinib, gefitinib, or a nuclear localization sequence-gefitinib conjugate (NLS-GEF) and then stimulated with EGF (50 ng/ml) for 30 minutes. These cells were subsequently analyzed for phosphorylation of EGFR, STAT3, STAT1, and ERK1/2. BSA (0) served as a protein stimulation control and total levels of EGFR, STAT1, and ERK1/2 were assessed as loading controls. **B.** NME-LM2 cells were stimulated with EGF (100 ng/ml) in the presence or absence of the indicated inhibitors (Tram = trametinib, GEF = gefitinib, NLS-GEF = gefitinib conjugated to a nuclear localization sequence). Twenty-four hours later, cells were assessed for caspase 3/7 activity. **C.** NME-LM2 cells were treated with trametinib (Tram; 5 nM) in the presence or absence of EGF (50 ng/ml) or IL6 for three days at which point cell viability was quantified. **D.** NME-LM2 cells were stimulated with EGF (50 ng/ml) in the presence or absence of the indicated inhibitors for 5 days at which point cell viability was quantified. All data are the mean ± SE of three separate experiments completed in triplicate resulting in the indicated P-values.

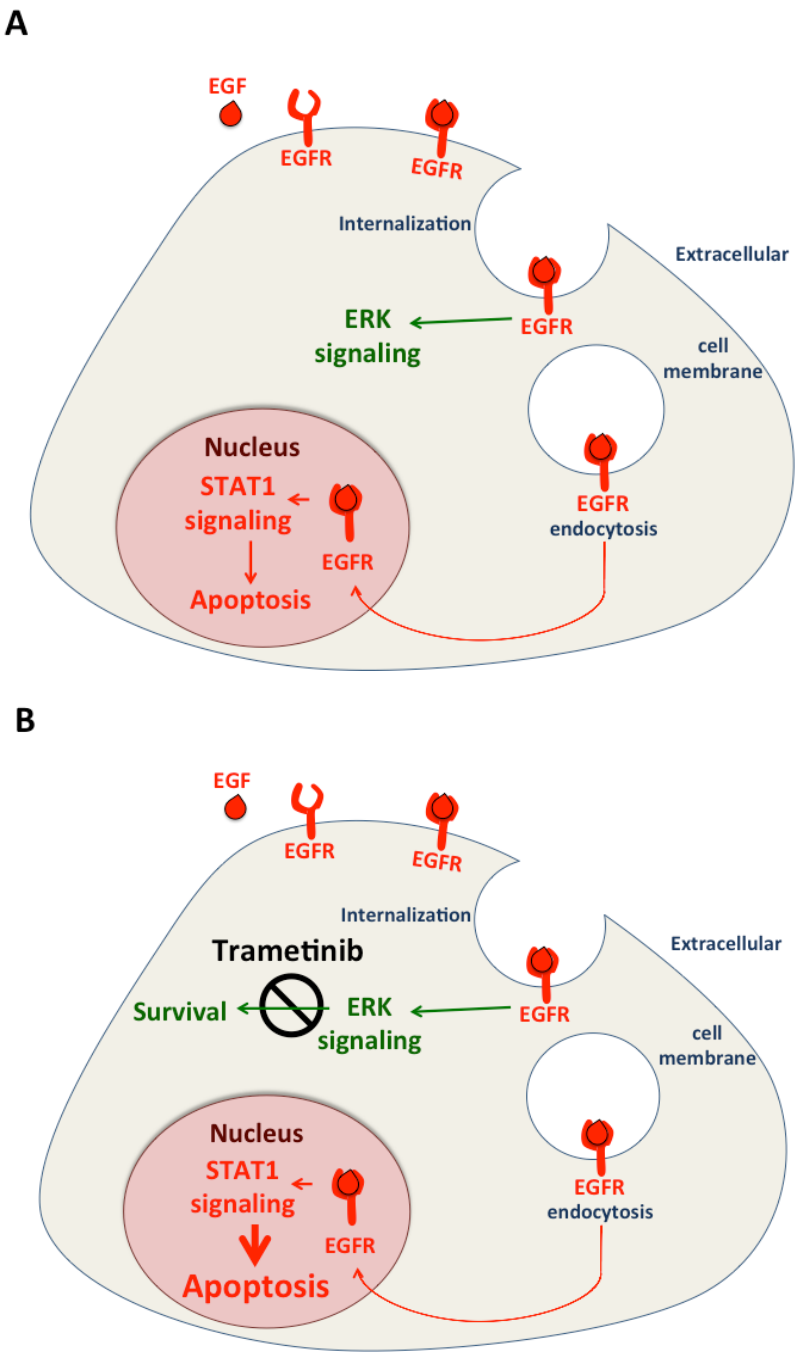


Figure 17. The role of nuclear EGFR in STAT1-mediated apoptosis. **A.** Schematic showing that in metastatic breast cancer, increased nuclear translocation of EGFR is responsible for STAT1 phosphorylation and induced apoptosis. This is because specifically blocking nuclear EGFR function (using NLS-gefitinib) blocks STAT1 phosphorylation and induced apoptosis. ERK1/2 phosphorylation, a cytoplasmic event, is consistently maintained whenever NLS-gefitinib is used, demonstrating that NLS-gefitinib does not target EGFR cytoplasmic signaling. **B.** Schematic showing that combining EGF with trametinib potentiates apoptosis by blocking survival cytoplasmic ERK1/2 signaling. Ultimately, this leads to dominant STAT1 phosphorylation and mediated apoptosis by nuclear EGFR.

2.7 Pharmacological Biasing of EGFR Signaling Fundamentally Changes the Response to EGF in Primary Tumor Cells

We next sought to determine if biasing EGFR signaling could change the EGF response of primary tumor cells from proliferative to apoptotic. Therefore, we treated the non-metastatic NME cells with EGF in the presence of NLS-gefitinib, unconjugated gefitinib, or trametinib. As observed in Figure 3, very little activation of STAT1 in NME cells occurred upon EGF stimulation, however, this pathway was greatly enhanced upon inhibition of MEK1/2 with trametinib (Figure 18A). Consistent with the notion that the ERK1/2:STAT1 activation ratio dictates the proliferative versus apoptotic outcome of EGF, we observed a drastic induction of apoptosis in NME cells upon co-administration of EGF and trametinib, whereas either treatment alone did not produce any caspase 3/7 activation (Figure 18B and C). Moreover, these effects could not be produced in control cells expressing normal amounts of EGFR, or in cells expressing the EGFR-AA variant that is deficient in STAT1 activation (Figure 19 and 18C). Finally, using our three-dimensional (3D) spheroid assay described in Figure 14 we could illustrate the non-metastatic nature of the NME cells as they fail to form any invasive structures (Figure 18D).

2.8 EGFR:STAT1 Signaling Augments Trametinib-induced Growth Inhibition

To extend our findings to other breast cancer models of EGFR signaling, we applied a similar EGF-trametinib treatment combination to our doxycycline (dox)-inducible model of EGFR expression in the metastatic Ca1a cells. When EGFR expression was induced with dox, we observed STAT1 phosphorylation in response to EGF stimulation (Figure 20A). Consistent with the Ca1a cells being transformed by a constitutively active form of RAS, we did not observe any further ERK1/2 phosphorylation upon EGF stimulation (Figure 20A). However, EGF-induced phosphorylation of STAT1 was enhanced in the presence of trametinib concentrations capable of completely blocking this constitutive phosphorylation of ERK1/2 (Figure 20A). Using these cells, we observed a dose-dependent induction of EGFR expression with dox (Figure 20B). This transient induction resulted in an EGFR-dependent inhibition of cell growth in the presence of EGF and trametinib that was not observed in the presence of trametinib alone (Figure 20C).

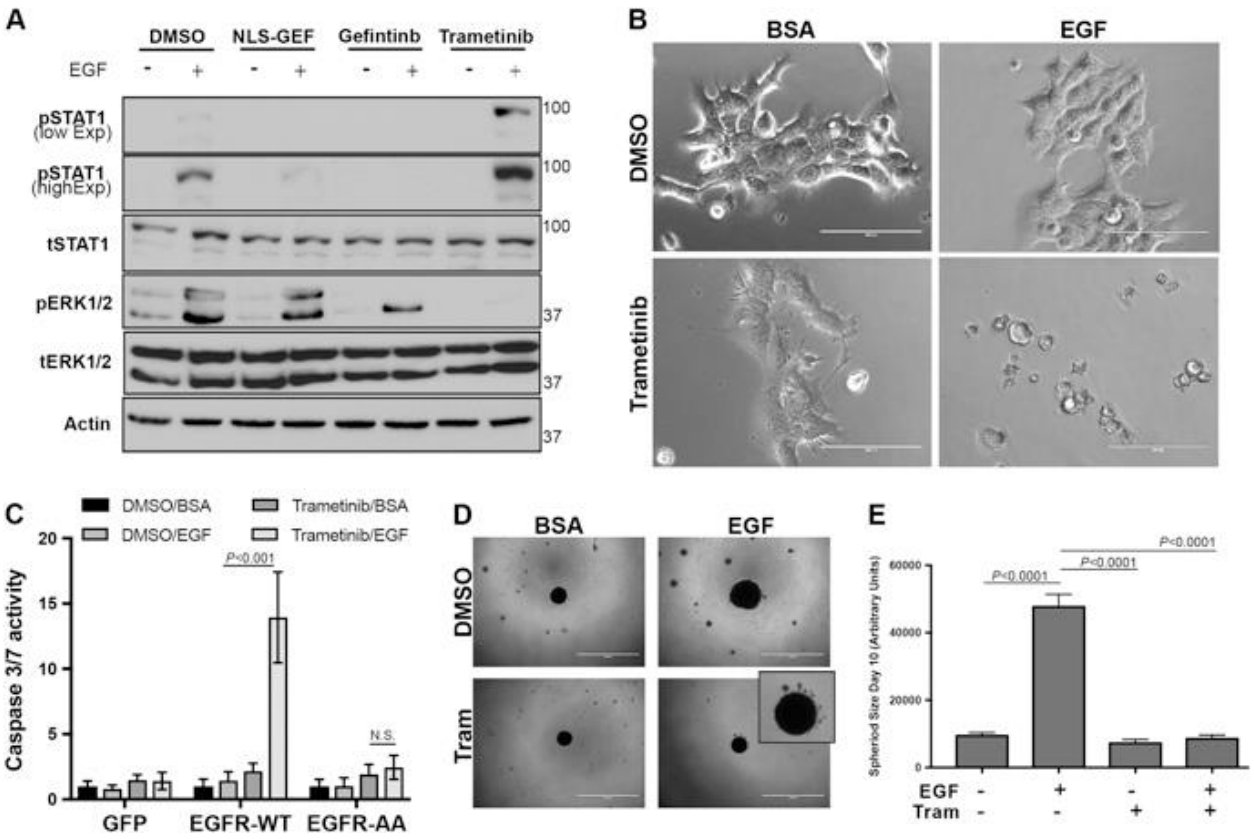


Figure 18. Inhibition of ERK1/2 signaling fundamentally changes the response to EGF in primary mammary tumor cells. **A.** NME cells were stimulated with EGF (50 ng/ml) for 30 minutes in the presence or absence of trametinib, gefitinib, or the gefitinib nuclear localization sequence-conjugate (NLS-GEF). These cells were then analyzed for phosphorylation of STAT1 and ERK1/2. Expression of total STAT1, ERK1/2, and actin served as loading controls. **B.** Representative brightfield photomicrographs of NME cells stimulated for 24 hours with EGF (50 ng/ml), trametinib (5 nM), or the combination. **C.** Control NMuMG-GFP (GFP) cells or those cells expressing wild type EGFR (EGFR-WT) or the 679–680-AA variant of EGFR (EGFR-AA) were stimulated with EGF in the presence or absence of trametinib as in panel B and assessed for Caspase 3/7 activity. **D.** Representative photomicrographs of NME tumorspheres cultured under 3D hydrogel conditions in the presence or absence of exogenous EGF (5 ng/ml) and trametinib (5 nM). **E.** Quantification of NME spheroid size under the conditions described in panel D. Data in panels C and C are the mean ± SE of three independent experiments completed in triplicate.

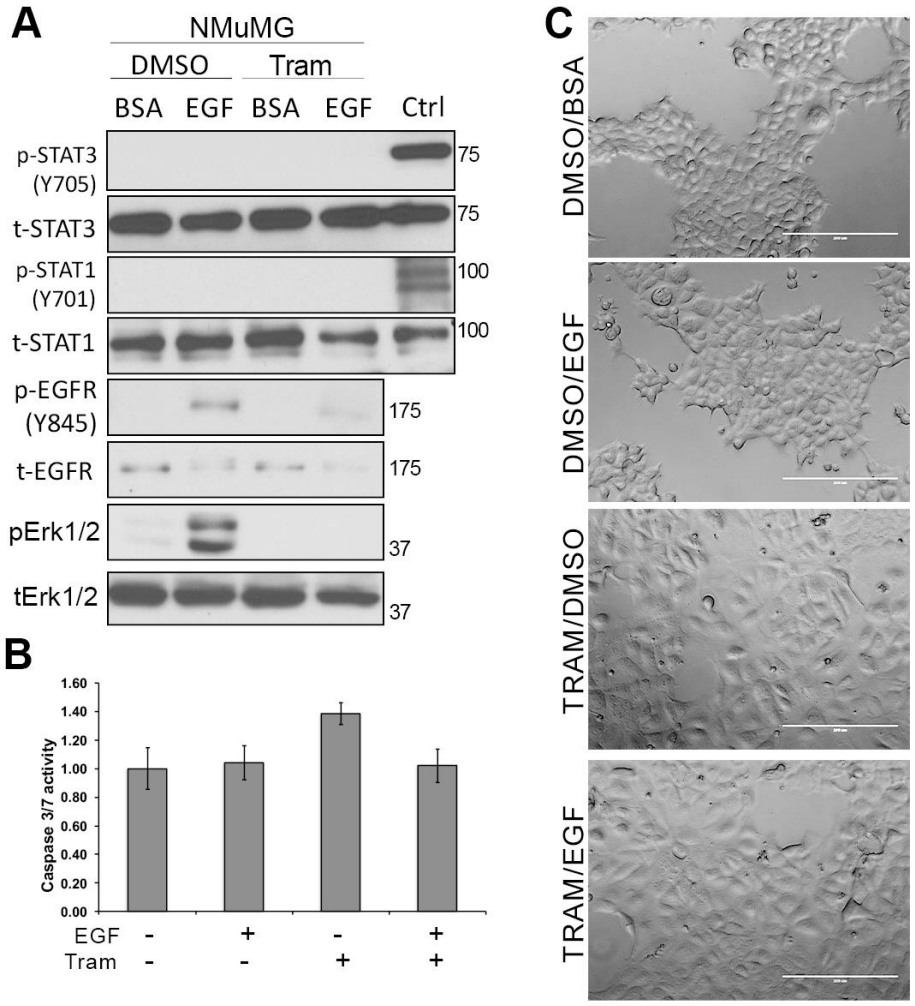


Figure 19. A combination of EGF and trametinib does not induce STAT1 phosphorylation or apoptosis in normal mammary epithelial cells. **A.** Non-transformed NMuMG cells were stimulated with EGF (50 ng/ml) for 30 minutes in the presence or absence of trametinib (Tram; 100 nM). These cells were lysed and analyzed for phosphorylation of STAT1, ERK1/2, and EGFR. Expression of total EGFR, STAT1, and ERK1/2 served as loading controls. **B.** NMuMG cells were stimulated with EGF (100 ng/ml) in the presence and absence of trametinib (Tram; 100 nM) for 24 hours and these cells were assessed for caspase 3/7 activity. Data are the mean ± SE of three separate experiments completed in triplicate. **C.** Phase-contrast photomicrographs of NMuMG cells treated as described in panel B.

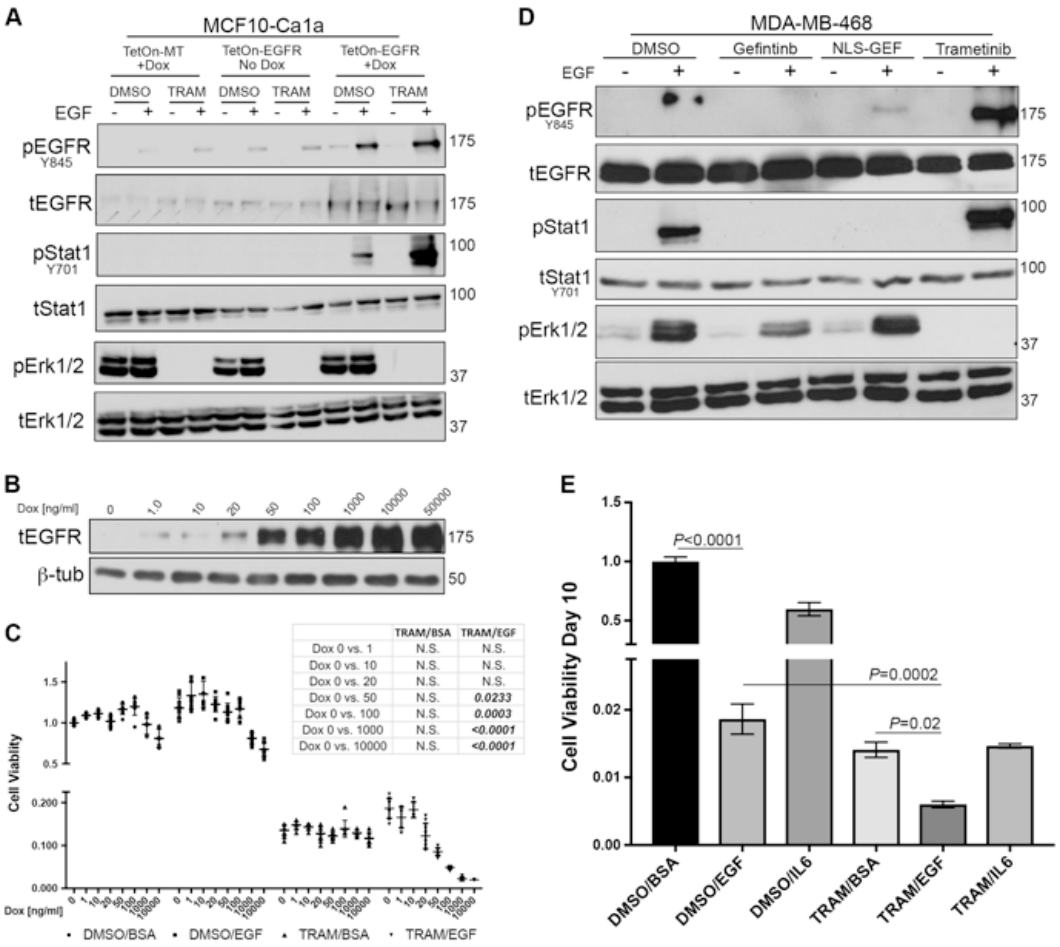


Figure 20. EGF enhances the growth inhibitory effect of trametinib. **A.** Metastatic MCF10-Ca1a cells were constructed to express a control (TetOn-MT) or EGFR (TetOn-EGFR) encoding vector under the control of doxycycline (Dox) inducible promoter. These cells were pretreated with Dox for 24 hours and subsequently stimulated with EGF (50 ng/ml) for 30-minute in the presence or absence of trametinib (TRAM). Phosphorylation of EGFR, STAT1, and ERK1/2 was assessed. Expression of total EGFR, STAT1, and ERK1/2 served as loading controls. **B.** MCF10-Ca1a TetOn-EGFR cells were stimulated with the indicated concentrations of Dox for 24 hours and assessed for expression of EGFR. β-Tub served as a loading control. **C.** MCF10-Ca1a TetOn-EGFR cells were stimulated with the indicated concentrations of Dox for 10 days in the presence or absence of EGF and trametinib and cell viability was quantified. Chart inset: The resultant P-values of ANOVA analyses comparing the indicated treatment groups under control (Dox 0) and Dox conditions. **D.** The MDA-MB-468 cells were stimulated with EGF (50 ng/ml) for 30 minutes in the presence or absence of trametinib and assessed for phosphorylation of EGFR, STAT1, and ERK1/2. Expression of total EGFR, STAT1, and ERK1/2 served as loading controls. **E.** The MDA-MB-468 cells were grown in the presence or absence of EGF (50 ng/ml), IL6 (20 ng/ml), trametinib (5 nM) or the indicated combinations for a period of 10 days at which point cell viability was quantified. The indicated groups were analyzed by T-test resulting in the indicated P-values. Data in panels A, B, and D are representative of three independent analyses, and data in panels C and E are the mean ± SE for three independent experiments completed in triplicate.

We similarly assessed the MDA-MB-468 (468) model of triple-negative breast cancer. As we observed in our metastatic cells and consistent with previous reports, treatment of the 468 cells with EGF induced phosphorylation of STAT1 (Figure 20D) (Andersen et al., 2008). EGF-induced phosphorylation of STAT1 was again completely blocked by our nuclear-localized EGFR inhibitor, a condition that has no effect on ERK1/2 phosphorylation (Figure 20D). Importantly, pretreatment with trametinib at a concentration that completely prevented ERK1/2 phosphorylation enhanced EGF-induced STAT1 signaling (Figure 20D). Finally, combined treatment with EGF and trametinib lead to potent inhibition of cell viability as compared to either treatment alone (Figure 20D). Such a combination effect was not observed with IL6, which has been shown to inhibit the growth of 468 cells by conferring stem-like properties but is unable to induce STAT1 phosphorylation.

