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INVESTIGATIONS OF NUCLEAR HER FAMILY RECEPTORS IN CANCER AND RESISTANCE TO CETUXIMAB THERAPY

By

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ABSTRACT

INVESTIGATIONS OF NUCLEAR HER FAMILY RECEPTORS IN CANCER AND RESISTANCE TO CETUXIMAB THERAPY

Toni Michel Brand

Under the supervision of Professor Deric L. Wheeler

At the University of Wisconsin-Madison

The HER family of receptor tyrosine kinases consists of four family members: epidermal growth factor receptor (EGFR/HER1/ErbB1), HER2 (ErbB2/Neu), HER3 (ErbB3), and HER4 (ErbB4). Ligand binding to HER family receptors initiates receptor dimerization, tyrosine kinase activation, and auto/trans phosphorylation of tyrosine residues located on each receptor's C-terminal tail. Classically, HER receptors are localized on the cell surface where they serve to initiate growth and survival signaling pathways.

Advances in HER family biology over the last decade have established that HER receptors can also be localized to intracellular organelles, one of which includes in the nucleus. Inside the nucleus HER family receptors can function as both co-transcription factors and nuclear kinases. These nuclear functions have been linked to three parameters of tumor biology: 1) inverse correlation with overall survival in numerous cancers, 2) resistance to both conventional and molecular targeted therapies, and 3) enhanced tumor growth.

Previous investigations into mechanisms of acquired resistance to the anti-EGFR monoclonal antibody therapeutic cetuximab demonstrated that resistant cells expressed increased levels of nuclear EGFR, which was regulated by Src family kinase (SFK) activity. To better understand how SFKs regulated EGFR nuclear translocation, we aimed to elucidate which SFK member(s) controlled this process as well as the EGFR tyrosine residues that were involved. Analyses of cetuximab resistant (Ctx^R) cells indicated that the SFKs Yes and Lyn were overexpressed and associated with the EGFR. Genetic ablation of either Yes or Lyn led to a loss of nuclear EGFR expression, and conversely, their overexpression resulted in increased nuclear EGFR expression. Interestingly, the stable overexpression of each SFK in MCF-7 cells resulted in an increase in nuclear EGFR expression, indicating that while Yes and Lyn function in this model other SFKs may regulate nuclear EGFR trafficking in other cell types. Further, site-directed mutagenesis of the SFK dependent phosphorylation sites on the EGFR, tyrosine 845 and tyrosine 1101, indicated that mutation of tyrosine 1101 (and not tyrosine 845) impaired nuclear entry of the EGFR. Collectively, these studies indicated that the SFKs Yes and Lyn could phosphorylate EGFR on tyrosine 1101 to influence its nuclear translocation in a model of acquired resistance to cetuximab.

Based on previous studies indicating that acquired resistance to cetuximab was mediated by nuclear EGFR, we hypothesized that nuclear EGFR may also promote intrinsic resistance to cetuximab in Triple-negative breast cancer (TNBC). To examine this question a battery of TNBC cell lines and human tumors were screened and found to express nuclear EGFR. Genetic ablation of the EGFR demonstrated that TNBC cell lines retained dependency on EGFR for proliferation, yet all cell lines were resistant to

cetuximab therapy. Further, SFKs influenced nuclear EGFR translocation in TNBC cell lines and in vivo TNBC xenografts, where inhibition of SFK activity led to potent reductions in nuclear EGFR expression and increases in membrane localized EGFR. Importantly, the inhibition of nuclear EGFR translocation greatly enhanced TNBC cell line sensitivity so cetuximab, suggesting that nuclear EGFR may be a functional molecular target in TNBC.

Recent studies indicate that all HER receptors can function as co-transcription factors in the nucleus; however, the specific domains on HER receptors that confer transcriptional potential remain poorly defined. To identify putative transactivation domains (TADs) of a HER receptor, various intracellular regions of HER3 were fused to the DNA binding domain of the yeast transcription factor Gal4 (Gal4DBD) and tested for their ability to transactivate Gal4 UAS-luciferase. Results from these analyses demonstrated that the C-terminal domain (CTD) of HER3 contained potent transactivation potential, and further mutational analyses resulted in the identification of a bipartite transactivation domain (TAD). Deletion of this bipartite TAD from the full-length HER3 receptor greatly reduced its ability to regulate the known target gene cyclin D1. These studies revealed that HER3 contains a bipartite TAD necessary for its ability to function as a co-transcription factor in the nucleus, and further suggests that all HER family receptors may contain homologous regions.

Collectively, the studies performed in this thesis indicate that nuclear EGFR translocation is dependent on SFK activation on tyrosine 1101 resulting in intrinsic resistance to cetuximab in TNBC. Further, nuclear HER receptors may be dependent on specific C-terminal TADs to function as co-transcription factors in the nucleus.

Dedicated to the people who have given me the love and support

to have made this journey possible:

In loving memory of my dad Terry Brand

My grandmother Joyce Brand

My two best friends Catherine Ramsey and Lauren Mulligan

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PREFACE

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ABBREVIATIONS

AJCC	American Joint Committee on Cancer
BCRP	breast cancer resistant protein
BTC	betacellulin
BP	base pair
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
COPI	coat protein complex I
COX2	cyclooxygenase-2
CRC	colorectal cancer
Ctx^S	cetuximab-sensitive
Ctx^R	cetuximab-resistant
CTD	C-terminal domain
DAPA	DNA affinity precipitation assay
DBD	DNA binding domain
DMSO	dimethyl sulfoxide
DM	double mutant
DNA	deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
ECL	enhanced chemiluminescence
EDTA	ethylenediamine-tetraacetic acid
EGTA	ethane dioxy-bis(ethylamine)tetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EM	electron microscopy
EPR	epiregulin
ER	endoplasmic reticulum
FBS	fetal bovine serum
FDA	food and drug administration
FGFR	fibroblast growth factor receptor

GAB1	GRB2-associated binding protein 1
GRB2	growth factor receptor bound 2
HB-EGF	heparin-binding EGF
HNSCC	head and neck squamous cell carcinoma
ICD	intracellular domain
IF	immunofluorescence
IHC	immunohistochemistry
INM	inner nuclear membrane
IP	immunoprecipitation
iNOS	inducible nitric oxide synthase
JKD	juxtamembrane and tyrosine kinase domain
mAB	monoclonal antibody
mRNA	messenger RNA
MAPK	mitogen-activated protein kinase
MEKK	MAPK kinase
nEGFR	nuclear EGFR
NLS	nuclear localization signal
NN	non-nuclear
NSCLC	non-small cell lung cancer
NRG	neuregulin
Nuc	nucleus
NT	non-targeting
OS	overall survival
PAGE	polyacrylamide gel electrophoresis
PCNA	proliferating cell nuclear antigen
PFS	progression free survival
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PLCg	phospholipase C-gamma
PDGFR	platelet-derived growth factor receptor

PNPase	polynucleotide phosphorylase
PTB	phosphotyrosine-binding domain
qPCR	quantitative polymerase chain reaction
RHA	RNA helicase A
RNA	ribonucleic acid
RTK	receptor tyrosine kinase
SFK	src family kinase
STAT	signal transducers and activators of transcription
TAD	transactivation domain
TKI	tyrosine kinase inhibitor
TNBC	triple-negative breast cancer
UAS	upstream activation sequence
WCL	whole cell lysate

Chapter I

General Introduction

THE HER FAMILY OF RECEPTOR TYROSINE KINASES

In the early 1960s Stanley Cohen isolated and characterized a protein from the salivary gland that induced eye-lid opening and tooth eruption in newborn mice[1]. Further experimentation with this protein showed that it could stimulate the proliferation of epithelial cells and was thus named the epidermal growth factor (EGF)[2]. It was not until a decade later that Graham Carpenter identified the presence of specific binding receptors for EGF on target cells, later termed the epidermal growth factor receptor (EGFR)[3,4]. In the 1980s, the EGFR was cloned and sequenced and subsequently recognized as a receptor tyrosine kinase (RTK)[5]. The identification of the EGFR as an RTK contributed to pivotal studies advancing our understanding of RTK activation and how RTK signaling can influence cancer formation and progression[6,7].