2.9 Discussion

EGFR activation is upstream of multiple signal transduction pathways. The differential activation of particular pathways in response to ligand leads to oncogenic versus apoptotic signals in specific cell types (Ali & Wendt, 2017; Högnason et al., 2001; Prasad & Church, 1991) (Figure 21). We have previously reported that EGFR function paradoxically changes from oncogenic to apoptotic after *in vivo* metastasis of BC cells (Wendt et al., 2015). In the current study, we demonstrate that ligand-mediated EGFR activation ultimately results in nuclear STAT1-dependent apoptosis of metastatic breast cancer (BC) cells. This fundamental change in response to EGF through breast cancer progression led us to address the hypothesis that pharmacological biasing of downstream signaling could reveal apoptotic EGFR signaling, even in early-stage breast cancer. This concept is supported by recent studies in the fields of G-protein coupled receptor signaling and receptor tyrosine kinase signaling, which indicate that differential ligand stimulation leads to biased downstream pathway activation and therefore unique biological outputs (Knudsen, Mac, Henriksen, van Deurs, & Grøvdal, 2014). These previous studies have focused on unnatural ligands and allosteric modulation of receptors. In contrast, our work herein demonstrates that unique cellular outcomes in response to an endogenous ligand can be manifested when specific downstream pathways are pharmacologically interdicted.

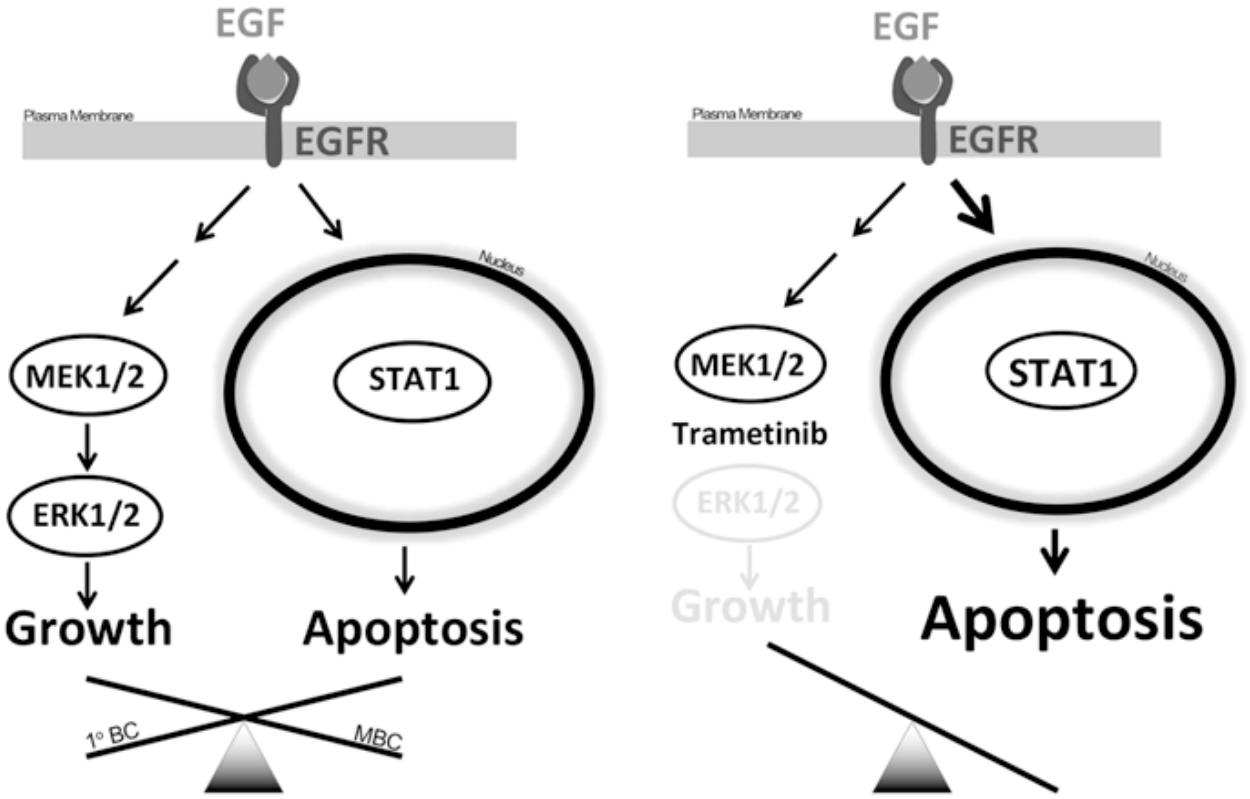


Figure 21. Schematic representation of biased EGFR signaling. **Left.** In primary breast cancer, EGFR signaling is dominated by pro-growth and pro-survival signaling pathways such as ERK1/2, driving several oncogenic processes. In contrast, in metastatic breast cancer, STAT1 signaling is enhanced, supporting EGF-induced apoptosis. **Right.** EGFR signaling can be biased toward STAT1-mediated apoptosis through pharmacologic inhibition of MEK (and thus ERK1/2) signaling using the allosteric inhibitor trametinib.

Recent studies indicate that constitutive EGFR signaling induced upon receptor mutations are distinct and mutually exclusive as compared to ligand-induced signaling (Chakraborty et al., 2014). Our findings indicate that direct pharmacological targeting of WT-EGFR using our nuclear-localized gefitinib conjugate completely prevents STAT1 activation, leading to enhanced cell growth as this compound has no effect on the ability of EGFR to signal to ERK1/2. Furthermore, in several instances, we observed unconjugated gefitinib prevents STAT1-mediated apoptotic signaling downstream of ligand-activated WT-EGFR at a much lower concentration than is required for inhibition of ERK1/2-mediated proliferative signaling. These data suggest that complete to near-complete blockade of EGFR function is required before an anti-tumorigenic response would be expected in breast cancer cells bearing high levels of WT-EGFR undergoing ligand-mediated signaling. Indeed, complete pharmacological blockade of a target molecule is

challenging, if not impossible, to achieve *in vivo*. This concept that incomplete inhibition of WT-EGFR is biasing signaling toward proliferative ERK1/2 signaling is completely consistent with the clinical failure of EGFR inhibitors in metastatic breast cancer (Dickler et al., 2008).

Our recent studies demonstrate that metastatic cells increase their nuclear pool of EGFR (Bartolowits et al., 2017). These data are consistent with the findings here demonstrating an enhanced ability of metastatic cells to access and phosphorylate nuclear STAT1 in response to EGF stimulation. The potential mechanisms by which metastatic cells increase their nuclear pool of EGFR are potentially numerous (Brand et al., 2014; Brand, Iida, Li, & Wheeler, 2011; Chakraborty et al., 2014). However, recent data suggest that more migratory cells undergo constant nuclear rupture, and these cells repair these events by using components of endosomal sorting complexes (Denais et al., 2016; Wang et al., 2010). Together with our findings using the EGFR-AA construct, these data suggest a mechanism in which more migratory and metastatic breast cancer cells undergo an increased rate of nuclear rupture and repair and thus sample more activated EGFR molecules from endosomes. These events would lead to an increased pool of nuclear EGFR and enhanced interaction of these receptors with the nuclear pool of STAT1. Finally, several of the model systems interrogated herein demonstrate that trametinib enhances EGFR:STAT1 signaling. Our recent studies indicate that EGFR signaling is regulated in metastatic cells via expression of the EGFR inhibitory molecule Mig6 (Wendt et al., 2015). Expression of Mig6 is driven via ERK1/2, constituting a physiologic negative feedback on EGFR activation (Fiorini et al., 2002; Keeton, Xu, Franklin, & Messina, 2004). Although not evaluated here, trametinib may serve to short-circuit this negative feedback by decreasing Mig6 and allowing unabated activation of alternate, apoptotic signaling downstream of EGFR such as STAT1.

In conclusion, our studies broadly illustrate the importance of understanding the cellular outcomes of cytoplasmic kinase inhibitors not only in terms of the pathway they are targeting, but also in terms of changes they insight to alternate signal transduction pathways induced from shared upstream receptors. These data support current clinical trials evaluating the efficacy of trametinib in the treatment of metastatic breast cancer (example clinical trials: NCT02900664 and NCT03065387). However, our results argue against the concurrent use of EGFR kinase inhibitors in these patients as this will block apoptotic, EGFR:STAT1 signaling, limiting the apoptotic effect of trametinib treatment. Current studies in our lab are exploring therapeutic approaches to enhance the antitumor effects of trametinib through specific augmentation of EGFR:STAT1 signaling.

2.10 Methods and Materials

2.10.1 Cell-lines and Reagents

Murine NMuMG and human MDA-MB-468 cells were purchased from the American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% or 5% fetal bovine serum (FBS), respectively. MDA-MB-468 passages 1–5 were used in this study. NMuMG cells and their metastatic variants also received 10 μg/ml of insulin. Construction of NMuMG cells expressing human mutant of EGFR (EGFR-AA) or the wild-type (EGFR-WT) construct (NME cells) and their metastatic variants are described elsewhere (Balanis et al., 2013). Cellular depletion of STAT1 cells was accomplished by vesicular stomatitis virus G protein (VSVG) lentiviral transduction of pLKO.1 shRNA vectors (Thermo Scientific). Sequences of shRNAs can be found in Table 2. The human MCF10-Ca1a cell-line was kindly provided by Dr. Fred Miller (Wayne State University) and cultured in DMEM supplemented with 10% FBS. A list of the chemical inhibitors and growth factors used throughout the study can be found in Table 3.

Table 2. Sequences of shRNAs used in Chapter 2.

Target	**TRC#**	**Sequence (mature antisense)**
mSTAT1#23	TRCN0000054923	ATTCTCTGGTATGTTCTCGGC
mSTAT1#24	TRCN0000054924	TAAGAGAGTGAAGTTCTTCGG
mSTAT1#25	TRCN0000054925	AAACGAGACATCATAGGCAGC
mSTAT1#26	TRCN0000054926	AATATCTGGGAAAGTAACAGC
mSTAT1#27	TRCN0000054927	ATCAGAGTGTTCTGAGTGAGC

Table 3. List of the chemical inhibitors and growth factors used in Chapter 2.

Name	Target	Concentration	Supplier
Trametinib	MEK1/2	Indicated	Selleckchem
Gefitinib	EGFR	500 nM	Selleckchem
Erlotinib	EGFR	Indicated	Selleckchem
NLS-gefitinib	EGFR	1μM	Davisson Lab
Leptomycin B	CRM1	20 nM	Cell Signaling Technologies
EGF	-	Indicated	GoldBio
IL-6	-	20 ng/ml	GoldBio

2.10.2 Immunoblot and Immunofluorescent Analyses

For immunoblot assays, equal aliquots of nuclear, cytoplasmic, or whole-cell lysates were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes using standard methods. Nuclear and cytoplasmic fractions were generated via a differential lysis Buffer A (10 mM HEPES; pH 7.9, 10 mM KCL, 0.1 mM EDTA, and protease and phosphatase inhibitors) to obtain a cytoplasmic fraction. Intact nuclei were pelleted from these lysates and washed twice in fresh Buffer A before being lysed in Buffer B (20 mM HEPES; pH 7.9, 0.4 M NaCl, 1 mM EDTA, 10% glycerol, and protease and phosphatase inhibitors). Immunofluorescent assays were conducted using primary antibodies in combination with fluorescently labeled secondary antibodies. Confocal images were captured using a Nikon A1Rsi inverted microscope. Super-resolution images are structure illuminations obtained on a Nikon Ti-E inverted microscope with N-SIM capability. Antibody concentrations and suppliers are listed in Table 4.

2.10.3 Apoptosis and Cell Viability Assays

Caspase 3/7 activity was quantified using the Caspase-Glo 3/7 assay (Promega) according to the manufacturer's instructions. Cell viability was measured using the CellTiter-Glo assay (Promega) according to the manufacturer's instructions. Floating cells were collected from all conditions, spun down, resuspended in trypsin to break clumps of cells into single-cell suspension. For Annexin V staining, adherent cells were trypsinized into single-cell suspensions and added to

the floating cell fraction and analyzed for Annexin V/PI staining using a FITC Apoptosis Detection Kit I (BD Pharmingen).

2.10.4 Three-dimensional (3D) Culture Methods

Primary and metastatic breast cancer cells (5×10^3) were plated into non-adherent round-bottom 96-well plates (Corning) in full growth media and cultured for 3 days. At this point, the tumorspheres were physically transferred with 50 µl of residual media and 150 µl of fresh media to a flat bottom 96-well plate coated with 50 µl of growth factor reduced basement membrane hydrogel (Trevigen) in the presence or absence of trametinib and EGF. These structures were allowed to grow for an additional 3 days for metastatic NM-LM1 cells or 10 days for non-metastatic NME cells at which point structure size was quantified using Image J analyses or invasive tips were enumerated.

2.10.5 Statistical Methods

Statistical analyses were carried out using unpaired Student's T-test or ANOVA where the data fit the parameters of the test. P-values < 0.05 were considered statistically significant. P-values for all experiments are indicated. All P-values were generated using Prism-GraphPad software.

Table 4. List of antibody concentrations and suppliers used in Chapter 2.

Antibody	**Dilution**	**Supplier**
pSTAT1(Y701)	1:1000	Cell Signaling Technologies
tSTAT1	1:1000, 1:100 (I.F.)	Cell Signaling Technologies
pEGFR(Y845)	1:1000	Cell Signaling Technologies
tEGFR	1:1000	Cell Signaling Technologies
EGFR	1:100 (I.F)	Santa Cruz Biotechnologies
pERK1/2	1:2000	Cell Signaling Technologies
tERK1/2	1:2000, 1:100 (I.F)	Cell Signaling Technologies
□-actin	1:1000	Santa Cruz Biotechnologies
Lamin A/C	1:500	Santa Cruz Biotechnologies
β-tubulin	1:1000	Developmental Studies Hybridoma Bank

CHAPTER 3. THE ROLE OF STAT3 IN PRIMARY AND METASTATIC BREAST CANCER

(Work-in-progress manuscript).

3.1 Abstract

Overexpression of EGFR in non-transformed mammary gland cells results in their oncogenic transformation and development of primary mammary tumors. EMT induction in these EGFR-transformed cells facilitates their systemic dissemination and metastasis to the lung and lymph nodes. Despite these findings, the role of STAT3 in these EGFR-transformed EMT-driven models of primary and metastatic breast cancer is unknown. This study demonstrates the necessity of STAT3 expression for EGFR-mediated transformation in the primary tumor setting. Moreover, metastasis of breast cancer is associated with mechanisms that upregulate STAT3 signaling. Indeed, breast cancer cells from EMT-driven and spontaneous metastatic models display increased STAT3 phosphorylation in basal conditions. In contrast to the EGFR:SRC:STAT3 signaling axis observed in mammary tumors, STAT3 activation in metastatic breast cancer is mediated by Janus activated kinases 1 and 2 (JAK1/2). Inhibition of JAK1/2 using the clinical drug ruxolitinib effectively blocks STAT3 phosphorylation but is unable to decrease the viability or invasive capacity of metastatic breast cancer cells. Further investigation of the role of STAT3 revealed its positive involvement in the pro-apoptotic function of EGFR in lung and lymph node models of metastatic breast cancer. Collectively, these investigations provide a plausible explanation for the lack of efficacy of JAK1/2 targeting in clinical and preclinical studies and warrant further investigation as to how to exploit this pathway for the treatment of metastatic breast cancer.

3.2 Introduction to EGFR-mediated Transformation of Mammary Cells

Signal transducer and activator of transcription 3 (STAT3) is a latent transcription factor that mediates signaling downstream of multiple receptor tyrosine kinases (RTKs) and cytokine receptors (Zhong, Wen, & Darnell, 1994). The canonical model of STAT3 activation as a transcription factor involves its phosphorylation, subsequent dimerization, and translocation to the nucleus to regulate gene expression (Yu, Lee, Herrmann, Buettner, & Jove, 2014). It has also been

shown that STAT3 molecules residing in the nucleus can be phosphorylated there by a subpopulation of EGFR constitutively trafficked to the nucleus (Bartolowits et al., 2017). Numerous studies examined the outcome of STAT3 activation on transformation, cell growth and metastasis in breast cancer. For example, substitution of two cysteine residues within the Src Homology 2 (SH2) domain of STAT3 results in spontaneous dimerization, nuclear translocation, and activation of transcription (Bromberg et al., 1999). Introduction of this constitutively-active STAT3 (STAT3-C) molecule into immortalized fibroblasts resulted in their oncogenic transformation and formation of subcutaneous tumors *in vivo* (Bromberg et al., 1999). A multitude of preclinical and clinical studies have supported the tumorigenic outcomes of constitutive STAT3 activation. For example, immunohistochemistry (IHC) analyses revealed increased staining for phosphorylated-STAT3 at tyrosine-705 (p-STAT3-Y705) in a wide variety of tumors, including those of the breast (Banerjee & Resat, 2016; Chung, Giehl, Wu, & Vadgama, 2014). Upstream cell-surface RTK activators of STAT3 are also commonly overexpressed in breast cancer, suggesting a potential role of STAT3 in RTK-mediated tumorigenesis (Wendt, Balanis, Carlin, & Schiemann, 2014). In addition to oncogenic transformation induced by persistent STAT3 activity, phosphorylated STAT3 induces a stem-like phenotype of breast cancer cells, thus supporting the maintenance of malignant phenotype (Foubert, De Craene, & Berx, 2010; Wendt, Allington, & Schiemann, 2009). Stem-like breast cancer cells represent subpopulations of cells within the tumor that are characterized by self-renewal potential. These cells are proposed to re-establish tumors after conventional or targeted therapies and are thus ultimately responsible for breast cancer recurrence and metastasis (Tudoran, Balacescu, & Berindan-Neagoe, 2016). The role of STAT3 in regulating cancer stem-cell properties is consistent with the long-established physiological role of STAT3 in normal stem-cell function (Raz, Lee, Cannizzaro, Eustachio, & Levy, 1999). Along these lines, depletion of STAT3 significantly decreased the tumor-initiating ability of breast cancer cells, further implicating STAT3 in their stem-like behavior (Ling & Arlinghaus, 2005). These abnormalities in STAT3 signaling and associated tumorigenic biology have therefore fueled intense investigations to develop inhibitors of STAT3 that could offer clinical benefits to breast cancer patients (Qin, Yan, Zhang, & Zhang, 2019).

The epidermal growth factor receptor (EGFR) is an established activator of STAT3 signaling (Shao et al., 2003). Overexpression of EGFR results in oncogenic transformation of otherwise non-transformed NIH3T3 fibroblasts and mammary gland cells (Di Fiore et al., 1987;

Wendt et al., 2010). Despite these findings, the functional significance of STAT3 in EGFR-mediated transformation of breast epithelium has not been explored. This study reports for the first time an essential role for STAT3 in EGFR-driven breast tumorigenesis localized to the mammary tissues. Moreover, metastasis of these cells is associated with upregulation of STAT3 signaling through mechanisms that utilize Janus kinases 1 and 2 (JAK1/2). These findings were demonstrated in both EMT-induced and spontaneous models of lung metastatic breast cancer. Inhibition of factors that drive hyper-phosphorylated STAT3 using the JAK1/2 inhibitor ruxolitinib did not reduce cell growth or survival in non-adherent conditions. Rather, this inhibition promoted the survival and proliferation of metastatic breast cancer cells in non-adherent and 3D organotypic cultures. Further investigation of the function of STAT3 revealed that it mediates the pro-apoptotic function of EGFR in the metastatic setting. These studies provide a model in which STAT3 signaling is essential for EGFR-driven transformation in the primary mammary tumors. However, following invasion and systemic dissemination, STAT3 operates downstream of EGFR to drive its pro-apoptotic function in metastatic cells. Collectively, findings herein highlight the need to further delineate the pro- and anti-tumorigenic nature of STAT3 signaling through the metastatic progression of breast cancer. Such studies are essential to appropriately design and assign therapeutics targeting relevant pathophysiology for the treatment of metastatic breast cancer.

3.3 EGFR Overexpression Activates STAT3 Signaling

Previous studies reported that overexpression of wild-type EGFR (EGFR-WT or EGFR) in normal murine mammary gland (NMuMG) cells leads to their oncogenic transformation and establishment of mammary tumors (Balanis et al., 2013; Wendt et al., 2010). Indeed, NMuMG-GFP or YFP cells remain dormant after engraftment onto the mammary fat-pad of female Nu/Nu mice while NMuMG-EGFR (NME) cells consistently form mammary tumors across these studies (Figure 22A and B).

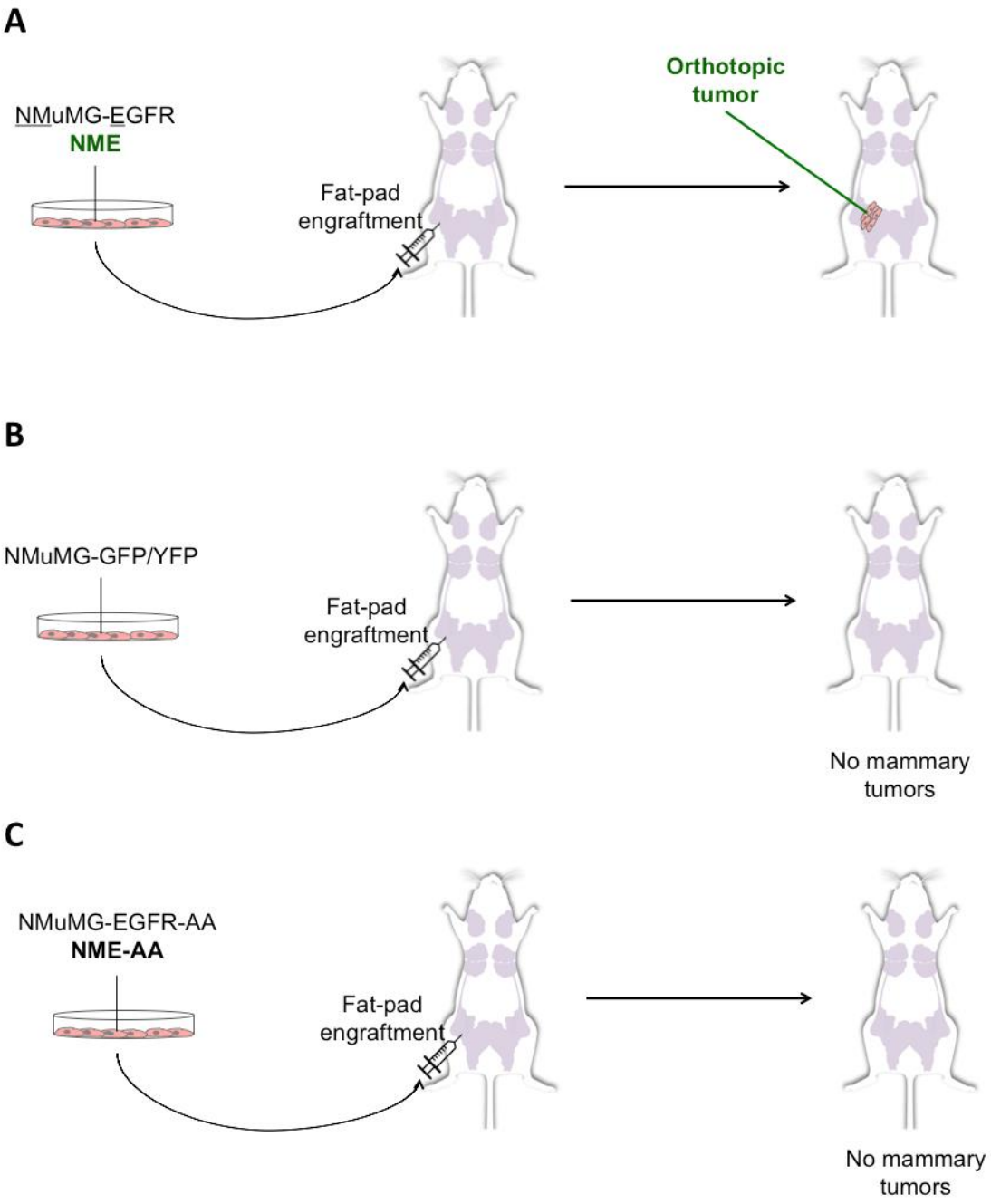
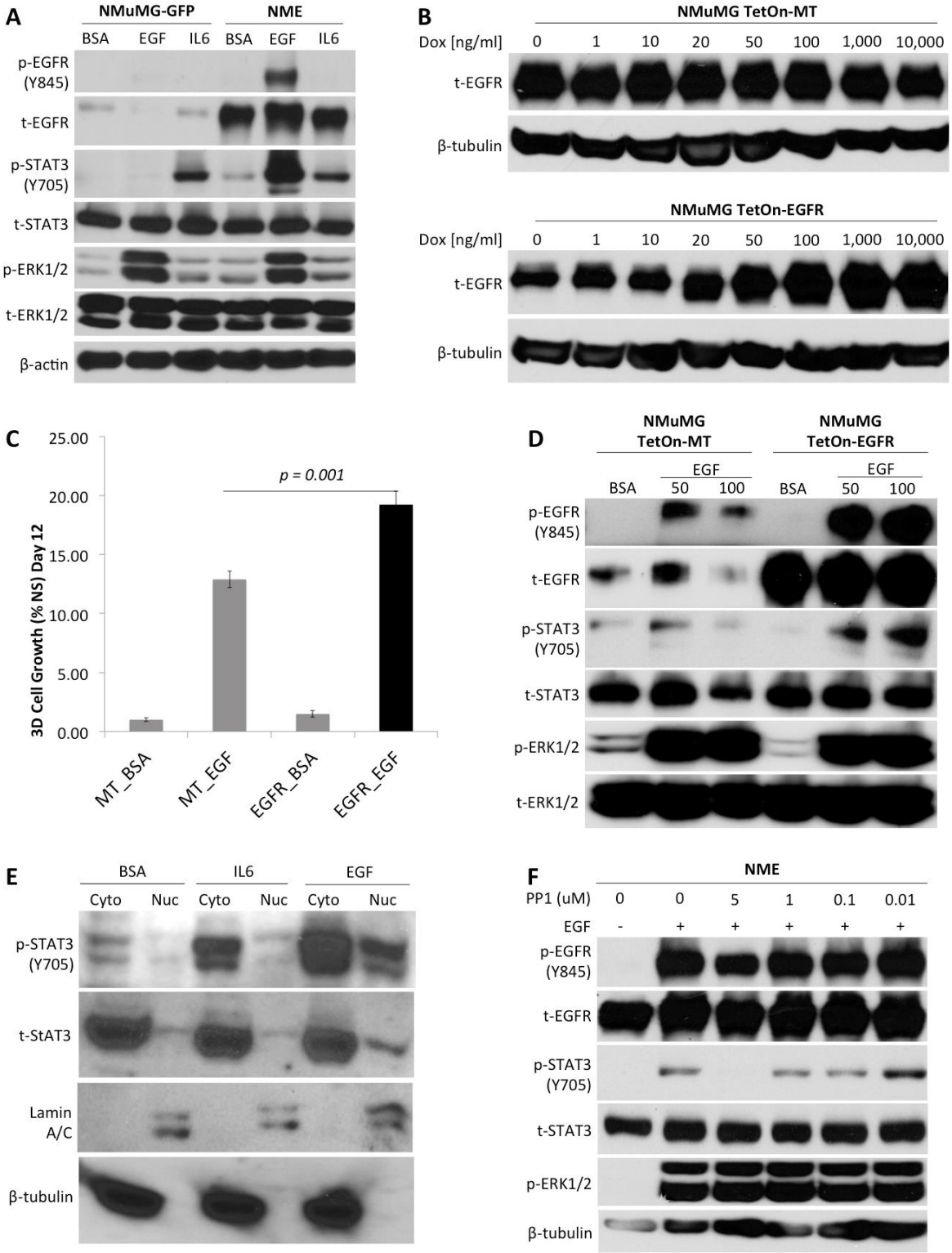


Figure 22. EGFR-mediated transformation of normal mammary epithelial cells. **A.** Schematic representation of EGFR-transformed NMuMG (NME) cells. Engraftment of NMuMG cells overexpressing WT-EGFR (NME cells) onto the mammary fat-pad of Nu/Nu mice results in mammary tumor formation. Engraftment of control NMuMG-GFP/YFP (**B**) or NMuMG cells overexpressing the EGFR-AA mutant (**C**) does not lead to mammary tumor formation (schematic is drawn to summarize findings from (Balanis et al., 2013; Wendt et al., 2010)).

This transformation event does not occur in NMuMG cells overexpressing an EGFR mutant with a di-alanine substitution for leucine-679 and leucine-680 (termed EGFR-AA) (Figure 22C) (Balanis et al., 2013). The EGFR-AA mutant is unable to undergo SRC-mediated phosphorylation at tyrosine-845 in the catalytic/kinase domain of EGFR (Balanis et al., 2011; Kil & Carlin, 2000; Song Jae Kil, Hobert, & Carlin, 1999). SRC-mediated phosphorylation of EGFR at tyrosine-845 is essential for STAT3 phosphorylation and activation as a transcription factor. Thus, the EGFR-AA mutant is defective for STAT3 phosphorylation in response to ligand stimulation (Balanis et al., 2013; Coffer & Kruijer, 1995). In contrast to NMuMG-GFP/YFP and NME-EGFR-AA cells, ligand stimulation results in robust phosphorylation of EGFR at tyrosine-845 and STAT3 at tyrosine-705 in NME-EGFR-WT (NME) cells (Balanis et al., 2013) (Figure 23A). As shown in Figure 23A, EGF stimulation leads to STAT3 phosphorylation in NME cells only, while ERK1/2 is comparably phosphorylated in NMuMG-GFP and NME cells. These results indicate that EGFR is a functional receptor in non-transformed NMuMG cells as it is capable of activating downstream MAPK pathway, but unable to phosphorylate STAT3. IL-6 is an established cytokine activator of STAT3 and was used in these experiments to ensure that EGFR overexpression does not alter the response to a STAT3 canonical inducer. Indeed, IL-6 stimulation results in comparable STAT3 phosphorylation in both NMuMG-GFP and NME cells (Figure 23A). Collectively, these results indicate the concomitance of EGFR-mediated oncogenic transformation with activation of the EGFR:STAT3 signaling axis. To expand these observations, a doxycycline (dox)-inducible expression system was utilized in addition to the constitutive overexpression model of EGFR used in NME cells. The doxycycline-inducible system offers the advantage of not exposing the cells to prolonged and sustained transgene expression. Thus, it allows for examining STAT3 signaling in the initial stages of EGFR-mediated transformation of NMuMG cells.

Figure 23B shows a dose-dependent induction of EGFR expression with dox treatment in NMuMG TetOn-EGFR but not in control NMuMG TetOn-MT cells. This transient induction of EGFR resulted in enhanced growth of NMuMG TetOn-EGFR cells using three dimensional (3D) organotypic cultures in response to EGF treatment as compared to NMuMG TetOn-MT cells (Figure 23C).

Figure 23. EGFR overexpression in mammary gland cells activates STAT3 signaling. **A.** NMuMG-GFP and NME-EGFR-WT (NME) cells were serum-starved for 6 hours and subsequently stimulated with EGF (50 ng/ml) or IL-6 (20 ng/ml) for 30 minutes. Whole-cell lysates were analyzed by immunoblot for phosphorylated STAT3 (p-STAT3 Y705), ERK1/2 (p-ERK1/2), and EGFR (p-EGFR Y845). BSA served as a protein stimulation control, and analysis of total levels of EGFR, ERK1/2, STAT3, and β-actin served as loading controls. **B.** NMuMG TetOn-MT (top) and NMuMG TetOn-EGFR (bottom) cells were stimulated with the indicated concentrations of dox for 24 hours and assessed for expression of EGFR. β-tubulin served as a loading control. **C.** NMuMG TetOn-MT (MT) and NMuMG TetOn-EGFR (EGFR) cells were stimulated with dox (1 ug/ml) in the presence or absence of EGF (50 ng/ml) or vehicle (BSA) in 3D-organotypic cultures. Cell viability was quantified on day 12. **D.** EGFR was ectopically expressed in NMuMG cells. These cells were stimulated with EGF (50 or 100 ng/ml) for 30 minutes. Whole-cell lysates were analyzed for phosphorylated STAT3 (p-STAT3 Y705), EGFR (p-EGFR Y845) and ERK1/2 (p-ERK1/2). BSA served as a protein stimulation control, and analysis of total levels of EGFR, STAT3, and ERK1/2 served as loading controls. **E.** NME cells were serum-deprived overnight and stimulated with EGF (50 ng/ml) or IL-6 (20 ng/ml) for 30 minutes. Cells were subjected to cell fractionation prior to immunoblotting with phospho-specific and total STAT3 antibodies. Cytosolic (*Cyto*) and nuclear (*Nuc*) fractions were confirmed by immunoblot for β-tubulin and Lamin A/C, respectively. BSA served as a protein stimulation control. **F.** NME cells were pre-treated with the indicated concentrations of SRC kinase-specific inhibitor (PP1) for 7 hours prior to stimulation with EGF (50 ng/ml) for 30 minutes. Whole-cell lysates were analyzed by immunoblot for phosphorylated STAT3 (p-STAT3 Y705), EGFR (p-EGFR Y845), and ERK1/2 (p-ERK1/2). BSA served as a protein stimulation control, and analysis of total levels of EGFR, STAT3, and β-tubulin served as loading controls. Immunoblots are representative of at least two independent experiments yielding similar results. Data in C are the mean ± SE for two independent experiments completed in triplicate resulting in the indicated P-value.


A
NMuMG-GFP
NME
BSA EGF IL6 BSA EGF IL6
p-EGFR (Y845)
t-EGFR
p-STAT3 (Y705)
t-STAT3
p-ERK1/2
t-ERK1/2
β-actin
B
NMuMG TetOn-MT
Dox [ng/ml] 0 1 10 20 50 100 1,000 10,000
t-EGFR
β-tubulin
NMuMG TetOn-EGFR
Dox [ng/ml] 0 1 10 20 50 100 1,000 10,000
t-EGFR
β-tubulin
C
3D Cell Growth (% NS) Day 12
25.00
20.00
15.00
10.00
5.00
0.00
p = 0.001
MT_BSA
MT_EGF
EGFR_BSA
EGFR_EGF
D
NMuMG TetOn-MT
NMuMG TetOn-EGFR
EGF
BSA 50 100 BSA 50 100
p-EGFR (Y845)
t-EGFR
p-STAT3 (Y705)
t-STAT3
p-ERK1/2
t-ERK1/2
E
BSA IL6 EGF
Cyto Nuc Cyto Nuc Cyto Nuc
p-STAT3 (Y705)
t-StAT3
Lamin A/C
β-tubulin
F
NME
PP1 (uM) 0 0 5 1 0.1 0.01
EGF - + + + + +
p-EGFR (Y845)
t-EGFR
p-STAT3 (Y705)
t-STAT3
p-ERK1/2
β-tubulin

As shown in Figure 23D, NMuMG TetOn-EGFR cells treated with dox and EGF display increased STAT3 phosphorylation in comparison with control NMuMG TetOn-MT cells. In contrast, ERK1/2 was similarly phosphorylated in control and EGFR-expressing cells. Overall, these data consistently indicate that EGFR overexpression is associated with increased STAT3 signaling, and suggest this pathway likely plays a critical role in EGFR-mediated transformation of mammary cells.

Consistent with STAT3 activation and role as a transcription factor, nuclear fractionation experiments show phosphorylated and total STAT3 to accumulate in the nuclei of NME cells when treated with EGF for 30 minutes (Figure 23E). Moreover, previous studies reported that EGF-stimulated STAT3 activation in NME cells was blocked by the SRC kinase inhibitor PP2 (Balanis et al., 2013). To extend these findings, the effect of a different SRC-kinase inhibitor (PP1) was examined. As shown in Figure 23F, EGF-induced STAT3 phosphorylation in NME cells was efficiently blocked by PP1 in a dose-dependent manner. ERK1/2 phosphorylation is not affected by SRC kinase inhibitors PP1 (Figure 23F) or PP2 (Balanis et al., 2013) treatment. These results further suggest that STAT3 phosphorylation downstream of EGFR is dependent on SRC kinase. Figures 24 and 25 are schematic representations of the above findings. Taken together, these results open the possibility to investigate the necessity of STAT3 in EGFR-mediated oncogenic transformation of mammary epithelial cells.

3.4 STAT3 Is Required for EGF-mediated Transformation of Mammary Gland Cells

To define the functional role of STAT3 downstream of EGFR activation, a genetic approach was utilized to specifically decrease STAT3 expression in NME cells. Depletion of STAT3 using two different shRNA sequences blocked EGF-induced phosphorylation of STAT3 in these cells (Figure 26A and B). Furthermore, STAT3 depletion prevented the growth of NME cells in three-dimensional (3D) organotypic cultures independent of exogenous EGF addition (Figure 26C). When propagated in 3D organotypic cultures, NME cells form compact filled organoids that resemble transformed cell morphology observed in tumor sections (Balanis et al., 2013; Kenny et al., 2007). In contrast, NMuMG-GFP cells form much smaller acinar ‘hollow’ organoids representative of differentiated mammary ducts when grown using the same conditions (Balanis et al., 2013; Kenny et al., 2007).

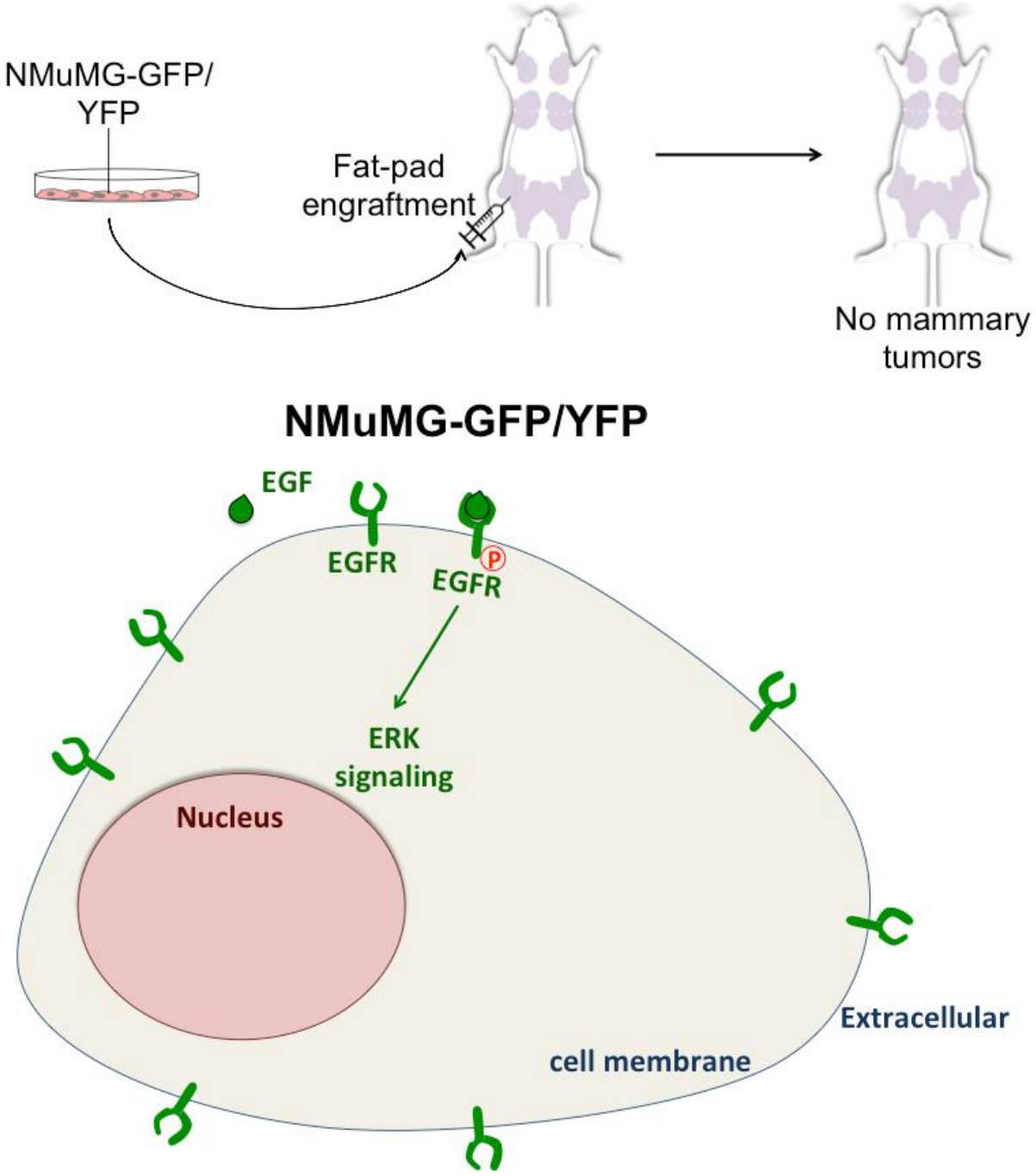


Figure 24. EGFR downstream signaling in non-tumorigenic NMuMG-GFP/YFP cells. EGF treatment results in phosphorylation of EGFR and activation of downstream MAPK pathway as evidenced by ERK1/2 phosphorylation.