The HER family of receptor tyrosine kinases consists of four members: EGFR (HER1/ErbB1), HER2/neu (ErbB2), HER3 (ErbB3) and HER4 (ErbB4). All family members contain an extracellular ligand-binding domain (domains I, II, III, IV), a single pass transmembrane domain, a juxtamembrane nuclear localization signal (NLS), an intracellular tyrosine kinase domain (TKD) and a C-terminal tyrosine rich signaling tail (**Figure 1**)[8,9]. While all HER receptors contain a NLS in the intracellular juxtamembrane region[9], several recent studies have identified alternative C-terminal nuclear localization sequences on HER3[10,11]. HER receptors are ubiquitously expressed in various cell types, but primarily include those of epithelial, mesenchymal and neuronal origin[12]. Under homeostatic conditions, receptor activation is tightly regulated by the availability of ligands, which collectively form the Epidermal Growth Factor family [12]. This family is divided into three distinct groups. The first includes EGF, transforming growth factor

alpha (TGFα) and amphiregulin (AR), which all bind specifically to the EGFR. The second group includes betacellulin (BTC), heparin-binding EGF (HB-EGF) and epiregulin (EPR), which bind to both the EGFR and HER4. The third group is composed of the neuregulins (NRG1-4) and are further subdivided based on their ability to bind HER3 and HER4 (NRG1 and NRG2) or only to HER4 (NRG3 and NRG4) [13]. HER2 binds to no known ligand[14].

EGFR, HER3, and HER4 receptors are activated on the cell surface through binding of cognate ligands via their extracellular ligand-binding domains I and III. Ligand binding initiates a major conformational change in the receptor ultimately exposing the dimerization loop on domain II[15]. The exposure of the dimerization arm allows for receptor homo- or hetero-dimerization on the plasma membrane. Interestingly, the HER2 receptor contains a dimerization domain II that is locked in the extended conformation, mimicking the conformation of ligand-bound EGFR[16-18]. The extended conformation of the HER2 receptor makes it poised to dimerize with other HER family receptors[16,17]. Both homo- and hetero- dimerization has been observed among HER family receptors, leading to the activation of each receptor's tyrosine kinase and subsequent phosphorylation of tyrosine residues located on each receptor's cytoplasmic tail. Another unique attribute of the HER family lies in the identification of kinase defective HER3, in which four residues within the kinase domain hinder HER3 from binding ATP and thereby makes HER3 severely diminished in tyrosine kinase activity[19,20]. While up for debate, a recent study using purified HER3 intraceullar domain clustered *in vitro* vesicles indicated that HER3 does contain low-level kinase activity, enough that it could trans-phosphorylate a partner receptor upon dimerization[21].

Nevertheless, HER3 serves as a signaling substrate through the recruitment of SH2 and phosphotyrosine-binding (PTB) domain containing proteins involved in cell signaling through its 14 C-terminal tyrosine residues (**Figure 1**)[22-24].

A link between HER receptor expression and cancer was first made upon the cloning of the EGFR (1,210 amino acids in length), where researchers identified high homology of EGFR's kinase domain to the erythroblastosis leukemia virus that induced malignant transformation of chicken cells[5,25]. This pivotal study marked the beginning of a new field of cancer research focused on the role of HER family receptors in cancer cell signaling pathways that regulate cellular differentiation, proliferation, survival, migration, invasion, angiogenesis, and metabolism[12,26-28].

Classically, HER family receptors are activated on the cell surface resulting in the propagation of a complex network of signaling pathways. The primary signaling pathways activated by HER receptors include the mitogen-activated protein kinase (MAPK also known as the RAS/RAF/MEK/ERK pathway) and the phophoinositide 3-kinase (PI3K)/AKT signaling pathways; however, Src family tyrosine kinases (SFKs), phospholipase C gamma (PLCg), protein kinase C (PKC) and signal transducers and activators of transcription (STATs) can also become activated in response to HER family signaling (**Figure 2**)[26,27]. Even though EGFR and HER2 can activate PI3K through adaptor proteins such as growth factor receptor bound 2 (GRB2) and GRB2-associated binding protein 1 (GAB1), HER3 and HER4 have docking sites for the p85 subunit of PI3K on their C-terminus. Due to differences in HER family structure, HER family specificity for ligand and heterodimerization serves to diversify growth and survival signals at any given time[26]. The most mitogenic HER family heterodimer is

thought to be the HER2:HER3 heterodimer, in which the rate of receptor mediated endocytosis is delayed and increased recycling of receptors to the cell surface is observed leading to prolonged and enhanced downstream signals[16,26]. While EGFR, HER2, and HER3 signaling have been implicated in tumorigenic processes, HER4 has been shown to be downregulated in numerous cancers and to potentially play non-tumorigenic functions in epithelial tumors[16].

To date, EGFR, HER2, and HER3 aberrant overexpression and hyperactivation have been implicated in the development and progression of several human cancers. While the mechanisms for this are still being unraveled, studies indicate that this can be attributed to overproduction of ligands, overproduction of receptors (due to dysregulated receptor downregulation or genomic amplification) or constitutive receptor activation (commonly due to mutations within the tyrosine kinas domain). Due to these findings, the HER family of receptors are now the most widely targeted family of RTKs in all of oncology. Two approaches have proven useful for the molecular targeting of HER receptors: 1) small molecule tyrosine kinase inhibitors (TKIs) that bind to the ATP-binding site in the tyrosine kinase domain, and 2) monocloncal antibodies (mABs) that bind the extracellular domain of HER receptors to either block ligand binding or sterically hinder dimerization with other receptors. To date, four anti-EGFR TKIs (erlotinib, gefitinib, lapatinib, and afatinib) and one anti-HER2 TKI (lapatinib) are FDA approved for use in oncology. Additionally, two anti-EGFR mABs (cetuximab and panitumumab) and two anti-HER2 mABs (trastuzumab and pertuzumab) are approved for use in oncology (**Figure 3**). While molecular agents targeting the HER3 receptor have been developed and are currently being evaluated in clinical trials, they are not

FDA approved at this time. Collectively, the therapeutic targeting of HER receptor activation and downstream signaling have been landmark advances for the treatment of cancer patients.

NUCLEAR HER FAMILY RECEPTORS

While HER family receptors were originally identified as cell surface receptors, recent reports have identified new and novel roles of HER receptors localized inside the nucleus. Nuclear EGFR was first reported in regenerating hepatocytes and in primary adrenocortical carcinomas more than two decades ago[29,30]. Nuclear expression of EGFR was further detected in other cell types and tissues, such as mouse uterus, developing mouse embryos, rat liver, placentas, thyroids and immortalized epithelial cells of ovary and kidney origins[31-33]. High levels of EGFR were also found in the nuclei of many tumors, including those of skin, breast, bladder, cervix, adrenocortical carcinoma, thyroid and oral cavity [29,32-37]. The nuclear counterpart of EGFR appears to be the full-length receptor and likely, in the phosphorylated form, as shown by a number of studies[31,32,34,35,38,39]. To date, all HER family members have been observed in the nuclei of different types of tumor specimens[40].

Intense efforts over the last decade have been undertaken by several research groups to better understand how nuclear HER receptors translocate to the nucleus, with the focus being on EGFR nuclear trafficking. With this effort, accumulating evidence suggests a novel pathway where EGFR is internalized and transported to the nucleus rather than degraded or recycled back to the cell surface. Studies using dynamin mutants that impair the formation of clathrin-coated pits at the cell membrane have

shown that inhibition of receptor endocytosis blocks nuclear import of EGFR[41,42]. In order to further elucidate proteins involved in EGFR's nuclear translocation, EGFR immunoprecipitation and subsequent mass spectral analysis identified phosphoinositide kinase, PIKfyve (synthesizes phosphatidylinositol 3,5-bisphosphate), to be associated with the EGFR[43]. PIKfyve has previously been linked to endosomal dynamics and intracellular trafficking[44-47], and thus PIKfyve RNA silencing studies led to impaired HB-EGF stimulated EGFR nuclear translocation[43]. Additionally, nuclear EGFR association with early endosomal antigen 1 (EEA1) further suggests the role of endocytosis in EGFR nuclear transport[42].

During classical cytoplasmic-nuclear transport, proteins containing NLS sequences form complexes with importin α/β that interact with nucleoporins within the nuclear pore complex (NPC) in order to transverse into the nucleus[48,49]. Lo et al. first verified that EGFR contains a functional NLS by generating an EGFR mutant where the NLS was mutated (^{645}RRR^{647} to AAA). This mutant, when expressed in EGFR-null CHO cells, was deficient in EGFR nuclear entry, verifying its role as a functional NLS[35]. A much more extensive analysis of putative NLSs in the EGFR and other HER family members identified a novel tripartite NLS containing three clusters of basic amino acids that are conserved near the juxtamembrane region of all HER family members. The NLS of EGFR (^{645}***RRR***HIV***RKR***TL***RR***^{657}) was capable of targeting cytoplasmic proteins into the nucleus. Current data suggests that this tripartite sequence is responsible for the nuclear localization of EGFR through association with importin β1 during internalization of the receptor[9,50]. Additionally, the deletion of this homologous region in the juxtamembrane region of HER2 also led to an inhibition of HER2 nuclear transport[51].