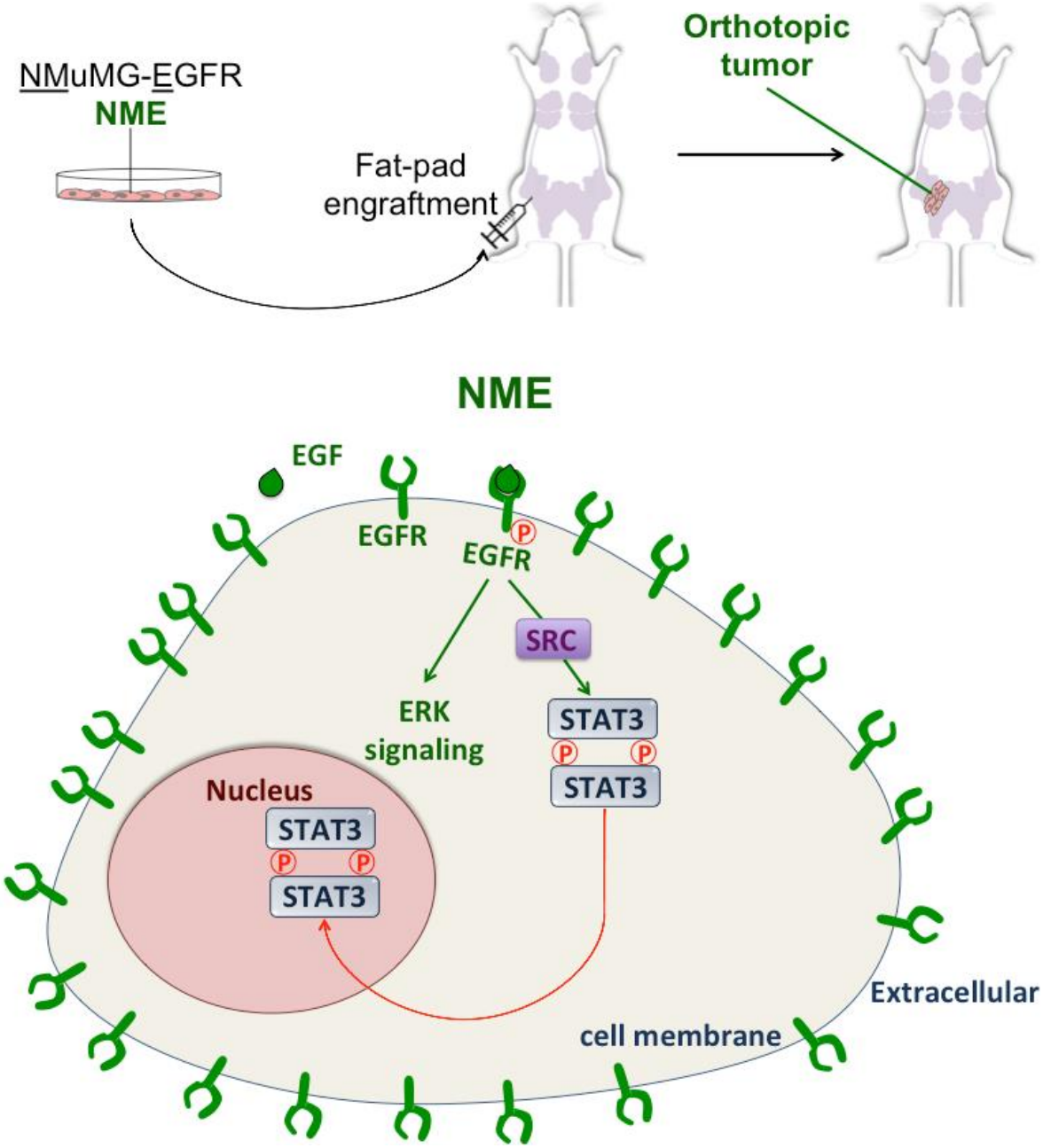
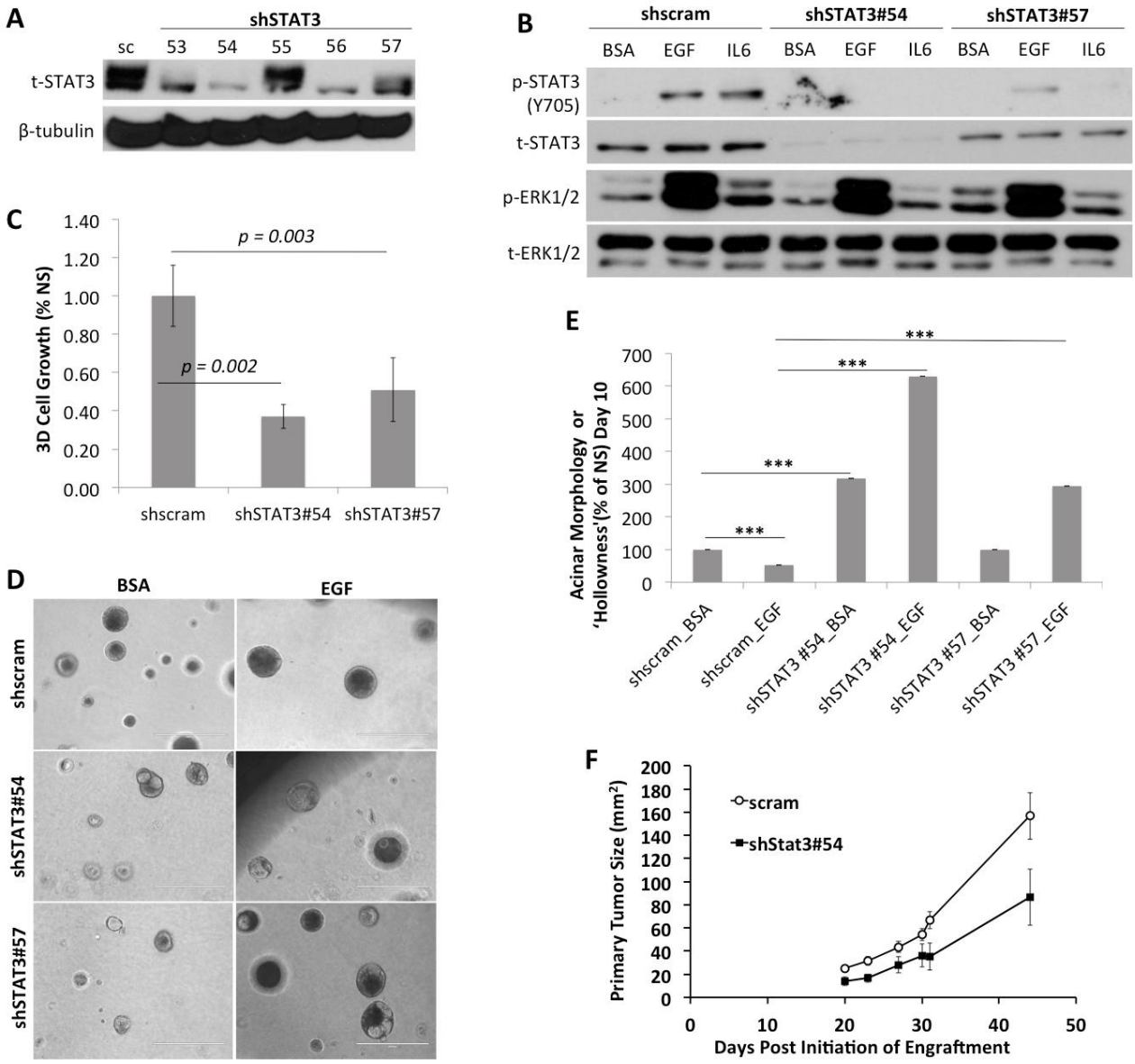


Figure 25. EGFR downstream signaling in NMuMG cells overexpressing EGFR (NME cells). EGF treatment of tumorigenic NME cells results in SRC-mediated phosphorylation and subsequent nuclear translocation of STAT3. The MAPK pathway is also activated in these cells in response to EGF stimulation.

Figure 26. STAT3 is required for EGFR-mediated transformation of mammary gland cells. **A.** NME cells were constructed to stably express a scrambled control (sc) shRNA or various shRNAs (53-57) targeting STAT3. These cells were analyzed for STAT3 expression by immunoblot. Expression of β-tubulin served as a loading control. **B.** Control (shscram) and STAT3 depleted (shSTAT3#54 and shSTAT3#57) NME cells were serum-starved and then treated with EGF (50 ng/ml) or IL-6 (20 ng/ml) for 30 minutes. Whole-cell lysates were analyzed by immunoblot for phosphorylated STAT3 (p-STAT3 Y705) and ERK1/2 (p-ERK1/2). BSA served as a protein stimulation control, and analysis of total levels of ERK1/2 and STAT3 served as loading controls. **C.** Control (i.e. shscram) and STAT3-depleted (shSTAT3#54 and shSTAT3#57) NME cells were incubated for 10 days in 3D-organotypic cultures and cell viability was quantified on day 10. Data are the mean ± SE of 2 independent experiments completed in triplicate resulting in the indicated P-values. **D.** Representative photomicrographs of control (i.e. shscram) and STAT3-depleted (shSTAT3#54 and shSTAT3#57) NME cells incubated for 10 days in 3D-organotypic cultures and supplemented with either EGF (50 ng/ml) or the vehicle (BSA). Photomicrographs are representative data from at least three independent experiments. **E.** Differences in organoid morphology in D were monitored by phase-contrast microscopy (40x). Hollow structures were quantified by counting 80-100 organoids in each well. Counted structures were classified as either hollow or dense and the percentage of hollow structures is shown. Data are the mean percentage ±SE of three independent experiments done in triplicate, ***P<0.001. **F.** Control (i.e. shscram) and STAT3-depleted (shSTAT3#54) NME cells (1×10^6) were engrafted onto the mammary fat-pad of female Nu/Nu mice. Mean tumor size over a 45-day period is plotted. Data are mean ± S.E. of 5 mice per group. Immunoblots are representative of at least two independent experiments yielding similar results.



Thus, this acinar characteristic (referred to as 'hollowness') was used to examine the effect of STAT3 depletion on NME cell morphology when grown in 3D cultures. In these investigations, control NME- shscram cells and those depleted for STAT3 were treated with vehicle or EGF and hollow and compact structures were counted in each well (Figure 26D). The percentage of hollow structures was plotted as shown in Figure 26E. Under basal conditions, NME cells depleted for STAT3 form smaller hollow organoids as compared to their control scrambled shRNA-expressing counterparts. EGF treatment of NME-shscram resulted in large filled structures while NME-shSTAT3 cells continued to grow as hollow organoids (Figure 26D and E). Importantly, the extent

to which NME organoid outgrowth was inhibited and hollowed was proportional to the level of STAT3 depletion. Overall, the above STAT3 genetic depletion data suggest a necessity for STAT3 expression in EGFR-mediated transformation. These results are consistent with a previous study showing NME cell growth to be potently inhibited with Stattic, a pharmacological inhibitor of STAT3 dimerization and activation (Balanis et al., 2013; McMurray, 2006). Consistent with the above *in vitro* findings, *in vivo* examination of the most effective STAT3 targeting sequence (shSTAT3#54) demonstrated that depletion of STAT3 inhibited mammary tumor outgrowth as compared to NME-shscram cells (Figure 26F). Altogether, these data clearly indicate the functional involvement of STAT3 in EGFR-mediated transformation of mammary gland cells.

3.5 Metastasis of Breast Cancer Is Associated with Increased STAT3 Signaling

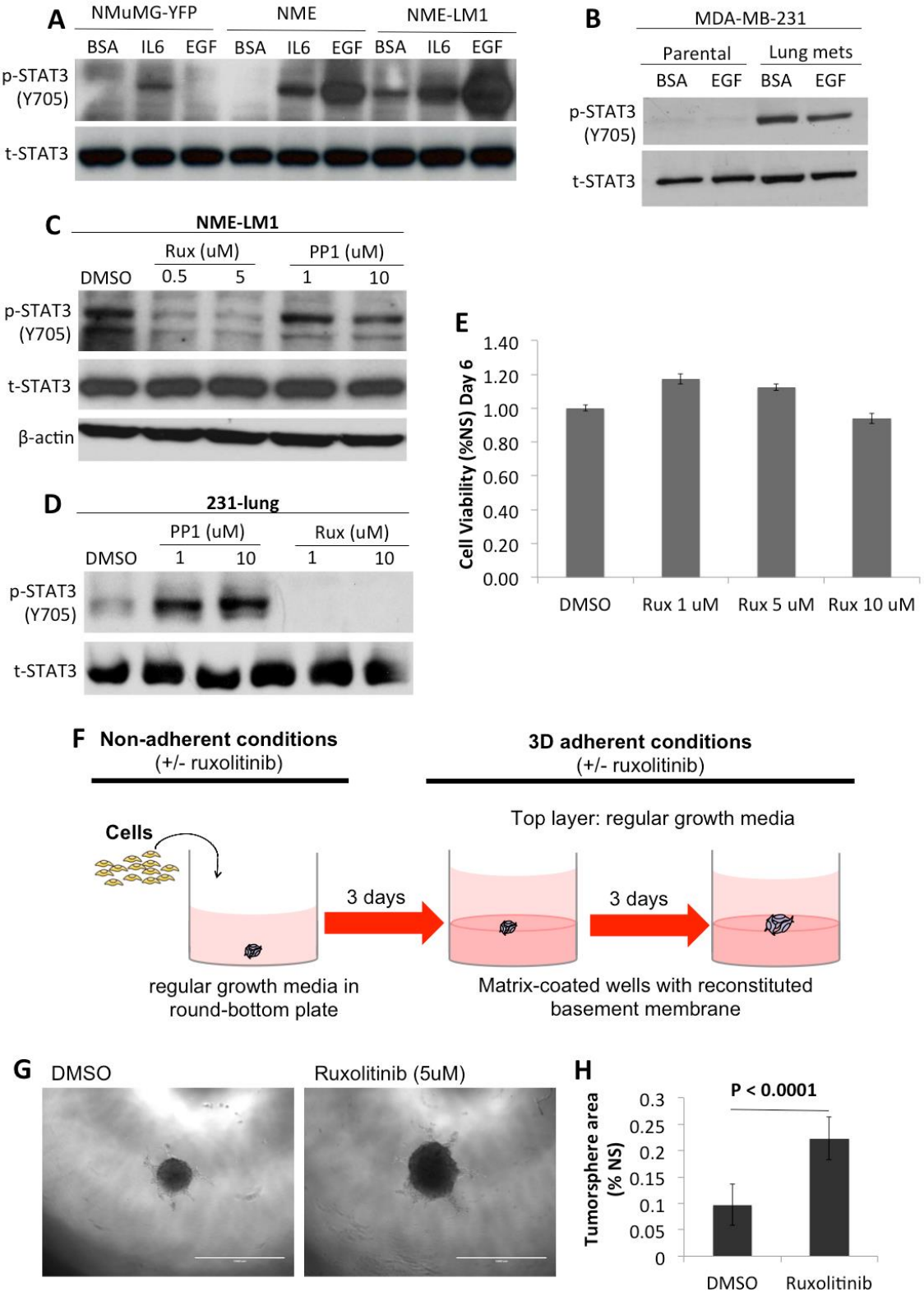
While overexpression of EGFR in NMuMG cells leads to mammary tumor formation, these cells do not metastasize when engrafted onto the mammary fat-pad of female Nu/Nu mice (Balanis et al., 2013). Transient induction of epithelial-mesenchymal transition (EMT) via transforming growth factor-beta (TGF-β) treatment prior to fat-pad engraftment facilitates the pulmonary and lymph node metastasis of NME cells (Ali, Brown, Purdy, Davisson, & Wendt, 2018; Wendt et al., 2010; Wendt, Taylor, et al., 2014). As discussed in these studies, the resulting pulmonary and lymph node metastases were harvested, subcultured, and termed NME-LM1 (lung metastasis), and NM- Lym1 and Lym2 (metastasis from two different lymph nodes).

Given the dependency of EGFR-mediated transformation on STAT3 expression in primary mammary tumors and the well-established correlation between hyperphosphorylated STAT3 and metastatic aggressiveness in breast cancer (Banerjee & Resat, 2016), the role of STAT3 in the above metastatic models was investigated. Figure 27A shows that lung metastatic NM-LM1 cells display basal STAT3 phosphorylation in non-stimulated conditions, in comparison with mammary tumor NME and non-transformed NMuMG-YFP cells. To confirm these findings in a different model, basal STAT3 phosphorylation was examined in patient-derived MDA-MB-231 breast cancer cells and their isogenic lung metastatic counterparts (Wendt et al., 2015). Lung-metastatic MDA-MB-231 (lung-231) cells were derived by harvesting and subculturing the spontaneous pulmonary metastases of MDA-MB-231 cells engrafted onto the mammary fat-pad of female Nu/Nu mice as discussed in (Wendt et al., 2015). Figure 27B shows that STAT3 phosphorylation was basally induced in lung-231 cells as compared to their parental MDA-MB-231 cell-line. These

data clearly show that the metastatic progression of breast cancer is associated with mechanisms that upregulate STAT3 phosphorylation. Paracrine and autocrine signaling loops are proposed to induce basal phosphorylation of STAT3 in metastatic breast cancer. Indeed, secreted factors from cancer cells or other cell types within the tumor microenvironment bind their respective receptors on cancer cells leading to STAT3 activation (Banerjee & Resat, 2016). The fact that NME-LM1 and lung-231 cells display basal STAT3 phosphorylation while being cultured *ex vivo* points to an autocrine rather than a paracrine mechanism.

While RTKs such as EGFR and vascular endothelial growth factor receptor (VEGFR) phosphorylate STAT3 through their intrinsic kinase domains or via a SRC-dependent mechanism, cytokines and interleukins employ Janus-activated kinases (JAKs) in this response (Wendt, Balanis, et al., 2014). Thus, the mechanisms that drive basal STAT3 phosphorylation in NME-LM1 and lung-231 cells were investigated. Figure 27C shows that treatment of NM-LM1 cells with the JAK1/2 inhibitor ruxolitinib effectively blocks p-STAT3 signal in these cells, while the SRC inhibitor PP1 had no effect. Analogous results were observed for the lung-231 cells (Figure 27D), thus suggesting a potential JAK1/2:STAT3 mechanism for the above models of lung metastatic breast cancer (Figure 28).

Figure 27. Metastasis of breast cancer is associated with activated STAT3 signaling. **A.** NMuMG-YFP, NME, and NME-LM1 cells were serum-starved for 7 hours and then stimulated with EGF (50 ng/ml) or IL-6 (20 ng/ml) for 30 minutes. Equal protein aliquots were immunoblotted with phospho-specific and total STAT3 antibodies. BSA served as protein stimulation control. **B.** Human MDA-MB-231 cells (parental) and their lung-metastatic counterparts (lung mets) were serum-starved for 7 hours and harvested under basal conditions (BSA) or following a 30-minute stimulation with EGF (50 ng/ml). Equal protein aliquots were immunoblotted with phospho-specific and total STAT3 antibodies. **C.** NME-LM1 cells were pretreated with vehicle (DMSO), JAK1/2 (0.5 or 5 uM ruxolitinib (Rux)), or SRC (1 or 10 uM PP1) kinase-specific inhibitors for 14 hours and subsequently harvested. Equal protein aliquots were immunoblotted with phospho-specific and total STAT3 antibodies. β-actin served as a loading control. **D.** 231-lung cells were pretreated with vehicle (DMSO), SRC (1 or 10 uM PP1), or JAK1/2 (1 or 10 uM ruxolitinib (Rux)) kinase-specific inhibitors for 7 hours and subsequently harvested. Equal protein aliquots were immunoblotted with phospho-specific and total STAT3 antibodies. **E.** NME-LM1 cells were grown on two-dimensional standard tissue culture plastic under control (DMSO) or ruxolitinib-supplemented conditions (1, 5, or 10 uM) and assayed for cell viability on day 6. Data are the mean ± SE of 3 independent experiments completed in triplicate. **F.** Schematic of assay investigating the effect of JAK1/2 inhibition on the growth of NME-LM1 tumorspheres in non-adherent and adherent conditions. NME-LM1 tumorspheres were formed in round-bottom wells in the absence (DMSO) or presence of ruxolitinib (5 uM) for 3 days and subsequently transferred to a hydrogel layer of basement membrane in the presence or absence of the same dose of ruxolitinib used in the non-adherent growth-phase for 3 more days. Representative photomicrographs (**G**) and sizes (**H**) of NME-LM1 tumorsphere assay described in F are shown. Data in H are the mean ± SE of two independent experiments completed in triplicate resulting in the indicated P-value. Immunoblots are representative of at least two independent experiments yielding similar results.


A
NMuMG-YFP
NME
NME-LM1
BSA IL6 EGF BSA IL6 EGF BSA IL6 EGF
p-STAT3 (Y705)
t-STAT3
B
MDA-MB-231
Parental
Lung mets
BSA EGF BSA EGF
p-STAT3 (Y705)
t-STAT3
C
NME-LM1
Rux (uM)
PP1 (uM)
DMSO 0.5 5 1 10
p-STAT3 (Y705)
t-STAT3
β-actin
D
231-lung
PP1 (uM)
Rux (uM)
DMSO 1 10 1 10
p-STAT3 (Y705)
t-STAT3
E
Cell Viability (%NS) Day 6
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
DMSO
Rux 1 uM
Rux 5 uM
Rux 10 uM
F
Non-adherent conditions
(+/- ruxolitinib)
3D adherent conditions
(+/- ruxolitinib)
Cells
Top layer: regular growth media
3 days
3 days
regular growth media in round-bottom plate
Matrix-coated wells with reconstituted basement membrane
G
DMSO
Ruxolitinib (5uM)
H
P < 0.0001
Tumorsphere area (% NS)
0.3
0.25
0.2
0.15
0.1
0.05
0
DMSO
Ruxolitinib

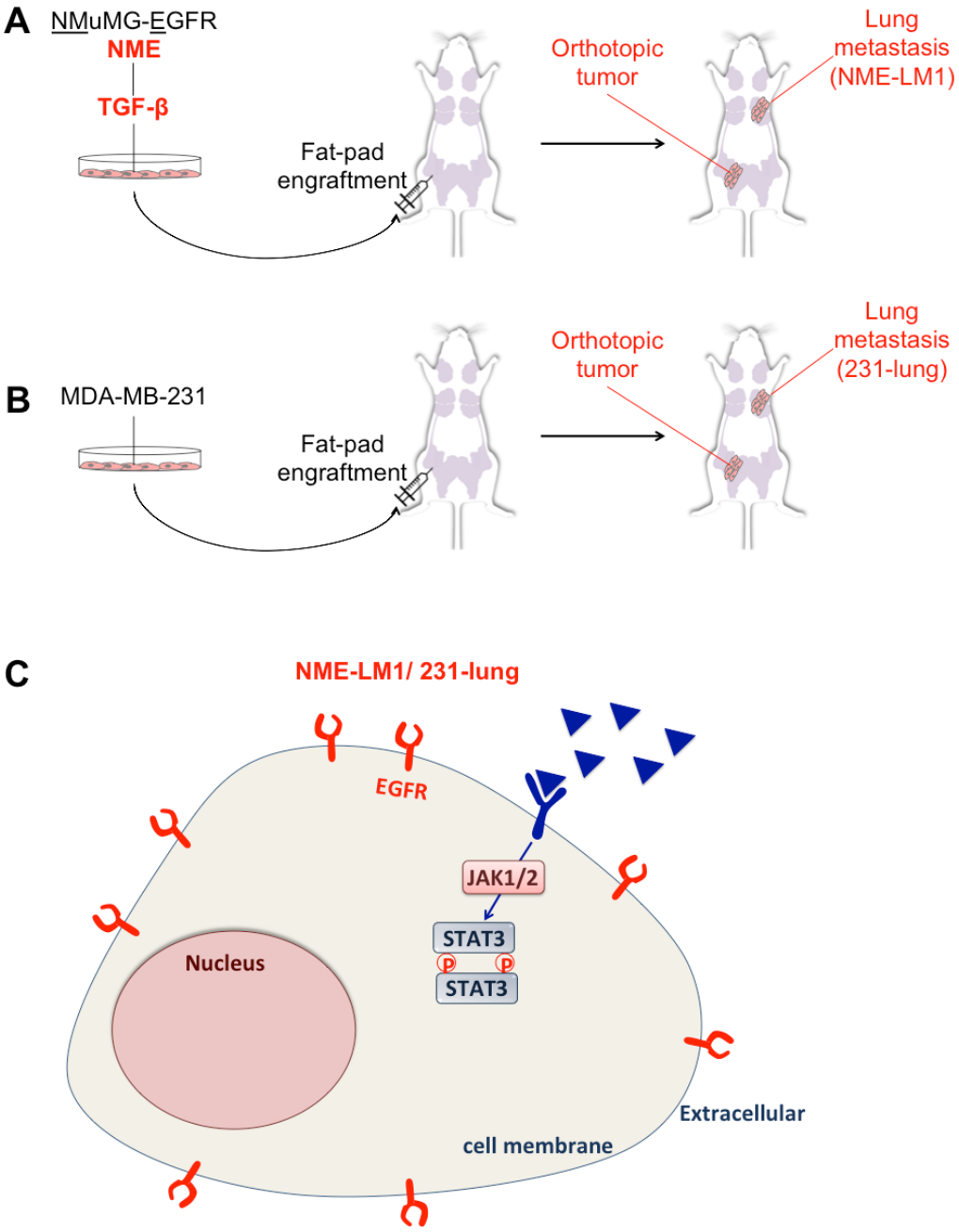


Figure 28. Activation of STAT3 signaling in metastatic breast cancer cells. Schematic showing the mechanism of increased STAT3 signaling in lung metastatic breast cancer cells from EMT-driven (**A**) or spontaneous (**B**) metastasis models. **C.** Basal activation of the STAT3 signaling axis in these cells occurs by a JAK1/2 mechanism, potentially via autocrine factors released from the tumor cells themselves.