Further studies pioneered by Dr. M.C. Hung's group have evaluated the movement of EGFR from endocytic vesicles to the Golgi apparatus, endoplasmic reticulum, and finally to the nucleus. Initially, investigators found that cells stimulated with EGF redistributed EGFR to the Golgi and endoplasmic reticulum (ER) [50]. Blocking of retrograde translocation from the Golgi to the ER using the drug brefeldin A or dominant mutants of the small GTPase ADP ribosylation factor (ARF) resulted in the disassembly of the coat protein complex I (COPI) to the Golgi and dramatically impairing EGFR transport to the ER and the nucleus. From these studies, the authors concluded that nuclear transport of the EGFR is regulated by COPI-mediated vesicular trafficking from the Golgi to the ER[50]. Next, researchers identified that the Sec61 translocon was localized on the inner nuclear membrane (INM) and was essential for the movement of EGFR complexed with importin β1 from the INM into the nucleus[52]. Most recently, researchers identified that EGF-induced EGFR travels from late endosomes to the Golgi via a microtubule-dependent pathway that interacts with the microtubule motor protein dynein and the SNARE protein syntaxin 6[53]. The movement of HER2 to the nucleus has also been shown to follow a similar path to that of EGFR[51,54]. Collectively, the trafficking of EGFR and HER2 to the nucleus has been termed the Integral Trafficking from the ER to the Nuclear Envelope Transport pathway (**Figure 4**).

THE NUCLEAR HER FAMILY SIGNALING PATHWAY

Advances in HER family biology over the last decade have established that HER receptors can perform several unique functions within the nucleus. These pioneering

studies have been focused on nuclear EGFR, however, recent reports have identified that both nuclear HER2 and HER3 can play similar roles. Three major functions of nuclear EGFR have been identified (**Figure 5**). First, nuclear EGFR can function as a co-transcription factor independent of its kinase activity. Although it was shown in 1994 that a kinase dead EGFR could enhance transcriptional expression of the c-fos gene[55], it was not until 2001 that a landmark paper provided direct evidence that EGFR could associate with and regulate the cyclin D1 promoter[56]. Since these initial findings, nuclear EGFR has been shown to co-regulate inducible nitric oxide synthase (iNOS), B-Myb, cyclooxygenase-2 (COX-2), aurora Kinase A, c-Myc, breast cancer resistant protein (BCRP), and STAT1[35,57-62]. Since EGFR does not contain a DNA binding domain, several nuclear co-factors have been found to facilitate EGFR association with target gene promoters. These factors include mucin-1 (MUC1)[63], RNA helicase A (RHA)[64], STAT3[35,58,60,62], STAT5 [59], E2F1[57] and c-Myc[65]. Additionally, nuclear HER2 has been identified to regulate cyclin D1[66] and COX-2[67], and nuclear HER3 has been shown to regulate cyclin D1[68]. Interestingly, a nuclear C-terminal splice variant of HER3 has been shown to regulate Schwann cell development through the regulation of the ezrin promoter, a component of Schwann cell microvilli, which are critical for the formation of the nodes of Ranvier[11]. Most recently, a genome-wide screen performed by ChIP-sequence analysis of chromatin sequences associated with nuclear EGFR-VIII (a kinase domain mutant of EGFR commonly overexpressed in glioblastoma) indicated that nuclear EGFR-VIII can associate with 2,294 potential target sequences, demonstrating the existence of a biologically relevant EGFR-VIII transcriptome[65].

Alternatively, nuclear EGFR has been shown to play essential roles in promoting DNA replication and repair by functioning as a nuclear kinase. The first study identified that nuclear EGFR can phosphorylate proliferating cell nuclear antigen (PCNA) on tyrosine 211, thereby increasing PCNA stability and ultimately enhancing cellular proliferation[69]. Finally, EGFR was shown to enter the nucleus upon radiation treatment and interact with DNA-dependent protein kinase (DNA-PK) leading to repair of radiation-induced DNA double strand breaks[39,70]. Collectively, the functions of nuclear EGFR (with less known about nuclear HER2 and HER3) have been implicated in enhanced cancer cell growth and resistance to both molecular and conventional cancer therapeutics[40].

NUCLEAR HER RECEPTORS AND CORRELATION WITH CLINICAL OUTCOME

Since the identification of nuclear HER receptors, many studies have quantified their expression status in human tumors and relationship to prognosis. In 1994, two independent reports documented nuclear EGFR expression in bladder and cervical cancers using immunohistochemical (IHC) methods. In the first study, 31 % of 234 transitional cell bladder cancer tumor samples stained positive for nuclear EGFR expression, which was further correlated with increased tumor grade, mitotic frequency, and cellular proliferation[37]. In the second study, Tervahauta and colleagues reported that 37 % of cervical cancer biopsies stained positive for nuclear EGFR[71]. While these early studies were the first to quantitate nuclear EGFR in patient tumors, they did not examine the prognostic value of nuclear EGFR expression.

In 2005, Lo et al. analyzed 130 breast carcinoma tumor samples via IHC for the

levels of nuclear EGFR[34]. They found nuclear EGFR staining in 38 % of these tumor tissues, 7 % of which had high levels of expression. Importantly, they demonstrated that patients with high levels of nuclear EGFR had poor overall survival (OS) as compared to patients with undetectable nuclear EGFR staining (33.4±14.6 vs. 47.8±15.3 months; P=0.009). They also found a positive correlation between expression of nuclear EGFR and two markers for cell proliferation, Ki67 and cyclin D1. Hadžisejdić et al. validated these findings in a separate cohort of invasive ductal breast tumors, where they detected nuclear EGFR expression in 40 % of 113 tumor samples[72]. Nuclear EGFR expression was statistically correlated with tumor size (P=0.0005) and lymph node involvement (P=0.0288). Additionally, patients with high levels of nuclear EGFR demonstrated worse OS compared to those with undetectable nuclear EGFR (0.58±0.249 vs. 0.83±0.053 months; P=0.0003), with a 3.4 times greater mortality risk (hazard ratio = 3.402; P=0.0026). One recent study recently identified that 33.6 % of 273 primary invasive breast carcinomas stained positive for nuclear HER2 and that nuclear HER2 was a significant independent predictor of worse OS in patients with membrane HER2 staining (hazard ratio =3.153, p= 0.03)[73].

Xia et al. further examined the levels of nuclear EGFR in a cohort of 221 ovarian cancer patient tissues using IHC followed by analysis with the ACIS III, automated cellular imaging system[74]. A high level of nuclear EGFR staining was detected in 28 % of tumor tissues, while 23 % had low levels of nuclear EGFR. Additionally, high nuclear EGFR expressing tumors were significantly correlated to have worse OS as compared to non-nuclear EGFR expressing tumors (P<0.01).

Lo et al. additionally analyzed 37 cases of oral squamous carcinomas for nuclear

EGFR using standard IHC methods. They found that 24 % had nuclear EGFR in more than 5 % of the tumor cells[34]. Patients with high nuclear EGFR expression in this cohort demonstrated poor OS compared to patients with no/low levels of nuclear EGFR (39.1±24.5 vs. 63.3±15.6 months; P=0.03). In 2005, Psyrri et al. quantitated EGFR protein expression via IHC and automated image acquisition and analysis (AQUA) on a tissue microarray composed of 67 oropharyngeal carcinomas [36]. They found nuclear EGFR staining in 49 % of these tumor tissues. Researchers determined that 88 % of patients recorded to have either a partial or no response to first line chemotherapies were more likely to express high levels of nuclear EGFR (P=0.025). Kaplan-Meier survival analysis demonstrated that high nuclear EGFR patients had poor 5-year disease-free survival (19 %; 24.3 months) compared to those with low nuclear EGFR expression (44 %; 35.8 months; P=0.0386). Most recently, research from our laboratory has identified that 26% of 88 non-small cell lung cancer patient cohort stained positive for nuclear EGFR expression, which was significantly correlated with shorter OS (14.1 months vs. 23.4 months)[75].