Given the effectiveness of ruxolitinib in blocking basal STAT3 phosphorylation and the established role of STAT3 in driving and maintaining breast malignancy, the effect of

ruxolitinib on metastatic cell growth was examined. Figure 27E shows that ruxolitinib treatment at doses that block STAT3 phosphorylation in NM-LM1 cells does not affect their 2D-growth. To examine the effect of JAK1/2:STAT3 inhibition on the invasive capacity of these cells, the invasion assay described in Chapter 2 was utilized (Figure 14E). As illustrated in Figure 27F, ruxolitinib was added in the non-adherent phase of growth to inhibit STAT3 signaling and stimulation of stem-cell properties of NME-LM1. The drug was added again as the clusters were transferred onto the bed of reconstituted basement membrane to maintain inhibition of stem-like NME-LM1 cells and prevent the switch from tumor dormancy to metastatic invasion and outgrowth (Figure 27F). Surprisingly, ruxolitinib treatment increased NM-LM1 spheroid size compared to control treatment (Figure 27G and H), thus suggesting an anti-tumorigenic role of STAT3 in these metastatic models of breast cancer driven from EGFR-transformed tumors.

3.6 STAT3 Is Required for EGF-induced Apoptosis in Metastatic Breast Cancer

As previously discussed, metastatic progression of breast cancer is associated with a switch in EGFR function from oncogenic in the primary mammary tumors to pro-apoptotic in metastatic ones (Ali et al., 2018; Wendt et al., 2015). As shown in Chapter 2, STAT1 is required for EGF-induced apoptosis in lymph node metastatic NM-Lym1 and NM-Lym2 cells. However, the role of STAT3 in mediating the pro-apoptotic function of EGFR in these metastatic models is unknown. Thus, genetic approaches were utilized to specifically deplete STAT3 expression in NM-Lym1 cells (Figure 29A). Figure 29B shows that EGF-induced apoptosis is significantly reduced in NM-Lym1 cells depleted for STAT3 expression. Additionally, pharmacological inhibition of STAT3 dimerization using the inhibitor Stattic effectively blocked EGF-induced apoptosis in NM-Lym1 (Figure 29C) and lung metastatic NM-LM1 (Figure 29D) cells. Previous studies demonstrated that metastatic progression of breast cancer is associated with increased nuclear translocation of EGFR (Bartolowits et al., 2017). In these studies, treatment with NLS-gefitinib (Chapter 2 Figure 13) specifically blocked EGF-induced STAT3 phosphorylation in NM-LM1 and MDA-MB-468 cells, suggesting STAT3 is phosphorylated by EGFR in the nuclei of these cells (Bartolowits et al., 2017). NLS-gefitinib treatment also blocked EGF-induced apoptosis in these cells as thoroughly discussed in Chapter 2. Thus, these data indicate the functional involvement of nuclear STAT3 in EGF-mediated apoptosis in metastatic breast cancer. These findings also provide a potential

explanation of the failure of drugs targeting the JAK1/2:STAT3 pathway in clinical trials of metastatic breast cancer (Figure 30).

3.7 Discussion

Since its designation as an oncogene in 1999, STAT3 has been linked to oncogenesis and metastatic progression of breast cancer by inducing oncogenic transformation, proliferation, invasion, and driving and maintaining stem-like character of cancer cells (Wendt, Balanis, et al., 2014). The current study delineates a change in the role of STAT3 that mirrors that of EGFR over the metastatic progression of breast cancer. Indeed, EGFR overexpression in mammary gland cells is tumorigenic, while induction of EMT to drive metastasis is associated with a pro-apoptotic switch in EGFR function in the metastatic cells. Findings herein show STAT3 expression is essential for EGFR-mediated oncogenic transformation of mammary tumors. Following invasion and dissemination, breast cancer cells upregulate mechanisms that hyper-phosphorylate STAT3 in a JAK1/2 dependent manner. This data is supported by similar findings herein utilizing the spontaneously-metastatic patient-derived MDA-MB-231 breast cancer cells and their lung-metastatic counterparts. Along these lines, numerous studies report the development of an inflammatory tumor microenvironment with metastatic progression of breast cancer (Banerjee & Resat, 2016). Elevated levels of released cytokines and interleukins have been ubiquitously shown to upregulate STAT3 signaling in breast tumor cells, resulting in STAT3-mediated metastatic progression, chemoresistance, and tumor-submissive immune response (Korkaya, Liu, & Wicha, 2011).

Despite the above findings, investigations herein utilizing the clinical JAK1/2 inhibitor ruxolitinib proved ineffective in inhibiting the growth and invasive capacity of metastatic breast cancer cells despite on-target activity. This data come in agreement with the lack of anti-tumor efficacy of ruxolitinib in clinical trials of TNBC (Stover et al., 2018).

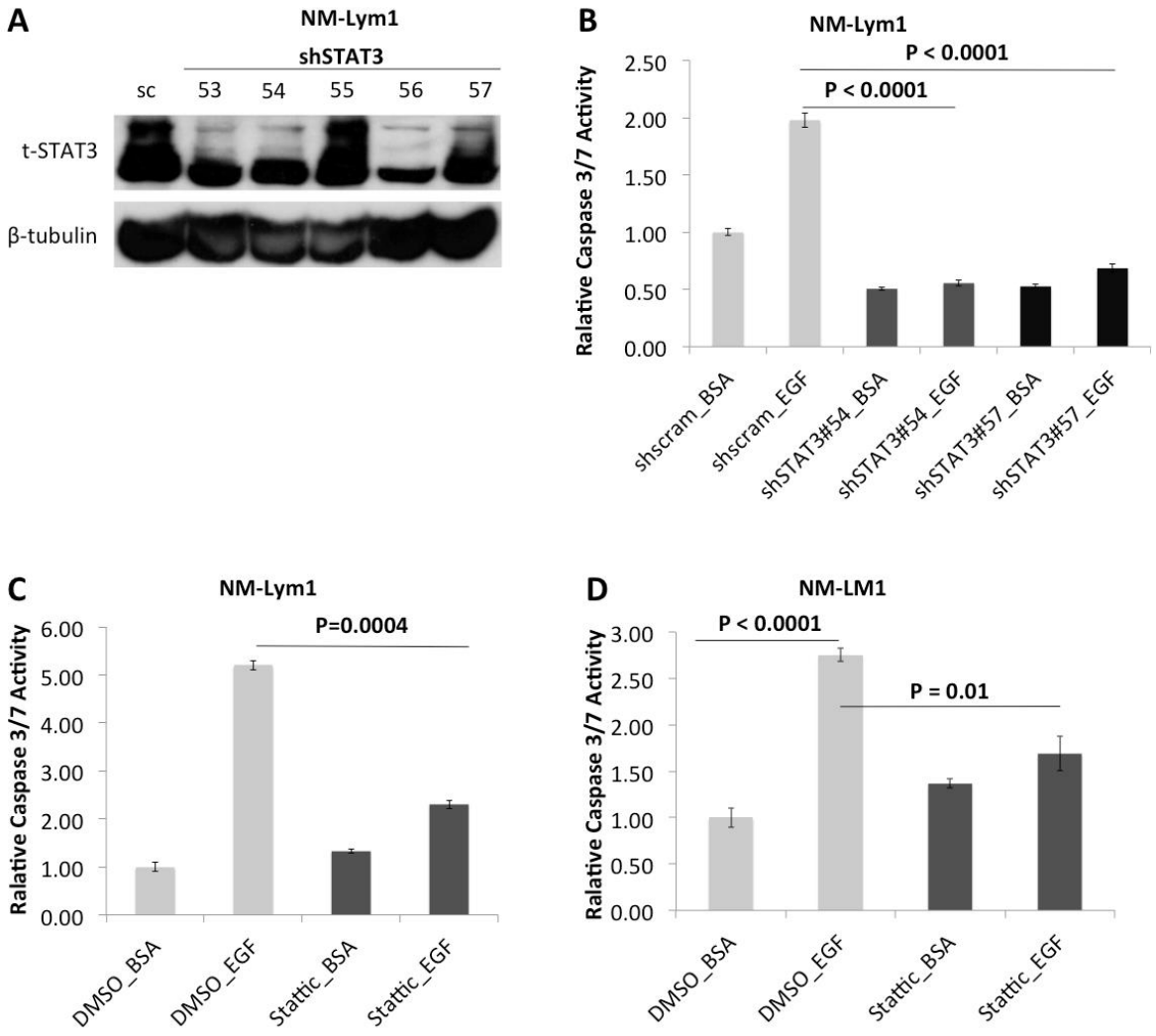


Figure 29. STAT3 is required for EGF-mediated apoptosis in metastatic breast cancer. **A.** Metastatic lymph node NM-Lym1 cells were constructed to stably express a scrambled control (sc) shRNA or various shRNAs (53–57) targeting STAT3. These cells were analyzed for STAT3 expression by immunoblot. Expression of β-tubulin served as a loading control. Immunoblots are representative of at least two independent experiments yielding similar results. **B.** Control (shscram) and STAT3 depleted (shSTAT3#54 and shSTAT3#57) NM-Lym1 cells were treated with EGF (100 ng/ml) or vehicle (BSA) for 36 hours and assayed for caspase 3/7 activity. Data are the mean ± SE of three independent experiments completed in triplicate resulting in the indicated P-values. **C.** NM-Lym1 cells were treated with the STAT3 inhibitor Stattic (1uM) in the presence of exogenous EGF (100 ng/ml) or vehicle (BSA) for 36 hours and these cells were assayed for caspase 3/7 activity. Data are the mean ± SE of two independent experiments completed in triplicate resulting in the indicated P-value. **D.** Lung metastatic NME-LM1 cells were treated for 36 hours with the STAT3 inhibitor Stattic (1uM) in the presence of exogenous EGF (100 ng/ml) or vehicle (BSA) and these cells were assayed for caspase 3/7 activity. Data are the mean ± SE of three independent experiments completed in triplicate resulting in the indicated P-values.

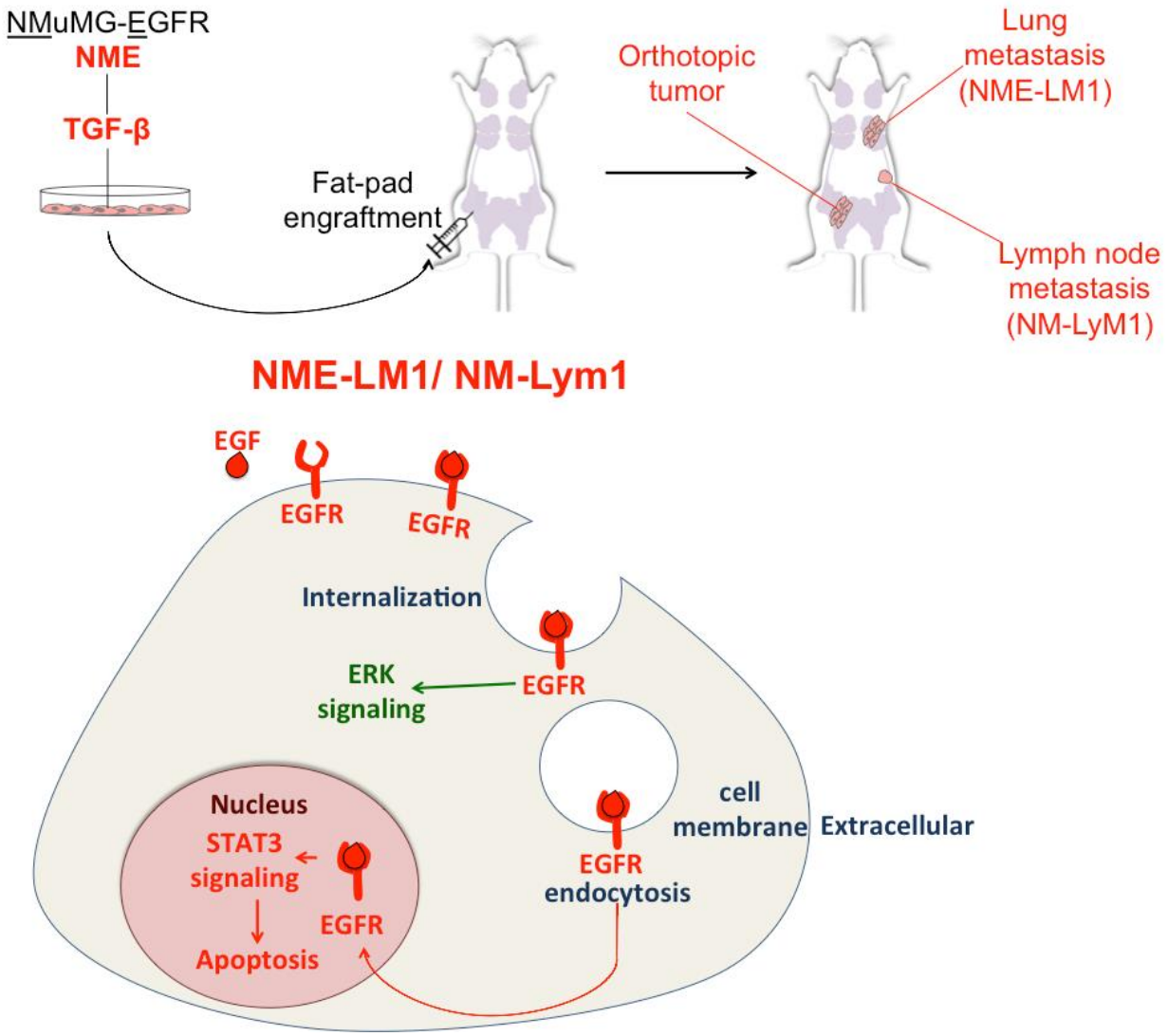


Figure 30. The role of nuclear STAT3 in EGF-induced apoptosis in metastatic breast cancer. Schematic showing the role of STAT3 in lung and lymph node metastatic breast cancer cells driven by EGFR transformation and EMT induction. In these cells, EGFR nuclear translocation and access to STAT3 molecules residing in the nucleus are increased as previously shown (Bartolowits et al., 2017). Genetic or pharmacological inhibition of STAT3 prevents EGF-induced apoptosis in these cells, thus implicating nuclear STAT3 in this response.

Furthermore, the breast cancer progression models used herein are characterized by intrinsic resistance to EGFR inhibitors. Data reported here are consistent with a study examining the clinical efficacy of ruxolitinib in patients with EGFR-mutant lung cancers who display acquired resistance to EGFR inhibitors (Yu et al., 2017). This study reported a similar lack of efficacy of ruxolitinib despite pre-clinical evidence of JAK1/2:STAT3 pathway involvement in acquired resistance to EGFR inhibitors (Yu et al., 2017). Since ruxolitinib inhibits JAK1/2

signaling, other STAT molecules may be targeted such as STAT1, which may contribute to the clinical inefficacy of ruxolitinib given the anti-tumorigenic function of STAT1. Along these lines, a recently developed pharmacological agent designed to degrade STAT3 with high selectivity over other STAT molecules resulted in sustained tumor regression in xenograft mouse models (Bai et al., 2019).

Given that EGFR functions as an apoptotic driver in EMT-driven EGFR-transformed metastatic breast cancer, the role of STAT3 in this response was further investigated. EGFR nuclear trafficking is increased in these metastatic cells, resulting in phosphorylation of nuclearly-residing STAT3 molecules in response to ligand stimulation (Bartolowits et al., 2017). Data in Figure 29 show that genetic and pharmacologic attenuation of STAT3 signaling blocks EGF-induced apoptosis in lung and lymph node metastatic breast cancer cells. These novel findings are contrasted by the widely assumed notion that STAT3 functions solely as an oncogenic driver. However, it should be noted that several studies reported the ability of STAT3 to drive apoptosis in cancer cells (Niu et al., 2001, 2005; Rozovski et al., 2016; Zammarchi et al., 2011). Additionally, a recent study established the positive involvement of STAT3 in EGF-induced apoptosis of patient-derived TNBC cell-line MDA-MB-468 (Jackson & Ceresa, 2017). Further, these cells have previously been established to undergo nuclear phosphorylation of STAT3 by nuclear EGFR in response to EGF treatment (Bartolowits et al., 2017). As shown in Chapter 2, EGF-induced apoptosis in these cells is also prevented by specific inhibition of nuclear EGFR kinase activity. Altogether, these data implicate nuclear STAT3 in the pro-apoptotic function of EGFR in metastatic breast cancer. As discussed in Chapter 2, STAT1 is also involved EGF-induced apoptosis of multiple pre-clinical and patient-derived metastatic breast cancer cells. These studies open the possibility to investigate STAT1-STAT3 interaction in facilitating the pro-apoptotic function of EGFR. Indeed, activated STAT3 in cooperation with EGFR was found to increase the transcription of STAT1 in breast cancer cell-lines (Han, Carpenter, Cao, & Lo, 2013). Additionally, EGF-induced apoptosis of A431 cells was characterized by the formation of DNA-bound STAT1-STAT3 heterodimers in contrast to IFN-γ treatment, which predominantly induced STAT1-STAT1 homodimers (Chin et al., 1997). Altogether, investigating the interplay of STAT1/3 signaling pathways in aggressive post-EMT breast cancers is essential to determine the appropriate context for pharmacologically inhibiting JAK1/2 and/or STAT3 and exploit this interaction to ultimately target metastatic breast cancer.

3.8 Methods and Materials

3.8.1 Cell-lines and Reagents

Murine NMuMG cells were purchased from the American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS). Construction of NMuMG cells expressing the human wild-type (EGFR-WT) or mutant EGFR (EGFR-AA) construct (NME cells) and their metastatic variants are described in a previous study (Balanis et al., 2013). Human MDA-MB-231 cells were purchased from ATCC and cultured as described previously (Balanis et al., 2013; Wendt, Taylor, et al., 2014) Lung-metastatic MDA-MB-231 cells were derived as previously described (Wendt et al., 2015). Cellular depletion of STAT3 in cells was accomplished by vesicular stomatitis virus G protein (VSVG) lentiviral transduction of pLKO.1 shRNA vectors (Thermo Scientific). Sequences of shRNAs can be found in Table 5. A list of the chemical inhibitors and growth factors used throughout the study can be found in Table 6.

3.8.2 Immunoblot Analyses

For immunoblot assays, nuclear, cytoplasmic, or whole-cell lysates were harvested and equal aliquots resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes using established methods. Nuclear and cytoplasmic fractions were generated via a different lysis protocol that uses Buffer A (10 mM HEPES; pH 7.9, 10 mM KCL, 0.1 mM EDTA, and protease and phosphatase inhibitors) to obtain a cytoplasmic fraction. After cytoplasmic fraction separation, intact nuclei were pelleted and washed at least twice in fresh Buffer A before being lysed in Buffer B (20 mM HEPES; pH 7.9, 0.4 M NaCl, 1 mM EDTA, 10% glycerol, and protease and phosphatase inhibitors). Antibody concentrations and suppliers are listed in Table 7.

Table 5. Sequences of shRNAs used in Chapter 3.

Target	**TRC#**	**Sequence (mature antisense)**
mSTAT1#53	TRCN0000071453	CCTAACTTTGTGGTTCCAGAT
mSTAT1#54	TRCN0000071454	CGACTTTGATTTCAACTACAA
mSTAT1#55	TRCN0000071455	CACCATTCATTGATGCAGTTT
mSTAT1#56	TRCN0000071456	CCTGAGTTGAATTATCAGCTT
mSTAT1#57	TRCN0000071457	GCAGGTATCTTGAGAAGCCAA

Table 6. List of the chemical inhibitors and growth factors used in Chapter 3.

Name	**Target**	**Concentration**	**Supplier**
Stattic	STAT3	Indicated	EMD Millipore
PP1	SRC	Indicated	Selleckchem
Ruxolitinib	JAK1/2	Indicated	Selleckchem
EGF	-	Indicated	GoldBio
IL-6	-	20 ng/ml	GoldBio

Table 7. List of antibody concentrations and supplies used in Chapter 3.

Antibody	**Dilution**	**Supplier**
pSTAT3(Y705)	1:1000	Cell Signaling Technologies
tSTAT3	1:1000	Cell Signaling Technologies
pEGFR(Y845)	1:1000	Cell Signaling Technologies
tEGFR	1:1000	Cell Signaling Technologies
pERK1/2	1:2000	Cell Signaling Technologies
tERK1/2	1:2000	Cell Signaling Technologies
β-actin	1:1000	Santa Cruz Biotechnologies
Lamin A/C	1:500	Santa Cruz Biotechnologies
β-tubulin	1:1000	Developmental Studies Hybridoma Bank

3.8.3 Apoptosis and Cell Viability Assays

Caspase-Glo 3/7 assay (Promega) was used to quantify caspase 3/7 activity according to the manufacturer's instructions. CellTiter-Glo assay (Promega) was used to measure cell viability according to the manufacturer's instructions. Floating cell fractions were collected from all conditions, spun down, and resuspended in trypsin to break the cell-clumps into single-cell suspensions.