Li et al. further demonstrated that 24 % of 104 cases of gallbladder carcinomas expressed nuclear EGFR[76]. They also found that nuclear-pEGFR^{Y1086} was overexpressed in 60 % of nuclear EGFR expressing cancers. Nuclear EGFR overexpression was also significantly associated with advanced stage cancer via American Joint Committee on Cancer (AJCC) standards (P=0.01) and vascular invasion (P=0.003).

Finally, nuclear HER3 expression has been quantified in 143 prostate cancer specimens and from 390 prostate cancer tumor cores on a tissue microarray.

Researchers found that nuclear HER3 staining was more frequent in hormone-refractory prostate tumors indicating that nuclear HER3 may play a role in prostate cancer disease progression[77]. Collectively, the inverse correlation of nuclear HER receptors with overall patient survival indicates their potential role in cancer formation and/or progression and thus provides strong rationale for the molecular targeting of these nuclear receptors.

THE ROLE OF NUCLEAR HER RECEPTORS IN RESISTANCE TO CANCER THERAPEUTICS

Intrinsic and acquired tumor cell resistance to both conventional and targeted cancer therapies remains one of the largest obstacles to overcome clinically. While nuclear HER receptors have been observed in cells of high proliferative origin, numerous reports describe increased nuclear EGFR levels in models of cancer resistance to different therapeutic regimes[70,78-81]. Unfortunately, the role of nuclear HER2 or HER3 in resistance to cancer therapeutics is unknown at this time. From our current knowledge, nuclear EGFR can enhance resistance by influencing DNA damage repair, DNA replication, and transcription of oncogenes[61,70,78-80]. Thus, nuclear EGFR is now emerging as a potent biomarker for response to numerous cancer therapies.

Nuclear EGFR and Radiation Resistance: Radiation therapy is one of the most common anti-cancer treatments used due to its ability to induce widespread DNA damage in tumor cells. Enhanced tumor cell DNA damage repair can lead to radiation resistance, a process that is mediated by DNA-PK[82]. In 1997, Schmidt-Ullrich and colleagues observed that radiation treatment of tumor cells led to EGFR activation and

internalization similar to growth factor stimulation of the EGFR[83]. Further research demonstrated that tumor cells could be radiosensitized upon inhibition of EGFR activation with cetuximab[84,85], suggesting that EGFR plays a role in promoting DNA damage repair pathways. Pioneering studies by Dittmann et al. demonstrated that EGFR and DNA-PK form a complex in the nucleus upon radiation treatment, and that this interaction led to enhanced DNA-PK activity and DNA repair[39,86]. Importantly, inhibition of nuclear EGFR localization led to the inactivation of DNA-PK, resulting in less DNA damage repair and increased radiation response[39,70].

To further understand the molecular requirements of nuclear EGFR transport, Dittmann et al. demonstrated that the phosphorylation of EGFR on tyrosine 654 was necessary for radiation induced nuclear transport and DNA damage repair[87,88]. Additional studies showed that nuclear EGFR participates in chromatin relaxation, a necessary step for recruitment of repair proteins to DNA double strand breaks[89]. Further, Liccardi et al. elaborated on these findings by demonstrating that EGFR mutants lacking nuclear localization (constitutively active EGFR L858R and EGFR lacking its NLS) had decreased repair of radiation induced DNA double strand breaks[78]. Collectively, this body of work supports the important role of nuclear EGFR in enhancing DNA-PK induced DNA damage repair upon treatment with radiation therapy.

Interestingly, two reports have identified two radioprotectors that enhance the nuclear transport of EGFR in tissues with wild-type p53. O-phospho-tyrosine and Bowman-Birk proteinase inhibitor were shown to induce nuclear EGFR localization and activation of DNA-PK in p53 wild-type cells, deeming these cells more resistant to radiation[88,90]. These two radioprotectors may protect normal tissues that have wild-type

p53 from the deleterious effects of radiation therapy. PKCε was also shown to play a role in the nuclear translocation of EGFR and O-phospho-tyrosine radioprotection, both of which were lost upon PKCε knockdown[91]. Overall, these studies support the role of nuclear EGFR in resistance to radiation therapy.

Most recently, a study identified that nuclear EGFR plays a key role in regulating the activity of an exoribonuclease termed polynucleotide phosphorylase (PNPase), which can function to degrade c-MYC mRNA in the cytoplasm[92]. Researchers show that radiation treatment of breast cancer cells promotes the nuclear association of EGFR and PNPase, and that this association was linked via DNA-PK phosphorylation of PNPase at Serine 776. The phosphorylation of PNPase at Serine 776 abolished its ribonuclease activity and led to the upregulation of c-MYC expression, thereby enhancing radioresistance. Upon inhibition of EGFR or DNA-PK activity, PNPase was no longer activated and c-MYC levels were downregulated, increasing radiosensitivity[92]. Overall, this study highlights a novel role of nuclear EGFR in the regulation of PNPase and augmentation of radiation response.

Nuclear EGFR and Cisplatin Resistance: Cisplatin is a mainstay chemotherapy used to treat a variety of cancers. Cisplatin elicits DNA damage through crosslinking DNA, preventing replication and cell division and thereby triggering apoptosis[93]. Treatment of tumor cells with cisplatin has also been shown to induce nuclear EGFR translocation much like radiation treatment[78,80]. In 2009, Hsu et al. demonstrated that wild-type EGFR stable cells were resistant to cisplatin and had enhanced DNA repair upon treatment, while EGFR deleted of its NLS exhibited hindered DNA repair capabilities and sensitivity[80]. Liccardi et al. further supported these findings by showing

that stable cell lines deficient in nuclear EGFR localization lacked DNA crosslinking repair mechanisms and association/activation of DNA-PK[78].

Nuclear EGFR and Anti-EGFR Therapy Resistance: Similar to radiation and chemotherapy, *in-vitro* models studying cancer cell resistance to both gefitinib and cetuximab have demonstrated that resistant cells often retain dependency on the EGFR for enhanced growth potential and express high levels of nuclear localized EGFR[61,79,94]. In the case of gefitinib resistance, nuclear EGFR was shown to function as a co-transcriptional activator for breast cancer resistant protein (BCRP/ABCG2), a plasma-membrane bound ATP dependent transporter that can extrude anti-cancer drugs from cells and thereby diminish their effects [61]. Authors hypothesize that this ATP dependent transporter may function to remove gefitinib from cells and thereby enhance resistance[61].

Cetuximab resistance has also been attributed to nuclear EGFR. Various researchers have demonstrated that cetuximab treatment can enhance the nuclear localization of EGFR[79,95,96], and that cell lines with intrinsic resistance to cetuximab contain high levels of nuclear EGFR[79]. In the setting of acquired resistance to cetuximab, the Wheeler laboratory demonstrated that resistant cells have enhanced nuclear EGFR levels, which were attributed to increases in Src Family Kinase (SFK) activity[79,94,97]. Inhibition of SFKs with the small molecule inhibitor dasatinib decreased nuclear EGFR and enhanced plasma membrane bound EGFR levels[79]. Importantly, dasatinib treatment sensitized resistant cells to cetuximab[79]. These findings were further validated via the use of a SV40-NLS sequence tagged EGFR, which enhanced cetuximab resistance in sensitive parental cells[79]. Collectively, these studies

demonstrate that nuclear EGFR plays a role in resistance to both gefitinib and cetuximab therapies.

TARGETING NUCLEAR HER RECEPTORS IN CANCER

The current body of work on nuclear HER family function and resistance to anti-cancer therapeutics has been predominately focused on nuclear EGFR. These studies suggest that targeting nuclear EGFR may be a powerful anti-cancer treatment option. Over the past decade, many studies have identified specific proteins and post-translational modifications of EGFR necessary for its nuclear translocation and/or function, and thus provide insight on how to therapeutically target nuclear EGFR in cancer cells (**Figure 6**).

Targeting nuclear EGFR with anti-EGFR therapies: In 2009, Kim et al. demonstrated that the small molecule EGFR inhibitor lapatinib could inhibit EGF induced nuclear EGFR translocation in two breast cancer cell lines; however basal levels of nuclear EGFR were not changed[98]. While this study provided evidence that anti-EGFR TKIs may prevent nuclear EGFR translocation, suggesting that the kinase activity of EGFR is necessary for its nuclear trafficking, the majority of current research suggests that anti-EGFR therapeutics actually enhance EGFR endocytosis and thereby promote its nuclear translocation[61,79,95,99,100]. Additionally, Weihua et al. further showed that a kinase-dead EGFR is capable of inhibiting autophagic cell death in cancer cell lines, demonstrating that some of EGFR's induced tumorigenic functions may be independent of its kinase activity[101]. Researchers have further demonstrated that a kinase-dead EGFR can undergo endocytosis[102], a critical step for EGFR nuclear

trafficking. Collectively, these data suggest that nuclear EGFR is not accurately targeted by kinase inhibiting anti-EGFR therapeutics and that nuclear EGFR translocation and function may be independent of kinase activity.