3.8.4 Three-dimensional (3D) Culture Methods

Breast cancer cells at a density of 2×10^3 were resuspended in DMEM supplemented with 10% FBS and 4% Cultrex (150 µl/well) and then plated into 96-well plates coated with Cultrex (50 µl/well). Media containing the indicated treatments was replaced every 2 days and organoid outgrowth was quantified using the CellTiter-Glo assay (Promega) according to the manufacturer's instructions.

For tumorsphere assays, breast cancer cells were seeded at a density of 5×10^3 in non-adherent round-bottom 96-well plates (Corning) in full growth media and cultured for 3 days. At this point, the tumorspheres were physically transferred with 50 µl of residual media and 150 µl of fresh media to a flat bottom 96-well plate coated with 50 µl of growth factor reduced basement membrane hydrogel (Trevigen) in the presence or absence of the indicated treatments. These structures were allowed to grow for an additional 3 days for metastatic NM-LM1 cells.

3.8.5 Tumor Growth Analysis

Orthotopic NME tumors were established and tumor volume was quantified as previously described (Wendt, Taylor, et al., 2014). All animal procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee for Case Western Reserve University (Cleveland, OH) and Purdue University (West Lafayette, IN).

3.8.6 Statistical Methods

Statistical analyses were carried out using unpaired Student’s T-test where the data fit the parameters of the test. P-values < 0.05 were considered statistically significant. P-values for all experiments are indicated.

CHAPTER 4. CONCLUSIONS

(Some paragraphs are extracted from the below publication:
Ali R, Wendt MK. The paradoxical functions of EGFR during breast cancer progression. Signal transduction and targeted therapy. 2017;2:16042.)

4.1 Future Directions Investigating the Role of STAT1 in EGFR-driven Primary and Metastatic Breast Cancer

EGFR:STAT1 signaling remains virtually unexplored in cancer treatment as compared to other STAT1-activating cytokines such as interferons, which are heavily used therapeutically (Schindler, Levy, & Decker, 2007; Thomasy & Maggs, 2016; Witthöft, 2008). Thus, EGFR agonism in combination with trametinib can be examined as a potential therapeutic strategy for metastatic breast cancer. Along these lines, a recent study using the A431 model of EGF-induced apoptosis has established proof-of-concept for *in vivo* tumor inhibition upon systemic administration of supra-physiologic levels of recombinant EGF (Lim et al., 2015). EGF administration was also reported to induce programmed cell death *in vitro* and regress tumors in a xenograft model of non-small cell lung carcinomas (NSCLC) (Ryu et al., 2017). EGF in topical forms is currently used therapeutically in human patients for cellular regeneration while trametinib is a MEK inhibitor clinically indicated for metastatic melanoma treatment (Bodnar, 2013; Thota, Johnson, & Sosman, 2015). Moreover, prolonged systemic administration of EGF in mouse studies did not result in generalized systemic transformation of major body organs as assayed by immunohistochemistry (IHC) (Lim et al., 2015; Ryu et al., 2017). Thus, the efficacy of EGF agonism in combination with trametinib will first be examined *in vivo* using previously developed breast carcinoma progression series discussed in Chapter 2. Subsequently, the combination can be tested using patient-derived xenografts (PDXs) from metastatic breast cancer patients resistant to currently available targeted therapies. Investigating the effect of EGF + trametinib combination on tumor burden and survival can be performed with isogenic cell-lines generated in previous studies as shown in Figure 31 (Ali et al., 2018; Wendt et al., 2015).

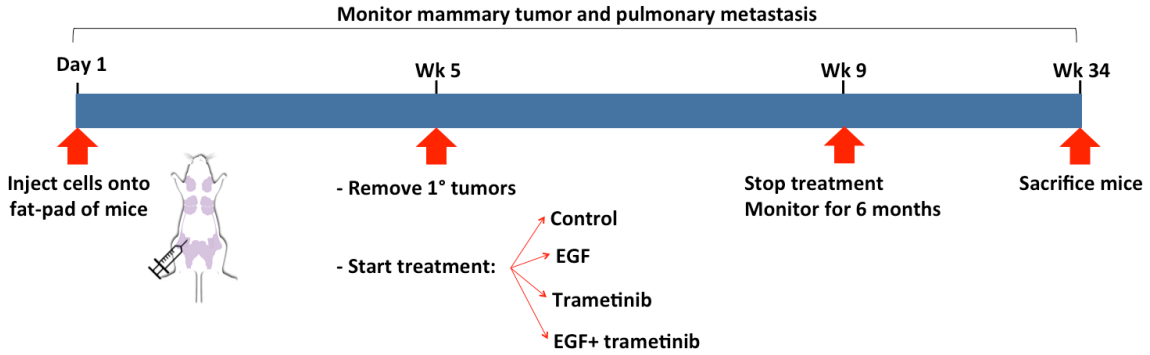


Figure 31. Schematic of an experimental design of *in vivo* studies examining the efficacy of EGF + trametinib using preclinical models of breast cancer metastasis. NME cells will be treated with TGF-β (5 ng/ml) for 48 hours to induce transient EMT. These cells will be engrafted onto the mammary fat-pad of twenty Nu/Nu mice (2 x 10^6 cells/mouse) as previously described (Balanis et al., 2013; Wendt et al., 2015). Mammary tumor outgrowth and pulmonary metastasis will be monitored over an initial period of five weeks. Once mammary tumor sizes reach < 200 mm^3, surgery will be performed to remove these tumors, and divide the mice into four groups (n = 5 mice per group): control group, EGF group, trametinib group, and EGF + trametinib combination group. Treatment will be performed for four weeks. Animals will be sacrificed at the end of the four-week treatment and the mammary tissues and lungs harvested. Immunohistochemistry (IHC) analysis will be performed using antibodies specific for Ki67 to detect proliferation, and anti-cleaved caspase-3 antibody (c-caspase3) to detect apoptosis.

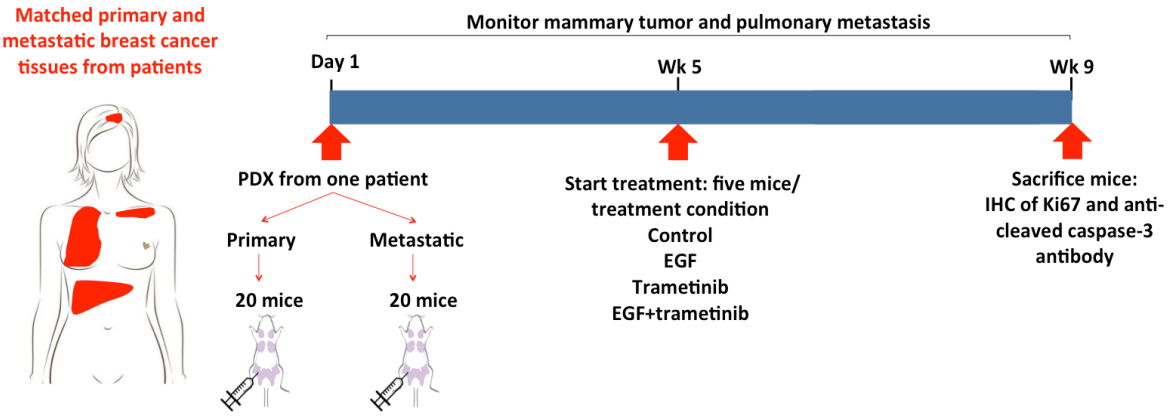


Figure 32. Schematic of an experimental design of *in vivo* studies examining the efficacy of EGF + trametinib using PDXs from TNBC patients with the disseminated disease.

Five metastatic tumors from TNBC patients will be obtained. Specifically, each tissue sample representing one patient will be divided into equivalent pieces and expanded *in vivo* by surgical implantation onto the mammary fat-pad of twenty NSG mice according to established protocols (Brown, Tan, Smith, Gray, & Wendt, 2016). When tumor volume reaches < 200 mm^3, mice will be divided into four cohorts (n=5 mice/cohort) and treatment initiated as follows: control group, EGF group, trametinib group, and EGF + trametinib combination group. Treatment will be performed for four weeks. Changes in mammary tumor growth as measured by digital calipers will the primary endpoint of these studies. In addition, remaining mammary and lung tumor tissues will be harvested for IHC analysis using antibodies specific for Ki67 to detect proliferation, and anti-cleaved caspase-3 antibody (c-caspase3) to detect on-going apoptosis.

Following studies with these preclinical models, the therapeutic utility of EGF + trametinib combination can be examined using patient derived xenografts (PDXs) from TNBC patients resistant to EGFR inhibitors. In these investigations, matched primary and metastatic tumors will be obtained from metastatic breast cancer patients. Subsequently, these PDXs will be implanted onto the mammary fat-pad of immunocompromised NSG (NOD scid gamma) mice and the efficacy of EGF + trametinib combination on tumor volume and survival will be established as shown in Figure 32.

Given the strong findings in Chapter 2 evaluating EGF + trametinib efficacy, this treatment combination is likely to emerge as a robust *in vivo* therapeutic strategy for the treatment of metastatic breast cancer. In addition to EGF, other activators of STAT1 may be explored in combination with trametinib. For example, interferon-gamma (IFN-γ) treatment of numerous cancer cell-lines induces STAT1-dependent apoptosis and has been investigated in clinical trials for the treatment of several cancers (Ni & Lu, 2018). Study designs analogous to those in Figures 31 and 32 can be performed using IFN-γ in combination with trametinib.

While the above investigations can be performed using recombinant EGF, progressing to human patient application requires careful consideration to avoid the potential pro-tumorigenic effects resulting from systemic EGF administration. Thus, data from the above experiments may provide a molecular underpinning for the concept of a novel EGFR agonist with high selectivity towards the EGFR:STAT1 signaling axis.

The design and use of such EGFR agonists are likely to result in STAT1-mediated apoptosis and bypass activation of pro-tumorigenic signaling such as the MAPK and AKT pathways. Indeed, newly developed EGFR inhibitors that possess favorable aspects of EGFR activation are proposed to have a therapeutic value for EGFR-expressing tumors. For example, compounds that phosphorylate EGFR at tyrosine-1045 without activating downstream MAPK/AKT pathways lead to EGFR degradation in NSCLC cells and subsequent apoptosis and tumor regression in multiple preclinical models (Huang et al., 2016; Joo et al., 2015; Lin, Hsu, Sun, Wu, & Tsao, 2017). Moreover, the natural compound galic acid blocked EGFR-associated AKT and MAPK pathway activation while maintaining STAT and p38 signaling in mesothelioma cell-lines, resulting in the apoptosis of these cells (Demiroglu-Zergeroglu, Candemir, Turhanlar, Sagir, & Ayvali, 2016). These studies will provide proof-of-concept for a novel class of anticancer

drugs defined as ‘partial EGFR agonists’ that have the ability to activate EGFR:STAT1 signaling while suppressing malignant MAPK/AKT signaling pathways associated with EGFR activation.

Despite the potential therapeutic utility of EGFR agonism in the treatment of metastatic breast cancer, predicting patient groups that might benefit from EGFR agonists versus inhibitors faces many challenges. Paramount to these challenges includes the design of effective biomarkers to predict the pro- versus anti-tumorigenic effect of EGFR. While EGFR expression and cellular localization can be assessed in primary mammary tumor biopsies, these types of analyses would need to be standardized into reproducible diagnostics that could be introduced to the clinic. Furthermore, these detection methods may not be feasible in metastatic breast cancer tissues. However, using the estrogen-paradox as a model, estrogen treatment has demonstrated growth inhibitory effects on breast cancer cells in culture and mouse models (Brünner, Spang-Thomsen, Vindeløv, & Nielsen, 1983; Brünner et al., 1985). Similarly, patients pretreated and resistant to endocrine inhibition therapies do show antitumor responses when switched to high dose estrogen (Lønning et al., 2001; Mahtani, Stein, & Vogel, 2009). Therefore, one potential course of therapy for patients who present with metastatic lesions and display EGFR expression in their primary tumor would be to initiate EGFR inhibitor treatment. At the point of disease progression, the treatment can abruptly be switched to a high dose EGFR agonist. Overall, more thorough preclinical and clinical studies will establish if we will be able to harness the power of the EGFR-paradox for the therapeutic benefit of metastatic breast cancer patients.

In addition to tumor cells, systemic administration of recombinant EGF or an EGFR partial agonist may drive tumor progression by affecting cell types in the tumor microenvironment. For example, potential activation of pro-tumorigenic signaling in stromal and tumor cells that have not undergone the EGFR paradox requires investigating alternative strategies to the whole-cell actions of EGF or an EGFR activator. One potential approach involves compartmentalizing the activity of the EGFR agonist by chemically linking it to a nuclear localization signal (NLS) as previously done for the EGFR inhibitor gefitinib (Bartolowits et al., 2017). Indeed, as shown in this study, following EMT and metastasis of breast cancer, nuclear trafficking of EGFR is increased to drive phosphorylation of STAT1/3 molecules residing in the nucleus. As discussed in Chapters 2 and 3, the nuclear EGFR pool is specifically responsible for driving the pro-apoptotic function of EGFR in a STAT1/3 dependent manner. Based on the above conclusions, a nuclear EGFR activator will have a greater tendency to induce apoptotic than proliferative signaling. Indeed, the canonical pro-

tumorigenic MAPK, PI3K, and PLC-γ pathways require activation of cytoplasmic components for subsequent translocation to the nucleus and induction of gene expression associated with tumorigenesis. While an NLS-linked EGFR agonist represents a novel treatment approach for metastatic breast cancer, it can be further optimized by the addition of trametinib as discussed for EGF + trametinib combination. This strategy would also allow for further reduction of the NLS-linked EGFR agonist and thus decrease the likelihood of oncogenic pathway activation while optimally inducing STAT1-mediated cell death.

Harnessing the full potential of EGFR's anti-tumorigenic function requires deducing the unique gene expression profiles involved in this response. Such investigations are optimally performed with specific activators of nuclear EGFR signaling to examine the transcriptional component of the pro-apoptotic function of EGFR. Han *et al.* showed that STAT1 gene expression is enhanced by nuclear EGFR and Her2 via cooperation with STAT3 (Han et al., 2013). In this study, EGFR phosphorylates STAT3 and subsequently forms a signaling complex involving Her2 and STAT3. This complex translocates to the nucleus to transcriptionally upregulate STAT1 expression in a STAT3-dependent manner (Han et al., 2013). Therefore, the use of a nuclear EGFR agonist may potentiate this mechanism in Her2-amplified breast cancer cells. These studies may allow for expanding the findings in Chapter 2 into the Her2-amplified subtype of breast cancer. In theory, the use of a nuclear EGFR agonist in Her2-expressing cells will phosphorylate nuclear STAT3, followed by EGFR-p-STAT3-Her2 complex formation, and ultimately upregulation of STAT1 gene transcription. The consequences of STAT1 transcriptional upregulation on the apoptosis of Her2-expressing breast cancer cells can be examined in the absence or presence of EGF. Given the involvement of p-STAT3 in the complex activating STAT1 transcription, this mechanism may also be analyzed in metastatic breast cancer cells that upregulate basal STAT3 signaling. Indeed, increased basal phosphorylation of STAT3 is a hallmark of post-EMT highly metastatic breast cancer. Altogether, the above investigations may ultimately provide novel mechanisms for utilizing EGFR agonism in the therapeutic targeting of metastatic breast cancer.

The therapeutic utility of a nuclear EGFR agonist can also be examined in NSCLC, where EGFR is commonly mutated to become constitutively active in the absence of ligand (Pines et al., 2010). Acquired resistance to EGFR inhibitors in NSCLC is associated with increased nuclear trafficking of EGFR (Li et al., 2009). However, the ability of constitutively active EGFR to phosphorylate nuclear STAT1 is unknown. Additionally, EGFR association with Her2, Her3, and

cMET is increased in cetuximab-resistant cells (Wheeler et al., 2008). However, the potential role of nuclear EGFR-Her2 complex in activating STAT1 transcription in NSCLC is unknown. These investigations would allow for expanding the therapeutic utility of nuclear EGFR agonists either alone or in combination with trametinib to NSCLC.

Studies in Chapters 2 and 3 involve metastatic breast cancer models characterized by an inherent resistance to EGFR inhibitors. In these models, the function of EGFR has been shown to change from oncogenic in the primary mammary tumors to apoptotic in their metastatic counterparts (Wendt et al., 2015). Despite these findings, fundamental gaps exist regarding the precise molecular drivers of growth and metastasis in these tumors. Particularly, the upstream oncogenic driver(s) that have emerged alternative to EGFR are unknown. There are two identified mechanisms of resistance to targeted cancer therapeutics (Rosell et al., 2013). The first involves reactivation of the inhibited pathway by secondary mutations to the drug target itself. Since EGFR function changes to pro-apoptotic in metastatic cells resistant to EGFR inhibitors, it is unlikely that mutations have emerged to drive EGFR activation. The second mechanism of resistance involves the reactivation of a core downstream survival-signaling pathway. This can occur either by activating mutations to components in this survival pathway, or the emergence of new parallel activators. Therefore, the oncogenic drivers of resistance to EGFR inhibitors in metastatic breast cancer can be characterized using screening assays of NME-LM1 tumorspheres described in Chapter 2 Figure 14E. To identify the potential upstream receptor(s) responsible for driving the growth of metastatic breast cancer cells, a genetic screen that comprises short-hairpin RNA (shRNA) vectors targeting all 518 human kinases can be performed as previously described (Manning, Whyte, Martinez, Hunter, & Sudarsanam, 2002). Potential gene candidates will be validated on an individual basis and the effects on oncogenic survival pathways, cell growth, and tumorsphere invasion determined. To establish the clinical relevance of these findings, IHC analysis of candidate receptors/kinases can be performed on metastatic breast cancer tissue resistance to EGFR inhibitors as described in Figures 31 and 32. Should the growth and/or invasion of NM-LM1 spheroids be unaffected by depletion of candidate kinases, a large-scale genetic screen that comprises shRNA vectors targeting 8000 human genes may subsequently be used (Berns et al., 2004).

4.2 Future Directions Investigating the Role of STAT3 in EGFR-driven Primary and Metastatic Breast Cancer

As shown in Chapter 3, ligand stimulation of NMuMG cells expressing EGFR constitutively or in a dox-inducible fashion results in the activation of STAT3 signaling and enhanced growth in 3D organotypic assays. However, EGFR-mediated oncogenic transformation of NMuMG cells has been established *in vivo* for the constitutive overexpression system only (Balanis et al., 2013; Wendt et al., 2010). Thus, the effect of dox-inducible EGFR on mammary tumor formation can be determined. In these investigations, NMuMG TetOn-MT or NMuMG TetOn-EGFR will be engrafted onto the fat-pad of female Nu/Nu mice and doxycycline will be administered to these animals. Primary tumor formation will be monitored by caliper measurements. Immunohistochemical analysis of EGFR, p-EGFR(Y845), pERK1/2 and p-STAT3-Y705 will be performed on harvested mammary tumors or fat-pad tissues from these mice, to confirm expression of EGFR and activation of STAT3. Additionally, staining for the proliferation marker Ki67 can be performed to examine the association of tumorigenesis with EGFR downstream effector activation.

Previous studies have established the downregulation of EGFR with metastatic progression of breast cancer (Choong et al., 2007; Wendt et al., 2015). Similar to EGFR, Her2 expression is reported to be decreased in metastatic versus primary mammary tumors (Niikura et al., 2012). Interestingly, a recent study reported the necessity of Her2 expression in mammary tumor formation using dox-inducible Her2 expression. However, following Her2 withdrawal, tumors cells survived and progressed to form distant metastasis, suggesting that Her2 is necessary for oncogenic transformation of mammary glands cells but not necessarily for disease recurrence (Goel et al., 2016). Given the pro-apoptotic function of EGFR in metastatic breast cancer, investigating dox-induction and withdrawal of EGFR can provide invaluable insight into its role in metastatic breast cancer. Thus, the doxycycline expression system of EGFR in NMuMG cells described in Chapter 3 can be used in studies analogous to the above Her2 study. The effect of turning off EGFR expression by withdrawing doxycycline treatment can be examined and is likely to reestablish primary tumors and result in metastases that employ EGFR expression-independent mechanisms.

Previous investigations of the mechanisms of EGFR:STAT3 signaling in breast cancer NME cells suggest the involvement of SRC (Balanis et al., 2013). Indeed, inhibition of SRC kinase

using the inhibitor PP2 prevented EGF-induced STAT3 phosphorylation in NME cells. Similar findings were shown in Chapter. To confirm SRC involvement, genetic approaches will be utilized to specifically deplete the expression of SRC in NME cells. SRC-depleted NME cells will be assayed for EGF-induced STAT3 phosphorylation and growth in 3D organotypic cultures. These cells can also be engrafted onto the fat-pad of Nu/Nu mice and the necessity of SRC expression in EGFR-mediated oncogenic transformation and metastasis can be established. Altogether, these investigations will establish the role of SRC in EGFR:STAT3 signaling axis in EGFR-driven mammary tumor formation.

As shown for metastatic NME-LM1 and 231-lung, progression of breast cancer is associated with activation of the STAT3 signaling pathway. While this basal STAT3 activity is inhibited by ruxolitinib and is thus likely to be JAK1/2 mediated, the upstream autocrine driver of STAT3 activation is unknown. ELISA (enzyme-linked immunosorbent assay) can be used to determine the cytokine(s) and/or interleukin(s) responsible for STAT3 activation in the above metastatic breast cancer models. Neutralizing antibodies can be used to sequester these released factors and the effect on basal STAT3 phosphorylation can be examined. These results will be confirmed using genetic depletion methods.

Persistent STAT3 activation is established to maintain stem cell-like character and mediate resistance to targeted therapies in breast cancer (Yu et al., 2014). Thus, after identifying the factors driving STAT3 phosphorylation in metastatic breast cancer cells, the potential role of STAT3 in maintaining a stem cell-like cancer (SCC) component will be studied *in vitro* and *in vivo* using the above models and patient-derived xenografts (PDXs).