Targeting nuclear EGFR via AKT Inhibition: The initial studies aimed at targeting nuclear EGFR were focused in cell lines that demonstrated resistance to both cetuximab and gefitinib therapies [61,79]. These cell lines were established by treating drug sensitive cell lines with increasing amounts of drug over several months until resistance was observed. Cell lines in both models of resistance demonstrated enhanced nuclear localization of the EGFR as compared to sensitive parental lines[61,79]. In the gefitinib resistant setting, Huang et al. demonstrated that the activity of AKT was enhanced, and that EGFR was specifically phosphorylated by AKT on serine 229 to promote EGFR nuclear translocation[61]. Importantly, the overexpression of EGFR mutated at serine 229 or the use of an AKT inhibitor rendered resistant cells more sensitive to gefitinib. Collectively, these data suggest that AKT inhibition may successfully inhibit nuclear EGFR transport and thereby sensitize cells to anti-EGFR therapies such as gefitinib[61].

Targeting nuclear EGFR via Src Family Kinase Inhibition: In the setting of acquired cetuximab resistance in NSCLC, work the Wheeler laboratory has indicated that cetuximab resistant cells have both enhanced nuclear EGFR and SFK activity[79,97]. These initial studies demonstrated that SFK inhibition of nuclear EGFR transport could enhance sensitivity of cetuximab resistant cell clones to cetuximab therapy[79].

Targeting nuclear EGFR via the selective COX-2 Inhibitor Celecoxib: The COX-2 inhibitor Celecoxib has demonstrated radiosensitizing effects in various tumors[103,104].

Interestingly, anti-tumor effects of Celecoxib treatment have been observed in cell lines that did not express COX-2 to a high degree[105,106]. To elucidate the mechanism of Celecoxib radiosensitization, Dittmann et al. demonstrated that Celecoxib could inhibit both basal and radiation-induced nuclear EGFR translocation in various cancer cell lines[107]. Celecoxib also inhibited radiation-induced phosphorylation of DNA-PK at threonine 2609 only in cell lines that were radiosensitized with this drug. Overall, this study highlights a COX-2 independent mechanism of Celecoxib radiosensitization through the direct inhibition of nuclear EGFR translocation [107]. Thus, this inhibitor may prove to be useful for treatment of nuclear EGFR expressing tumors, even for tumors lacking COX-2 expression.

Targeting nuclear EGFR via PCNA inhibition: Another method of targeting nuclear EGFR is to directly inhibit the activity of its effector molecules. One main effector molecule of nuclear EGFR is PCNA[69]. In a recent study by Yu et al. researchers demonstrate that treatment of triple negative breast cancer (TNBC) cells with an anti-PCNA peptide (targeting Tyrosine 211) can inhibit growth of cells *in-vitro* and *in-vivo* mouse models[108]. Additionally, researchers established both geftinib and erlotinib resistant cell lines, both of which contained enhanced nuclear EGFR and activated PCNA levels. Treatment of these resistant cell lines with the anti-PCNA peptide sensitized all cell lines to their respective therapies. These studies suggest that the inhibition of the nuclear EGFR effector molecule PCNA may enhance the dependency of cells on classical EGFR signaling pathways and thereby re-sensitize them to EGFR inhibitors.

Prospective Targets of Nuclear EGFR Translocation and/or Function: There have been several other proteins identified to associate with nuclear EGFR to influence its nuclear function and/or nuclear trafficking. The transmembrane protein MUC1[63] and RHA[64] have been shown to be instrumental for EGFR association with target gene promoters in the nucleus. Additionally, the transcriptional co-factor Tat interacting protein (TIP3) has been shown to suppress nuclear EGFR translocation, while conversely its loss enhances nuclear EGFR translocation and function[109]. The latent membrane protein 1 (LMP1) has also been shown to promote the interaction of nuclear EGFR with transcriptional intermediary factor 2 (TIF2) to upregulate cyclin D1 expression[110]. Finally, Wanner et al. demonstrated that PKCε could phosphorylate EGFR on threonine 654 to induce EGFR's nuclear translocation in response to radiation therapy[91]. PKCε inhibitors such as midostaurin (PKC 412) and enzastaurin (LY317615) have been available since the early 2000s and have shown radiosensitizing capabilities, however, their ability to target nuclear EGFR has yet to be examined[111]. Collectively, the proteins MUC1, RHA, TIP2, LMP1, and PKCε may be potential future targets for the inhibition of both nuclear EGFR translocation and function.

QUESTIONS FORMING THE BASIS OF THIS THESIS

While met with initial skepticism, the field of nuclear HER receptors is now flourishing, with over 50 publications on the role of nuclear EGFR in cancer alone. However, this field is still in its infancy and thus the focus of this thesis was to answer three main questions to bridge the gap in our knowledge of how HER receptors translocate and function in the nucleus with the overall goal of targeting nuclear HERs in

hopes of developing novel therapeutics regimes that can be translated to the clinic. First, while several steps of EGFR trafficking from the membrane to the nucleus have been described, the specific phosphorylation events on EGFR necessary to stimulate its nuclear trafficking were still ill defined. Thus, the first aim of this thesis was to identify critical tyrosine sites on the EGFR that may initiate nuclear EGFR trafficking (**Chapter II**). Next, pioneering studies have indicated a role for nuclear EGFR in both resistance to conventional cancer therapeutics as well as anti-EGFR agents. These studies indicate that nuclear EGFR may propagate resistance and thus be a molecular target. Accordingly, the second aim of this thesis was to identify if cancers that are intrinsically resistant to cetuximab, such as triple-negative breast cancers (TNBCs), can be sensitized through the targeting of nuclear EGFR translocation (**Chapter III**). Finally, while HER receptors function as co-transcription factors in the nucleus, the specific domains necessary for their transactivation potential were ill defined. Therefore, the third aim of this thesis was to identify the specific transactivation domains on a nuclear HER receptor (focus was on nuclear HER3) that enable the receptor to function as a co-transcription factor (**Chapter IV**). Collectively, the studies presented in this thesis broaden the knowledge of the nuclear HER family field by identifying novel processes necessary for nuclear HER family trafficking, targeting, and function.

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Figure 1: Domain Structure of the HER family. The HER family consists of four closely related transmembrane receptor tyrosine kinases. EGFR, HER2, and HER3 are aberrantly overexpressed and/or activated in several human cancers, while the role of HER4 (not shown) in cancer is less clear[26,27]. Ligand binding (excluding HER2), receptor dimerization, intrinsic tyrosine kinase activation, and auto/trans phosphorylation of tyrosine residues on each receptor's C-terminal tail initiates a multi-faceted signaling network. Here, specific amino acid residues known to play a role in the initiation of receptor signaling pathways are shown, in addition to the identified NLS sequences on each HER receptor.

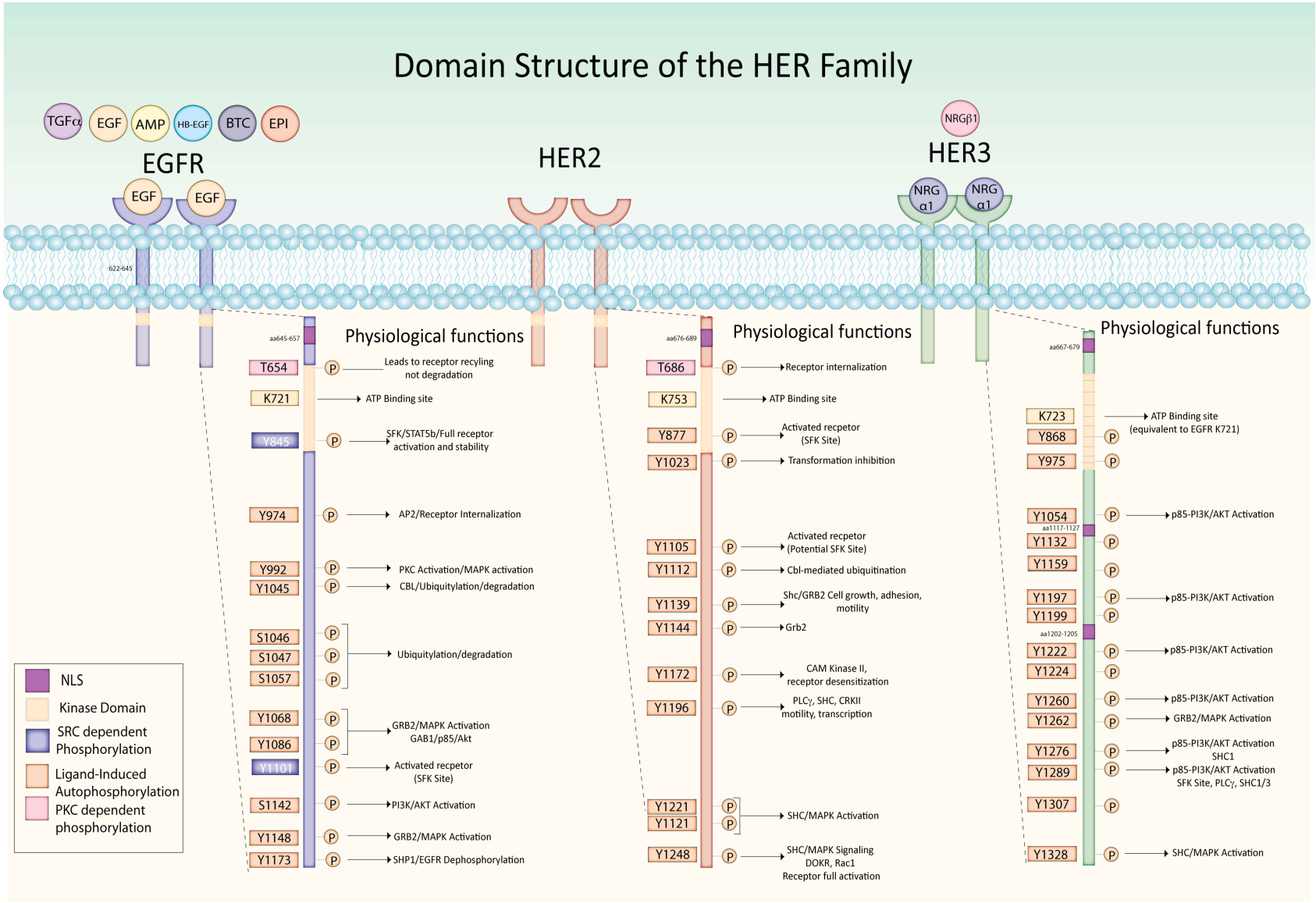

Domain Structure of the HER Family
TGFα
EGF
AMP
HB-EGF
BTC
EPI
EGFR
EGF
EGF
HER2
NRGβ1
HER3
NRG α1
NRG α1
622-645
aa645-657
Physiological functions
T654
Leads to receptor recyling not degradation
K721
ATP Binding site
Y845
SFK/STAT5b/Full receptor activation and stability
Y974
AP2/Receptor Internalization
Y992
PKC Activation/MAPK activation
Y1045
CBL/Ubiquitylation/degradation
S1046
S1047
S1057
Ubiquitylation/degradation
Y1068
Y1086
GRB2/MAPK Activation GAB1/p85/Akt
Y1101
Activated recpetor (SFK Site)
S1142
PI3K/AKT Activation
Y1148
GRB2/MAPK Activation
Y1173
SHP1/EGFR Dephosphorylation
aa676-689
Physiological functions
T686
Receptor internalization
K753
ATP Binding site
Y877
Activated recpetor (SFK Site)
Y1023
Transformation inhibition
Y1105
Activated recpetor (Potential SFK Site)
Y1112
Cbl-mediated ubiquitination
Y1139
Shc/GRB2 Cell growth, adhesion, motility
Y1144
Grb2
Y1172
CAM Kinase II, receptor desensitization
Y1196
PLCγ, SHC, CRKII motility, transcription
Y1221
Y1121
SHC/MAPK Activation
Y1248
SHC/MAPK Signaling DOKR, Rac1 Receptor full activation
aa667-679
Physiological functions
K723
ATP Binding site (equivalent to EGFR K721)
Y868
Y975
Y1054
p85-PI3K/AKT Activation
aa1117-1127
Y1132
Y1159
Y1197
p85-PI3K/AKT Activation
Y1199
aa1202-1205
Y1222
p85-PI3K/AKT Activation
Y1224
Y1260
p85-PI3K/AKT Activation
Y1262
GRB2/MAPK Activation
Y1276
p85-PI3K/AKT Activation SHC1
Y1289
p85-PI3K/AKT Activation SFK Site, PLCγ, SHC1/3
Y1307
Y1328
SHC/MAPK Activation
NLS
Kinase Domain
SRC dependent Phosphorylation
Ligand-Induced Autophosphorylation
PKC dependent phosphorylation

Figure 2: Classical EGFR Signaling. Several key signaling pathways are regulated by EGFR. When unregulated, these signaling pathways can lead to the formation and progression of cancer. Two main signaling pathways emanating from the EGFR are the PI3K/AKT and RAS/RAF/MEK/ERK pathways. In addition to EGFR, HER2 and HER3 have also been shown to signal down these pathways leading to a diversification of signal intensity and longevity. Ultimately, these pathways can augment cellular proliferation, survival, migration, differentiation, angiogenesis, and metabolism[12,26-28]. Thus, the aberrant overexpression or hyperactivation of HER receptors can result in tumor formation and cancer. While EGFR and HER2 activate PI3K/AKT signaling indirectly through RAS, HER3 can activate PI3K directly by associating with PI3K's p85 regulatory subunit. Grb2: Growth factor receptor-bound protein 2, adaptor protein that recruits SOS to activated HER family receptors. SOS: son of sevenless homolog 1, guanine nucleotide exchange factor responsible for facilitating GTP binding to the small GTPase RAS.