Given the lack of efficacy of ruxolitinib on inhibiting NM-LM1 cell growth and invasion, direct STAT3 inhibitors may be investigated instead. The candidate molecule for these studies is a recently reported novel degrader of STAT3 that has a minimal effect on other STAT members (called SD-36) (Bai et al., 2019). Niclosamide, an FDA-approved drug under investigation as an anticancer drug due to STAT3 inhibition properties, can also be used in these investigations. Additionally, the STAT3 inhibitor S31-201 may be used as a reference molecule since it has previously been proven effective in inhibiting STAT3 in various *in vivo* models (Ahmad et al., 2018; Lin et al., 2009).

NM-LM1 spheroids will be generated in the presence or absence of a STAT3 inhibitor. The invasive behavior of these spheroids will be examined after transfer onto 3D-organotypic growth

cultures that recapitulate the pulmonary microenvironment. To determine the effect of STAT3 inhibition on SCC properties, spheroids from the above experiments can be dissociated and stained for flow-cytometry analysis with murine stem markers CD49f and CD29. To examine tumor-initiating ability *in vivo*, these spheroids can be generated for limiting dilution tumorigenicity experiments *in vivo* using 10, 100 or 1,000 cells for tail-vein injection in Nu/Nu mice. In these experiments, NM-LM1 spheroids generated in the presence or absence of a STAT3 inhibitor can be dissociated into single-cell suspensions and then injected through the tail-vein of Nu/Nu mice for pulmonary seeding. The effect of STAT3 inhibition on metastatic tumor growth can be monitored by bioluminescence.

4.3 Summary

Breast cancer continues to be a significant health problem as it is the most commonly diagnosed and the second most lethal malignancy in women (Torre et al., 2015). Metastasis is responsible for morbidity and mortality in breast cancer. Indeed, the five-year survival rates for breast cancer patients diagnosed with tumors confined to the mammary tissues are much higher than those of patients presenting with the metastatic disease (Jemal et al., 2011). Genetically, breast cancer is classified into the luminal A and B subtypes which express estrogen receptor alpha (ER-α), Her2-amplified, and triple-negative (TNBC) categories (Britten, Rossier, Taright, Ezra, & Bourgier, 2013). The TNBC subtype accounts for approximately 20% of all breast cancer diagnoses and lacks the expression of ER-α, progesterone receptor (PR), and Her2 amplification (Chavez, Garimella, & Lipkowitz, 2010; Lehmann et al., 2011). Thus, being a diagnosis of exclusion, TNBC lacks biomolecules for targeted therapy and patients are limited to radiation and chemotherapy. Many patients become resistant to these limited therapeutic options and eventually develop metastasis at which point the disease is highly lethal (Dent et al., 2007).

The process of breast cancer metastasis can be described as a sequential cascade of events starting with oncogenic transformation and development of primary mammary tumors in the context of the normal epithelium (Steeg, 2016). Subsequently, primary tumor cells will temporarily shed epithelial characteristics and acquire motility through epithelial-mesenchymal transition (EMT) (Christiansen & Rajasekaran, 2006). Following invasion and survival in the blood and/or lymphatic circulation, tumor cells will seed into distant sites and re-acquire an epithelial phenotype through the reverse mesenchymal-epithelial-transition (MET). This secondary epithelial growth

will eventually predominate and impede the function of a vital organ resulting in the morbidity and mortality associated with metastatic breast cancer (Steeg, 2016).

Recent advances in breast cancer research has facilitated a fundamental shift in therapeutic targeting from highly toxic chemotherapy into pharmacologic and biologic agents that target components specifically needed for tumor cell survival and progression, hence the term 'targeted' therapy. By targeting the specific pathophysiology, such therapies are associated with milder side effects and are better tolerated than conventional chemotherapy that non-specifically targets proliferating cells. However, in many instances, the response to these newer agents is short-lived due to the emergence of resistance mechanisms soon after the onset of therapy (adaptive resistance) or the failure of patients to respond in the first place (intrinsic resistance). Indeed, newly developed anti-cancer therapeutics are ultimately unable to efficiently inhibit tumor growth once tumor cells escape the mammary gland into metastatic sites. Thus, targeting late-stage metastatic disease requires understanding the unique molecular characteristics of metastatic tumors compared with the primary tumors from which they arise (Masoud & Pagès, 2017).

Epithelial-mesenchymal transition (EMT) is the process during which adherent epithelial cells lose their tight junctions, acquire an apolarized phenotype and become loosely attached to the extracellular matrix, leading to tissue invasion and migration (Kalluri & Weinberg, 2009). EMT is a physiological process involved in fetal development, wound healing, and tissue regeneration. For example, during embryonic development, EMT facilitates the formation of mesoderm from the primitive ectoderm (Kim et al., 2017). In this evolutionarily conserved process, migrated cells eventually differentiate into different cell types through MET, and this secondary epithelial state is distinct from the arising tissue. This concept has been reported to be hijacked by breast cancer cells during metastasis (termed pathologic EMT) (Yao, Dai, & Peng, 2011). Indeed, numerous lines of evidence suggest that primary tumors change their intrinsic subtypes during the metastatic cascades. As discussed above, breast cancer is not a single disease and it can be categorized molecularly into several different subtypes. EMT has been implicated in subtype switching of breast cancer where metastases can change drastically compared to the primary tumor from which they evolve (McAnena et al., 2018). Since therapeutic regimens are based on primary tumor analysis, EMT lies at the heart of the failure of therapeutic targeting of metastatic breast cancer due to primary-metastatic tumor discordance.

One such example of primary-metastatic tumor discordance in breast cancer concerns the epidermal growth factor receptor (EGFR) (Ali & Wendt, 2017). EGFR is a critical signaling molecule involved in a multitude of biological processes and carcinogenic events (Lurje & Lenz, 2009). Inhibition of EGFR's activity via kinase inhibitors and monoclonal antibodies has resulted in fundamental changes in patient care for some tumor types (Ali & Wendt, 2017). Elevated expression levels of EGFR in TNBC correlate with poor prognosis and decreased survival for breast cancer patients due to metastasis (Tischkowitz et al., 2007; Ueno & Zhang, 2011). However, numerous attempts to apply EGFR inhibitor therapies to metastatic TNBC patients have been unsuccessful (Ali & Wendt, 2017). As previously reported, the function of EGFR changes from tumorigenic in the mammary tumor to pro-apoptotic in metastatic cells whose invasion and dissemination were driven by EMT induction (Ali et al., 2018; Wendt et al., 2015). These studies show that ligand treatment that elicits pro-tumorigenic outcomes in primary mammary tumors paradoxically induces apoptosis in metastatic breast cancer cells derived from these tumors. Consistent with the growth-inhibitory function of EGFR in metastatic breast cancer, EGFR is reported to be down-regulated with breast cancer metastasis both clinically and in multiple pre-clinical models (Choong et al., 2007; Wendt et al., 2015).

As thoroughly discussed in Chapter 2, fundamental changes in downstream signaling mediate the pro-apoptotic function of EGFR. Indeed, EGFR signaling in metastatic breast cancer is dominated by STAT1 activation through increased nuclear trafficking of EGFR. STAT1 expression is also essential for mediating the pro-apoptotic function of EGFR in these cells. Further, metastatic breast cancer cells from various mouse models and patient-derived cell-lines activated STAT1 and underwent growth inhibition in 2D and 3D growth assays *in vitro* upon dual treatment with EGF and trametinib. Importantly, this treatment combination resulted in apoptosis of breast cancer cells that respond to EGF in a proliferative fashion but did not induce apoptosis in normal mammary epithelial cells. Altogether, these data support a paradoxical shift in EGFR function to a STAT1-dominated pro-apoptotic signaling mechanism and provide a rationale to further investigate the clinical utility of EGF and trametinib combination in the treatment of metastatic breast cancer (Figure 33).

Similar to STAT1, STAT3 is phosphorylated in the nucleus by EGFR in the above metastatic breast cancer models (Bartolowits et al., 2017). In Chapter 3, the function of STAT3 in both primary and metastatic breast cancer was investigated. STAT3 expression was found to be

essential for EGFR-mediated oncogenic transformation of primary mammary tumors. Metastasis of breast cancer is associated with mechanisms that upregulate STAT3 phosphorylation. Unexpectedly, STAT3 expression was found to be necessary for the pro-apoptotic function of EGFR in metastatic breast cancers derived from these tumors. The overall conclusions from Chapters 2 and 3 represented by the model in Figure 34 justify the need to further characterize and explore the therapeutic utility of STAT1 and STAT3 signaling in the treatment of metastatic breast cancer.

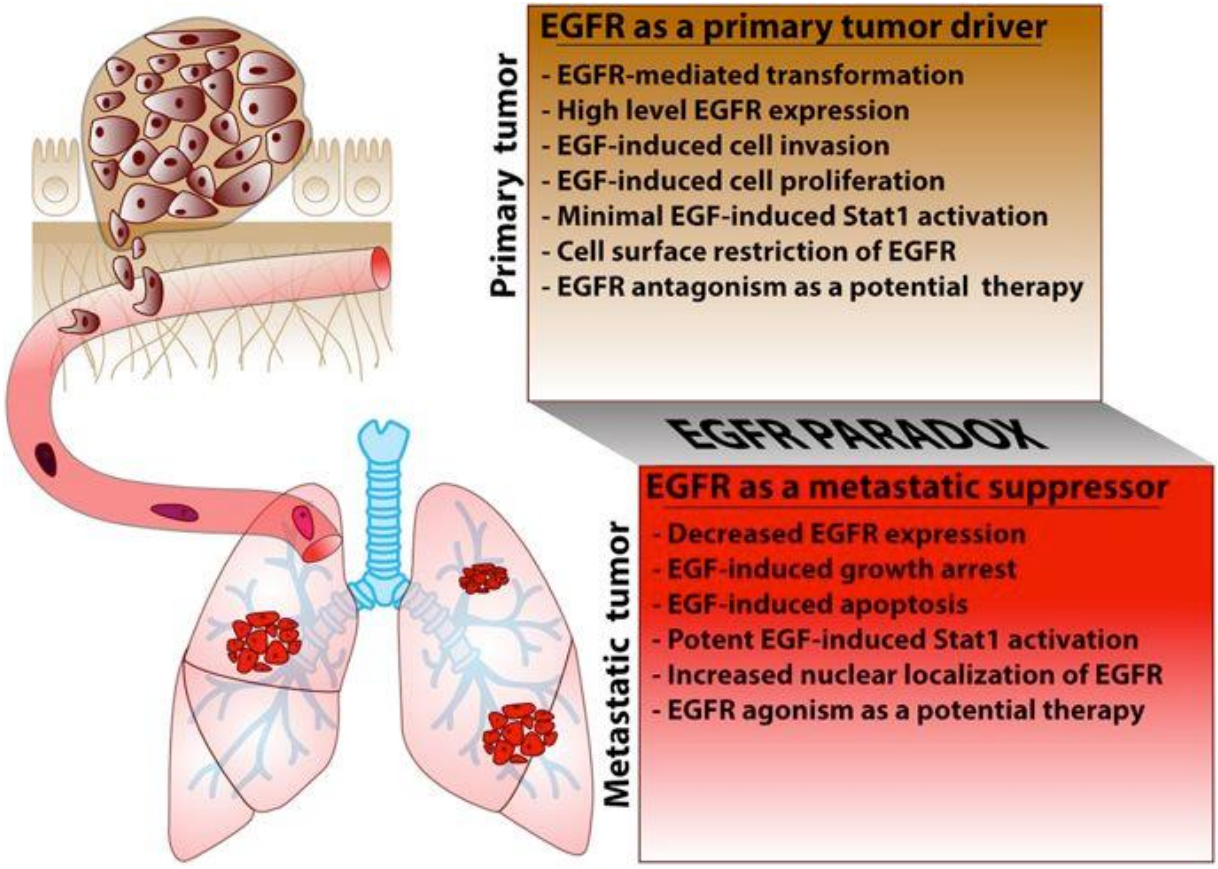


Figure 33. The 'EGFR paradox' model describing EGFR behavior in primary versus metastatic breast cancer. As tumor cells invade and disseminate, numerous selective pressures drive fundamental changes in cell signaling and growth versus death stimuli (noted by the changing colors of the tumor cells). These selective pressures and the unique microenvironment of the metastatic destination (depicted here as the lungs) yield metastatic tumors that can be quite diverse from the primary tumor. These events contribute to the listed fundamental changes in EGFR signaling in metastases as compared with primary breast tumors, constituting the 'EGFR paradox.' Overall, these events likely contribute to the failure of EGFR inhibitor therapy for the treatment of metastatic disease. In addition, these events point to EGFR agonism as a potential therapeutic strategy in metastatic breast cancer (Ali & Wendt, 2017).

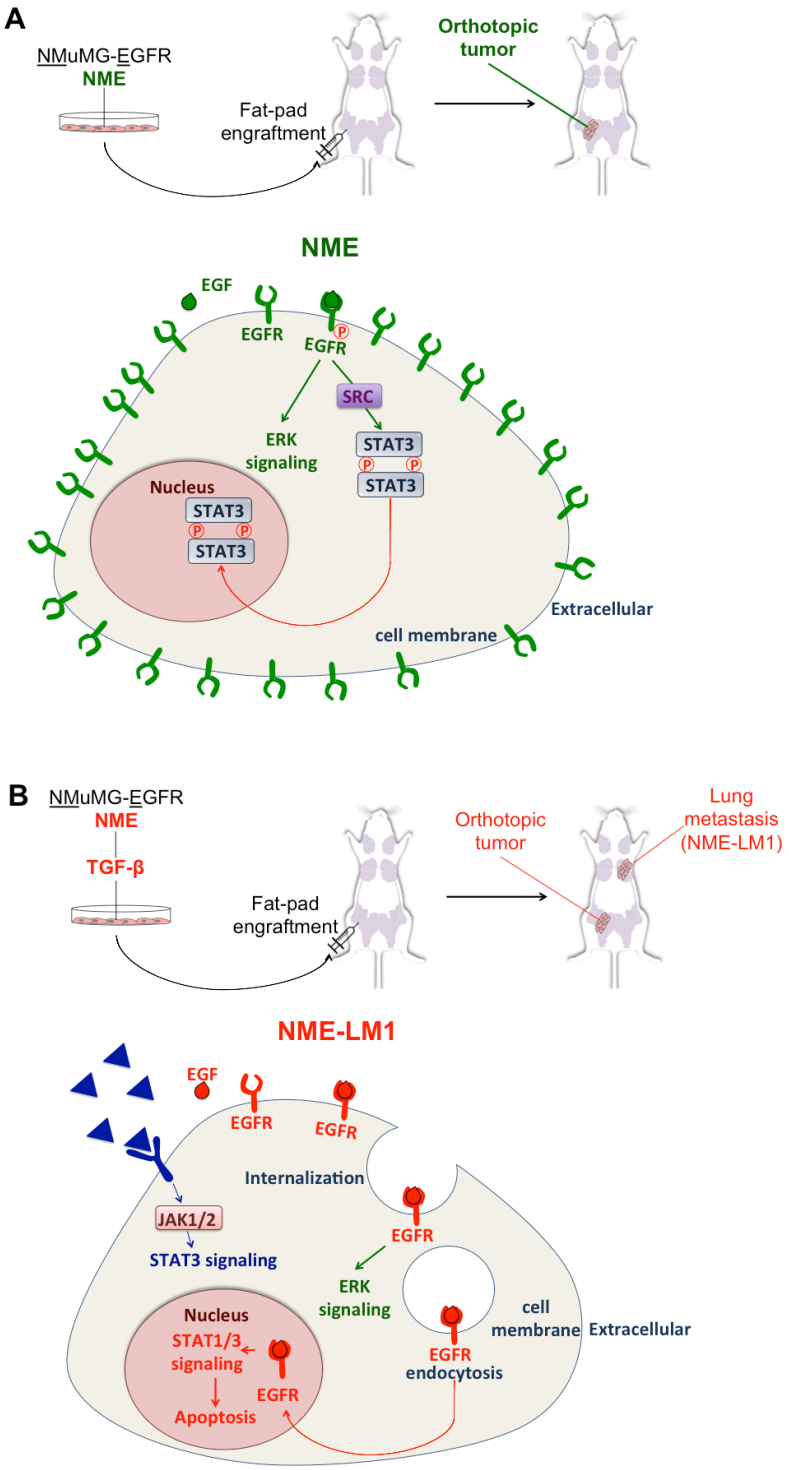
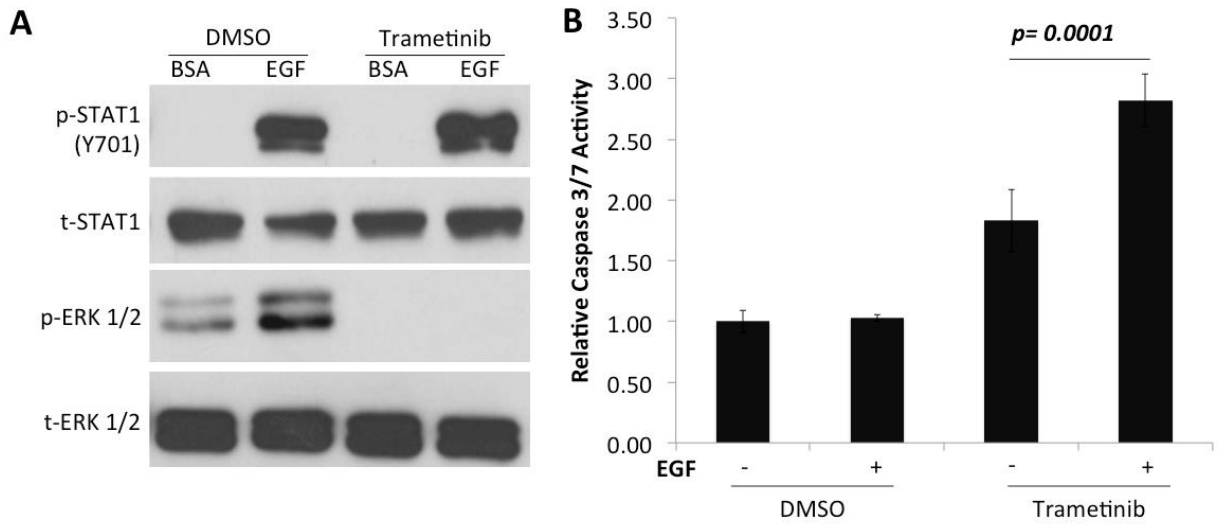


Figure 34. The role of STAT1 and STAT3 signaling in primary versus metastatic breast cancers. **A.** Overexpression of EGFR is associated with activation of STAT3 signaling axis, which is essential for EGFR-mediated transformation of mammary gland cells. **B.** In EMT-driven metastatic breast cancer, EGF-induces activation of STAT1 and STAT3 signaling occurs in the nucleus of these cells by EGFR molecules that constitutively translocate to the nucleus. EGF-induced apoptosis is mediated by the nuclear EGFR:STAT1/3 signaling.