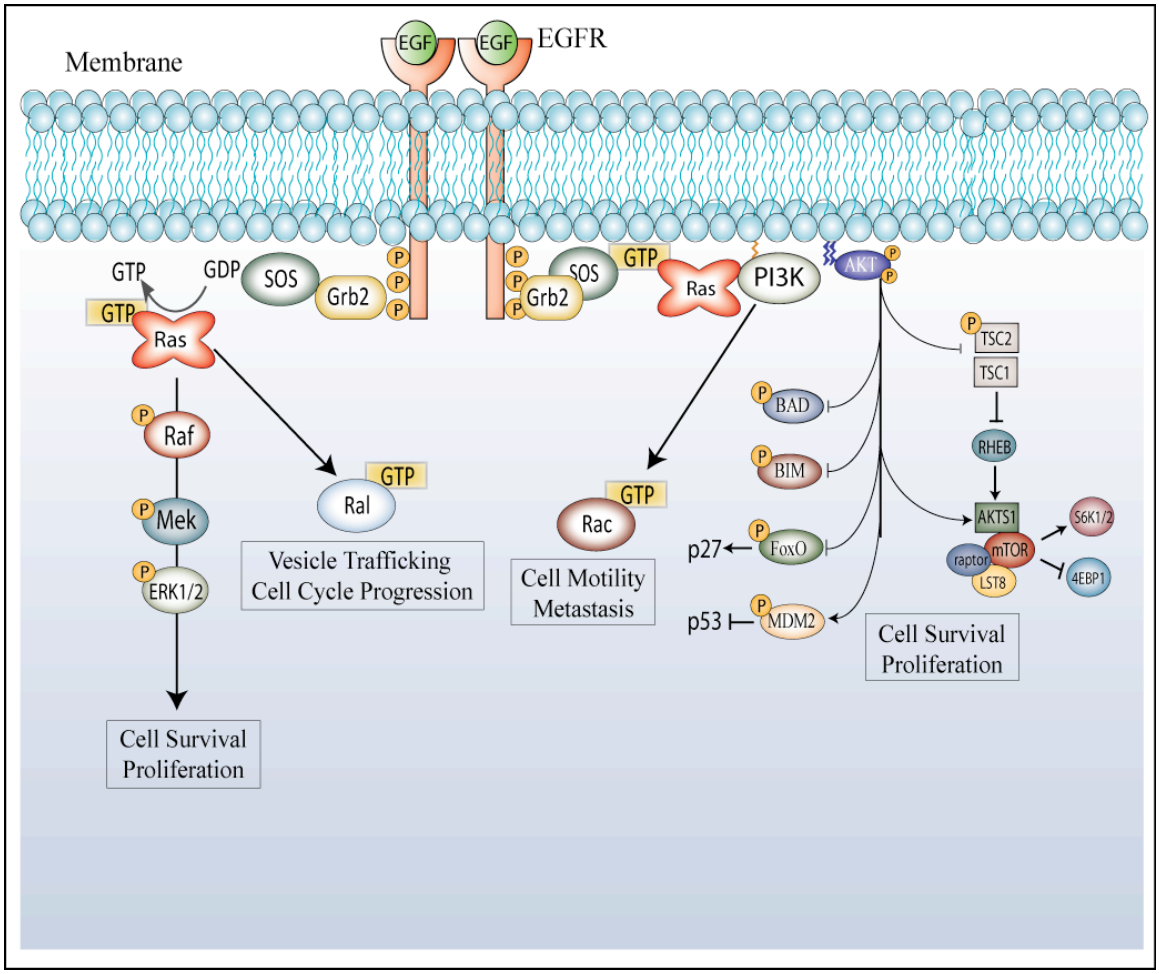

Membrane
EGF
EGF
EGFR
GTP
GDP
SOS
Grb2
GTP
Ras
Raf
Mek
ERK1/2
Cell Survival
Proliferation
GTP
Ral
Vesicle Trafficking
Cell Cycle Progression
SOS
GTP
Grb2
Ras
PI3K
AKT
GTP
Rac
Cell Motility
Metastasis
BAD
BIM
p27
FoxO
p53
MDM2
TSC2
TSC1
RHEB
AKTS1
mTOR
raptor
LST8
S6K1/2
4EBP1
Cell Survival
Proliferation

Figure 3: FDA approved molecular agents targeting the EGFR. Targeting EGFR has been intensely pursued over the last three decades as a treatment strategy for cancer. From these efforts two fundamental approaches have proven useful. One approach involves the use of small molecule tyrosine kinase inhibitors (TKIs) that bind to the ATP-binding site in the tyrosine kinase domain (TKD) of EGFR. The use of TKIs prevents the activation of EGFR's intrinsic tyrosine kinase and thereby prevents autophosphorylation and downstream signaling. The second approach is the use of monoclonal antibodies that can bind to specific extracellular regions of the EGFR to block natural ligand binding, receptor dimerization, and promote receptor internalization and degradation[112]. Additionally, mABs can effectively recruit immune cells to tumor cells in a process termed antibody-dependent cellular cytotoxicity (ADCC). Cetuximab has been the most successful of all anti-EGFR mABs, and is now FDA approved for the treatment of patients with head and neck squamous cell carcinoma (HNSCC)[113,114,115] and metastatic colorectal cancer (mCRC)[116-118].

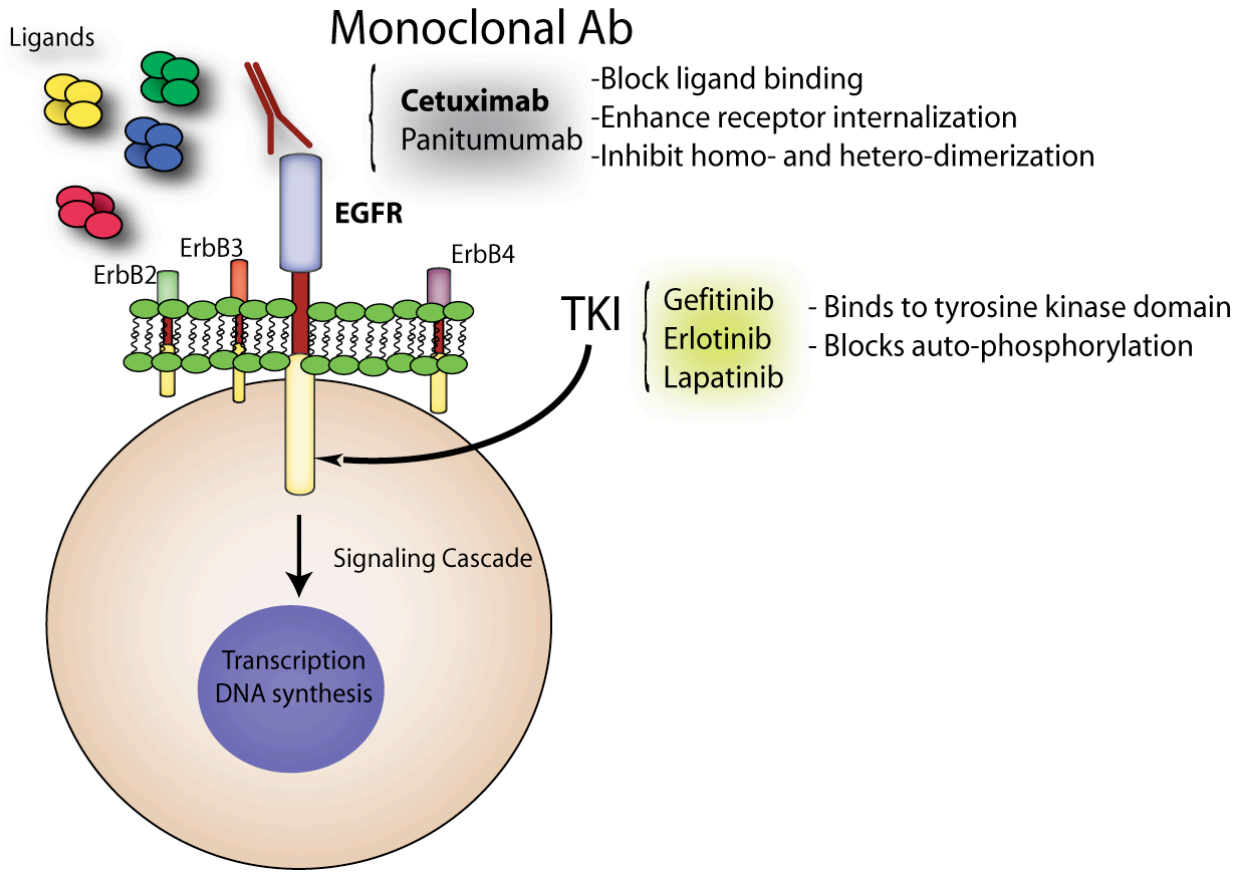

Ligands
Monoclonal Ab
Cetuximab
Panitumumab
-Block ligand binding
-Enhance receptor internalization
-Inhibit homo- and hetero-dimerization
EGFR
ErbB2
ErbB3
ErbB4
TKI
Gefitinib
Erlotinib
Lapatinib
- Binds to tyrosine kinase domain
- Blocks auto-phosphorylation
Signaling Cascade
Transcription
DNA synthesis

Figure 4: EGFR trafficking from the plasma membrane to the nucleus. Ligand activation of the EGFR stimulates receptor dimerization and internalization to endocytic vesicles (EV)[42]. EGFR localized in the endosome moves along microtubules powered by the motor protein dynein[53]. EGFR localized in the late endosome fuses with the Golgi apparatus via interaction with SNARE protein syntaxin-6[53]. EGFR then undergoes COPI-mediated retrograde translocation through the Golgi apparatus to the endoplasmic reticulum (ER)[50]. At the ER, EGFR moves from the outer nuclear membrane (ONM) to the inner nuclear membrane (INM) via interaction between importin β1 and the nuclear pore complex (NPC)[42,51]. In the INM, EGFR interacts with Sec61 for removal from the membrane and release into the nucleus[52]. The HER2 receptor has also been found to follow a similar route to the nucleus[51,54]. Collectively, nuclear HER family trafficking has been termed the integral trafficking from the ER to the nuclear envelope transport pathway[54].