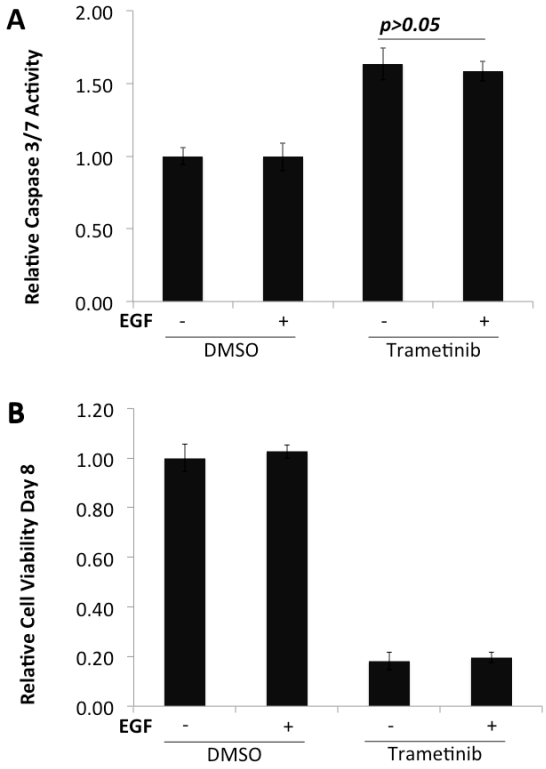
APPENDIX

Appendix Figure 1



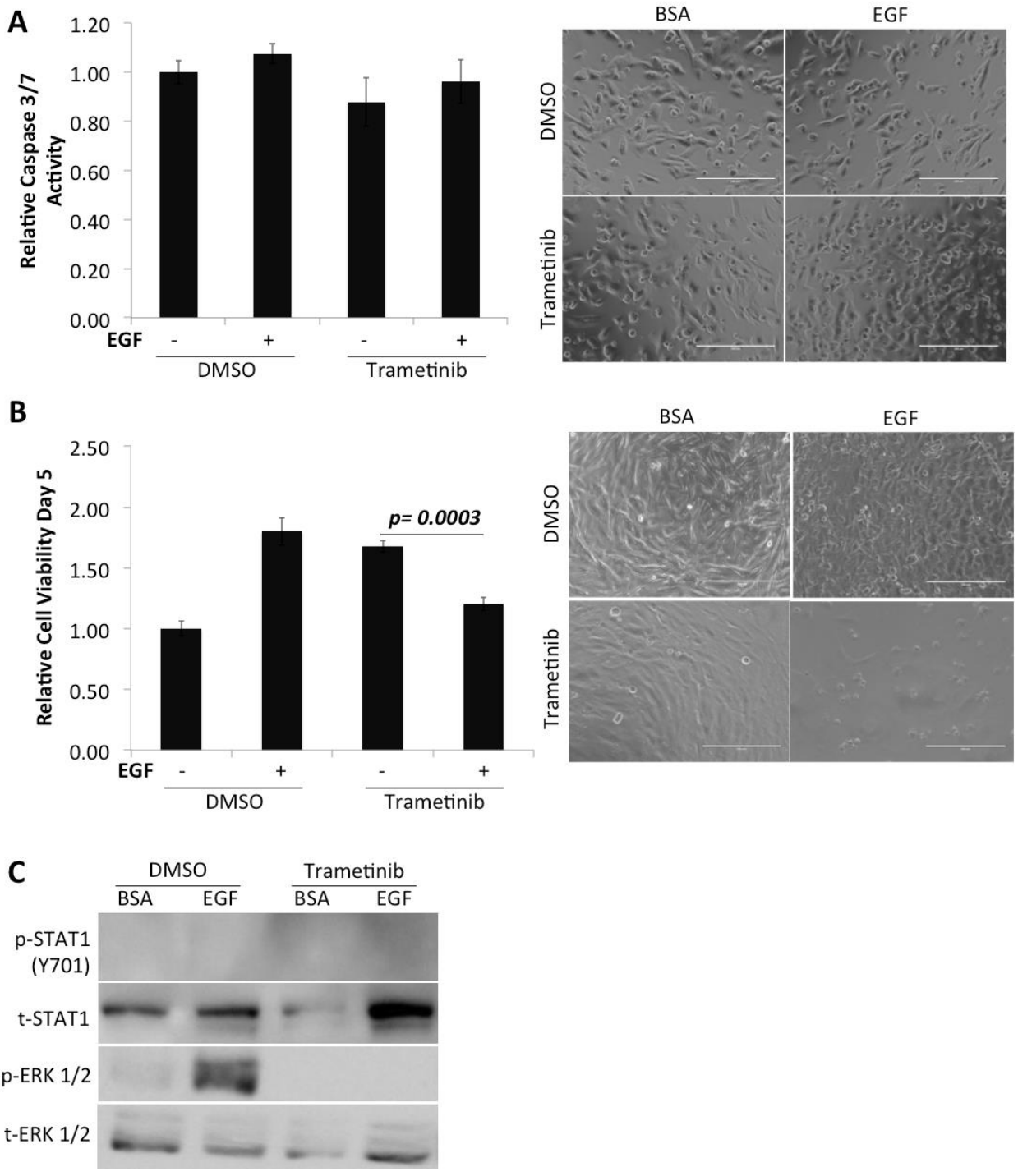
Appendix 1. EGF + trametinib combination induces apoptosis of patient-derived TNBC BT-20 cell-line. **A.** BT-20 cells (passages 1-2) were serum-starved for 7 hours in the presence of vehicle (DMSO) or trametinib (100 nM) and then stimulated with EGF (50 ng/ml) for 30 minutes. These cells were subsequently analyzed for phosphorylation of STAT1 (p-STAT1 Y701) and ERK1/2 (p-ERK1/2). BSA served as a protein stimulation control and total levels of STAT1 and ERK1/2 were assessed as loading controls. EGF treatment in the absence or presence of trametinib results in STAT1 phosphorylation, while trametinib effectively blocks EGF-induced MAPK pathway activation in these cells. Immunoblots are representative of at least two independent experiments yielding similar results. **B.** BT-20 cells were stimulated with EGF (100 ng/ml) in the presence of vehicle (DMSO) or trametinib (100 nM). Following 24 hours of treatment, these cells were assayed for caspase 3/7 activity. EGF + trametinib co-treatment results in a significant increase in apoptosis as assayed by caspase 3/7 activity when compared to either treatment alone. Data are the average ± SD of a single experiment completed in triplicate.

Appendix Figure 2



Appendix 2. EGF + trametinib combination does not induce apoptosis of patient-derived TNBC MDA-MB-231 cell-line. **A.** MDA-MB-231 cells were stimulated with EGF (100 ng/ml) in the presence of vehicle (DMSO) or trametinib (100 nM). Following 24 hours of treatment, these cells were assayed for caspase 3/7 activity. EGF treatment alone or in combination with EGF does not affect the apoptosis of these cells. Data are the average ± SD of a single experiment completed in triplicate. **B.** MDA-MB-231 cells were stimulated with EGF (50 ng/ml) in the presence of vehicle (DMSO) or trametinib (100 nM) for 8 days at which point cell viability was quantified. Data are the average ± SD of a single experiment completed in triplicate. MDA-MB-231 cells express high levels of Mitogen Induced Gene-6 (Mig6), an inhibitory protein that mitigates EGFR signaling following ligand stimulation (Wendt et al., 2015). Indeed, depletion of Mig6 in MDA-MB-231 reactivates EGFR resulting in pronounced cell death and inhibition of tumor growth (Wendt et al., 2015). These studies suggest that metastatic MDA-MB-231 cells neutralize EGFR-driven apoptosis by Mig6 expression, which may explain their lack of response to EGF + trametinib combination.

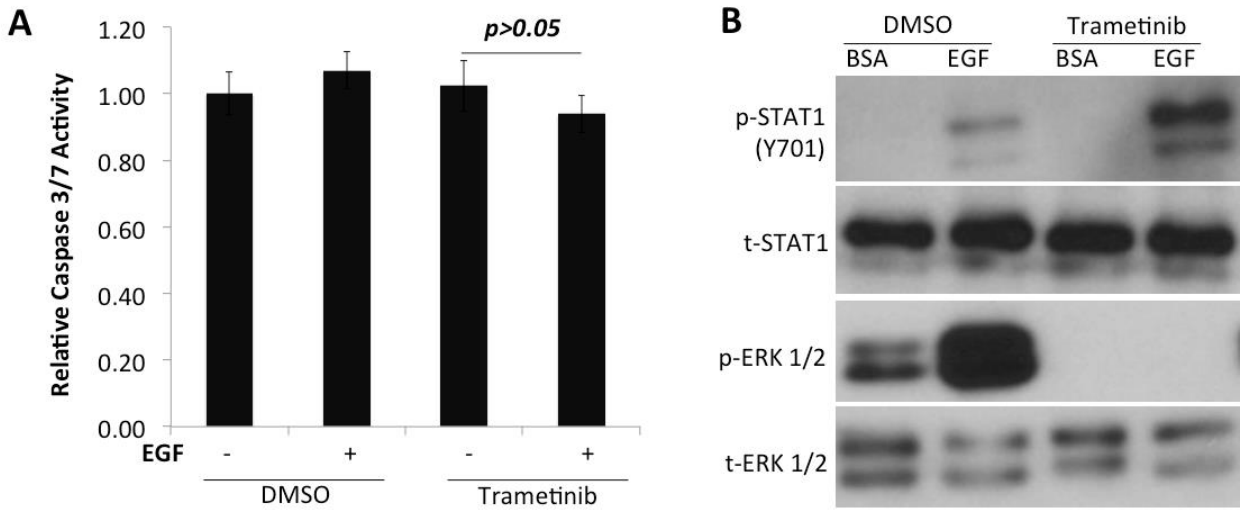
Appendix Figure 3



Appendix 3. EGF + trametinib combination induces growth inhibition but not apoptosis or STAT1 phosphorylation of patient-derived TNBC SUM159 cell-line. **A.** SUM159 cells were stimulated with EGF (100 ng/ml) in the presence of vehicle (DMSO) or trametinib (100 nM). (Left) Following

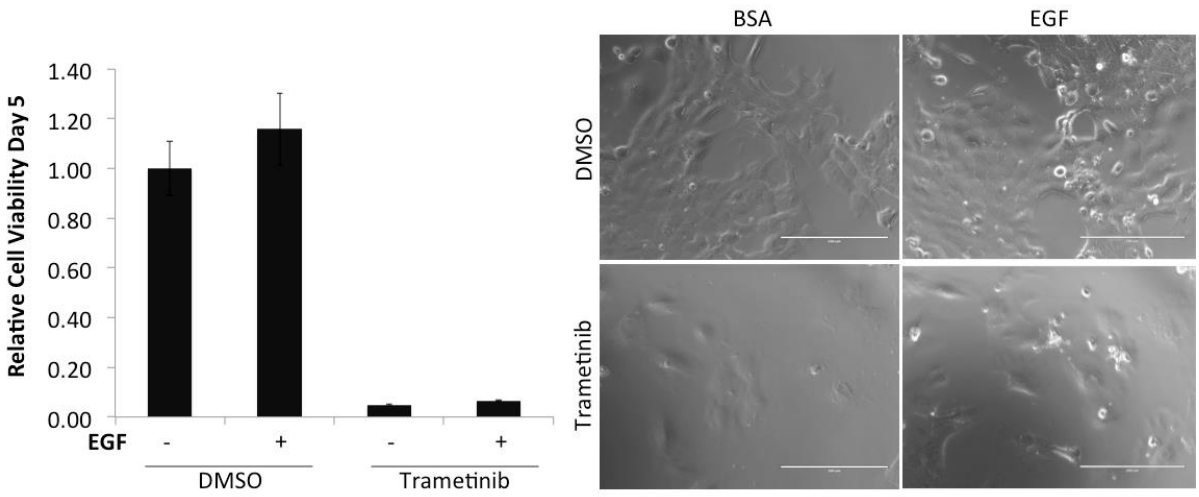
24 hours of treatment, these cells were assayed for caspase 3/7 activity. The EGF + trametinib combination does not result in apoptosis. Data are the average ± SD of a single experiment completed in triplicate. (Right) Representative photomicrographs of treatment conditions are shown. **B.** SUM159 cells were stimulated with EGF (10 ng/ml) in the presence of vehicle (DMSO) or trametinib (10 nM) for 8 days at which point cell viability was quantified (Left). Data are the average ± SD of a single experiment completed in triplicate. (Right) Representative photomicrographs of treatment conditions are shown. **C.** SUM159 cells were serum-starved for 7 hours in the presence of vehicle (DMSO) or trametinib (100 nM) and then stimulated with EGF (50 ng/ml) for 30 minutes. These cells were subsequently analyzed for phosphorylation of STAT1 (p-STAT1 Y701) and ERK1/2 (p-ERK1/2). BSA served as a protein stimulation control and total levels of STAT1 and ERK1/2 were assessed as loading controls. EGF treatment in the absence or presence of trametinib does not result in STAT1 phosphorylation. Immunoblots are representative of at least two independent experiments yielding similar results. The response of SUM159 cells switches from proliferative when treated with EGF alone to inhibitory when EGF is combined with trametinib. While the mechanism of growth inhibition does not seem to involve apoptosis or STAT1 phosphorylation, EGF + trametinib combination elicits a senescent morphology in these cells. Indeed, senescence has previously been involved in EGF-induced growth inhibition (Ali & Wendt, 2017).

Appendix Figure 4



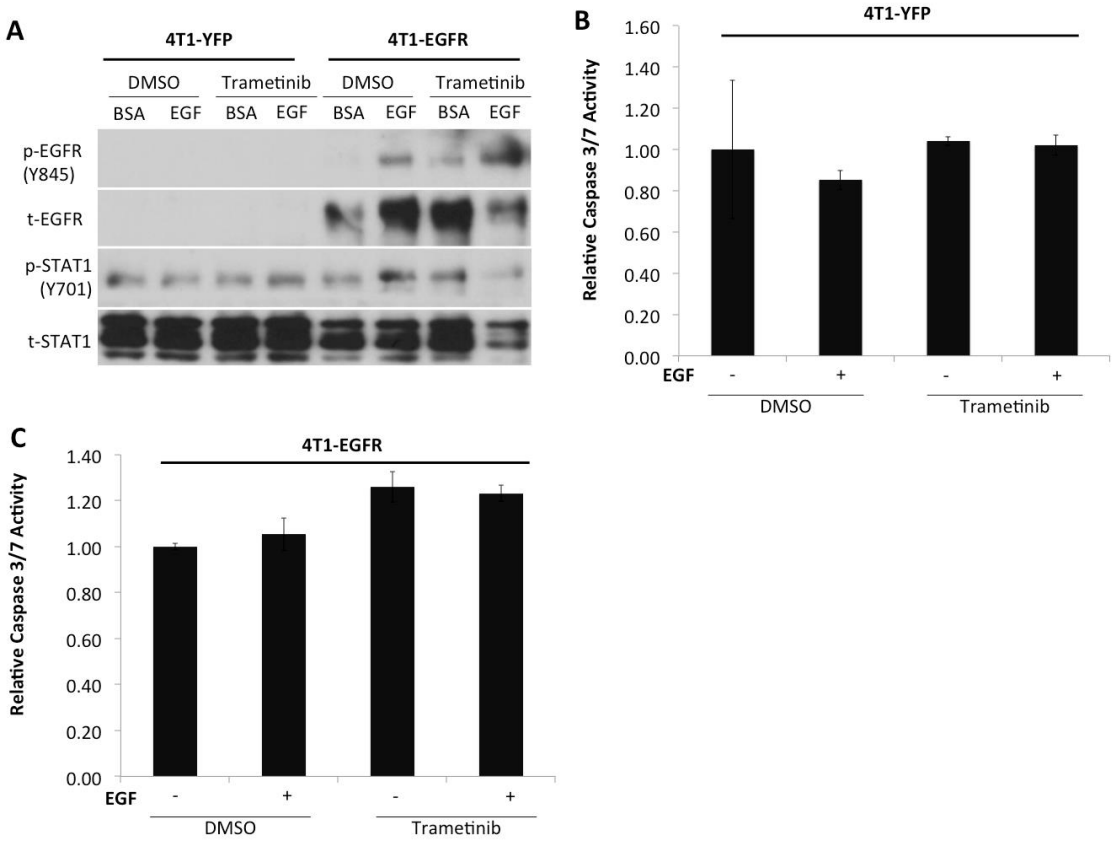
Appendix 4. EGF + trametinib combination results in STAT1 phosphorylation of patient-derived Her2-amplified SKBR3 cell-line. However, the combination does not induce apoptosis. **A.** SKBR3 cells were stimulated with EGF (100 ng/ml) in the presence of vehicle (DMSO) or trametinib (100 nM). Following 24 hours of treatment, these cells were assayed for caspase 3/7 activity. Data are the average ± SD of a single experiment completed in triplicate. **B.** SKBR3 cells were serum-starved for 7 hours in the presence of vehicle (DMSO) or trametinib (100 nM) and then stimulated with EGF (50 ng/ml) for 30 minutes. These cells were subsequently analyzed for phosphorylation of STAT1 (p-STAT1 Y701) and ERK1/2 (pERK1/2). BSA served as a protein stimulation control and total levels of STAT1 and ERK1/2 were assessed as loading controls. Immunoblots are representative of at least two independent experiments yielding similar results.

Appendix Figure 5



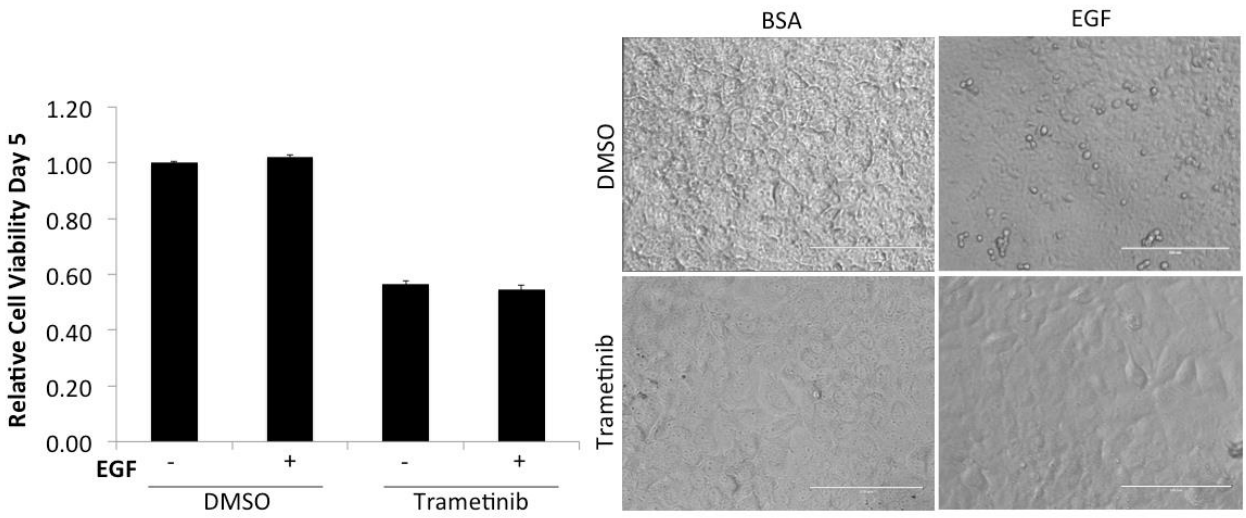
Appendix 5. EGF + trametinib combination does not result in apoptosis of metastatic TNBC 4T1 cell-line. 4T1 cells were stimulated with EGF (50 ng/ml) in the presence of vehicle (DMSO) or trametinib (100 nM) for 5 days at which point cell viability was quantified (Left). Data are the average ± SD of a single experiment completed in triplicate. (Right) Representative photomicrographs of treatment conditions are shown. Consistent with the growth inhibitory function of EGFR in metastatic breast cancer, 4T1 cells have decreased expression of EGFR as compared to the primary breast cancer cells from which they were derived (unpublished observation). Lower protein levels of EGFR may explain the lack of response to EGF + trametinib shown in this growth assay.

Appendix Figure 6



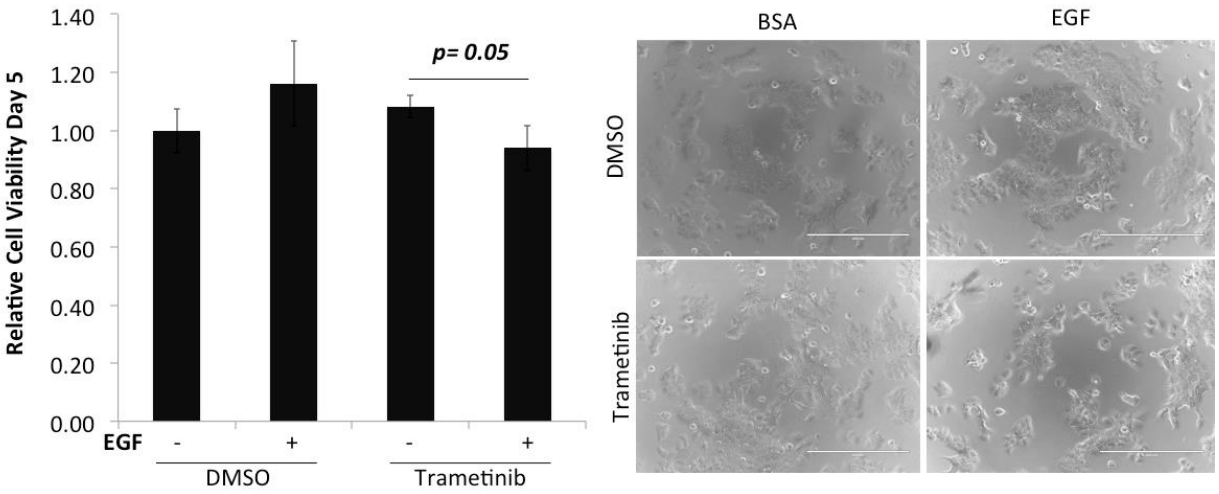
Appendix 6. EGF + trametinib combination does not result in apoptosis of metastatic TNBC 4T1 cell-line engineered to re-express EGFR using the stable expression system used to re-express EGFR in Ca1a cells in Chapter 2. **A.** Metastatic TNBC 4T1 cells were stably transfected to express YFP or EGFR. EGFR expression was verified in stable polyclonal 4T1 cell populations by serum-starving 4T1-YFP and 4T1-EGFR cells for 7 hours in the presence of vehicle (DMSO) or trametinib (100 nM) and then stimulating with EGF (50 ng/ml) for 30 minutes. Whole-cell lysates were subsequently analyzed for phosphorylation of STAT1 (p-STAT1 Y701) and EGFR (p-EGFR Y845). BSA served as a protein stimulation control and total levels of STAT1 and EGFR were assessed as loading controls. Immunoblots are representative of at least two independent experiments yielding similar results. 4T1-YFP (**B**) and 4T1-EGFR (**C**) cells described in A were stimulated with EGF (100 ng/ml) in the presence of vehicle (DMSO) or trametinib (100 nM). Following 24 hours of treatment, these cells were assayed for caspase 3/7 activity. These results may be attributed to the inability of 4T1 cells to express a high levels of EGFR when compared to NMuMG or Ca1a cells, due to the potential potent growth-inhibitory role of EGFR in 4T1 cells. Data in B and C are the average ± SD of a single experiment completed in triplicate.

Appendix Figure 7



Appendix 7. EGF + trametinib combination does not result in growth inhibition of metastatic breast cancer D2A1 cell-line. D2A1 cells were stimulated with EGF (50 ng/ml) in the presence of vehicle (DMSO) or trametinib (100 nM) for 5 days at which point cell viability was quantified (Left). Data are the mean ± SE of three independent experiments completed in triplicate. (Right) Representative photomicrographs of treatment conditions are shown. Similar to 4T1 cells, metastatic D2A1 cells downregulate EGFR expression as compared to the primary tumor cells from which they arise (Wendt, et al., 2010). Insufficient expression levels of EGFR may explain the lack of response to EGF + trametinib treatment.

Appendix Figure 8



Appendix 8. EGF and trametinib combination induces growth inhibition of patient-derived Her2-amplified BT474 cell-line. BT-474 cells were stimulated with EGF (10 ng/ml) in the presence of vehicle (DMSO) or trametinib (10 nM) for 5 days at which point cell viability was quantified (Left). Data are the mean ± SE of three independent experiments completed in triplicate. (Right) Representative photomicrographs of treatment conditions are shown. BT-474 cells have previously been shown to undergo growth inhibition when treated with high concentrations of EGF (Imai et al., 1982). The EGF dose used here is much lower than the one used in the above study.

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