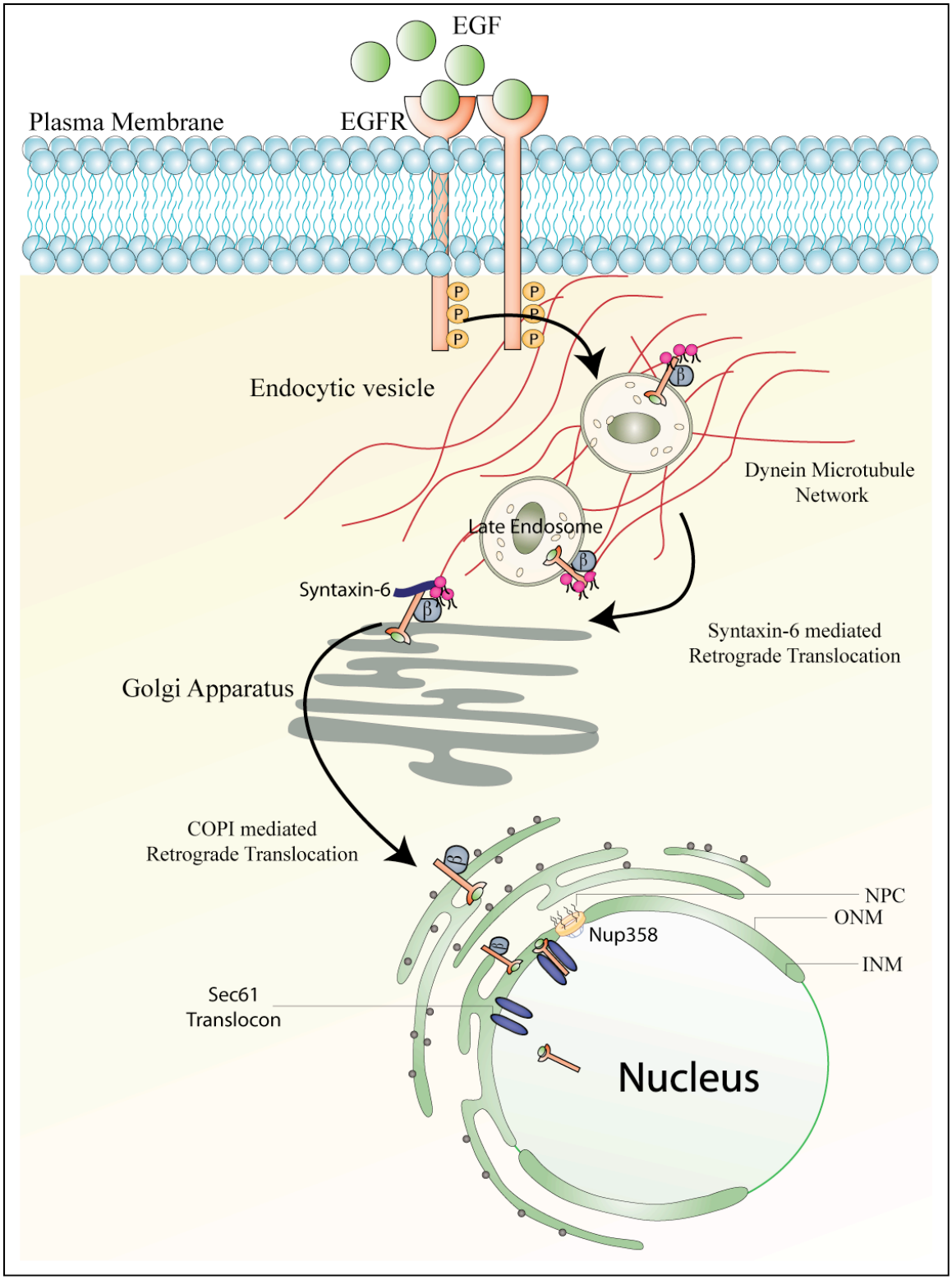
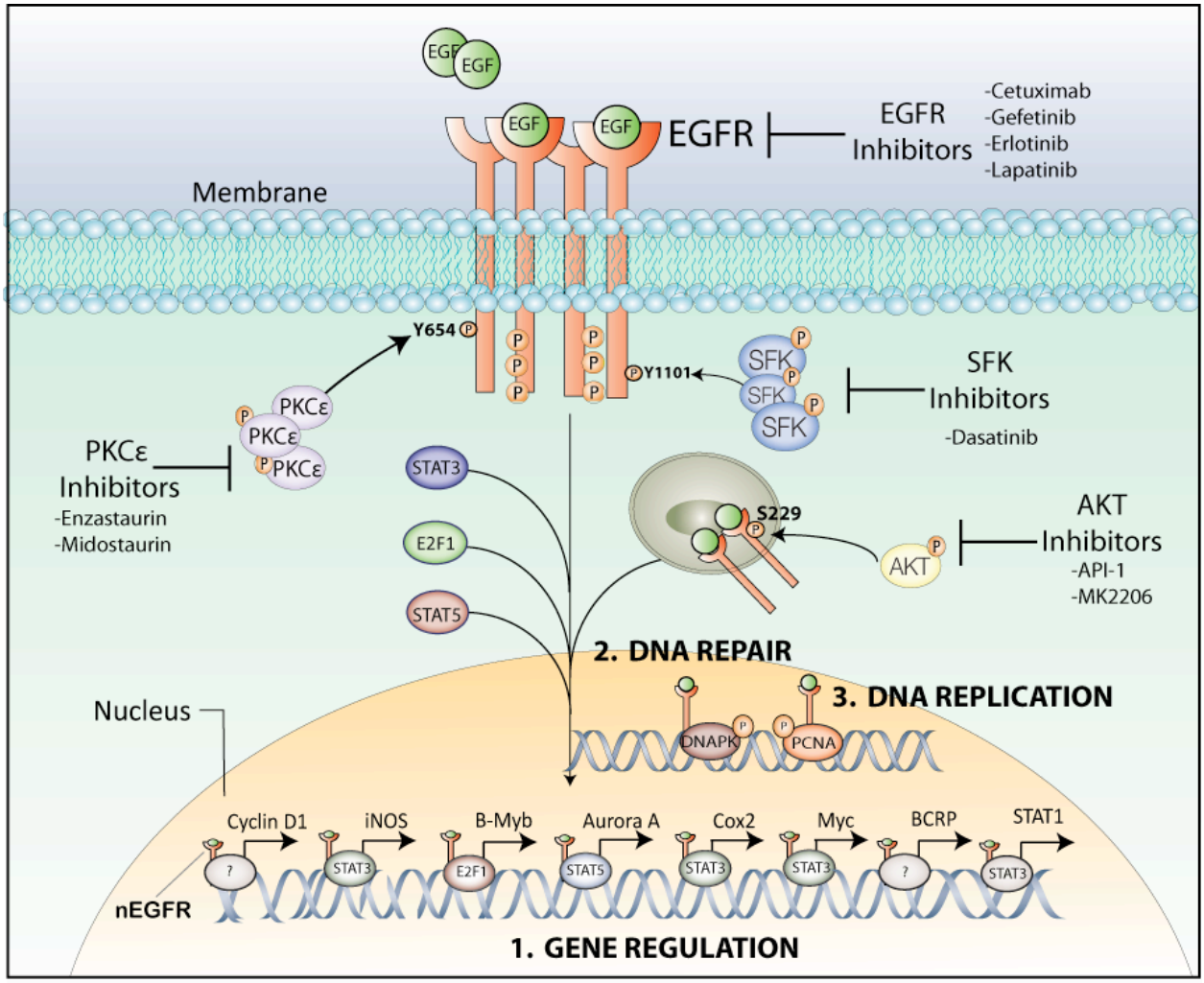

EGF
Plasma Membrane
EGFR
P
Endocytic vesicle
Dynein Microtubule Network
Late Endosome
Syntaxin-6
Syntaxin-6 mediated Retrograde Translocation
Golgi Apparatus
COPI mediated Retrograde Translocation
NPC
ONM
Nup358
INM
Sec61 Translocon
Nucleus

Figure 5: **Targeting the Nuclear EGFR Signaling Network.** EGFR has been consistently detected in the nuclei of cancer cells, primary tumor specimens and highly proliferative tissues. There have been three identified functions of nuclear EGFR: **1)** Regulation of gene transcription, including cyclin D1[32], iNOS[35], B-Myb[57], Aurora Kinase A[59], COX2[58], Myc[60], BCRP[81] and STAT1[62], **2)** promotion of DNA replication through the phosphorylation and stabilization of PCNA[69,108], and **3)** phosphorylation of DNA-PK to promote DNA repair[39]. Collectively, these functions have been inversely correlated with patient survival, resistance to conventional cancer therapeutics and anti-EGFR agents, and enhanced *in vitro* and *in vivo* tumor growth[40]. Research over the last decade has identified several intracellular kinases (SFKs[79], AKT[81], and PKCε[87]) and specific phosphorylations of the EGFR (phosphorylation at serine 229[81] and threonine 654[87]) that mediate EGFR nuclear transport in response to different stimuli. Nuclear EGFR functions may be effectively inhibited through drugs that target these intracellular kinases, and thereby sensitize nuclear EGFR expressing cancer cells to both conventional and anti-EGFR therapeutics.


EGF
EGF
EGF
EGF
EGFR
EGFR Inhibitors
-Cetuximab
-Gefetinib
-Erlotinib
-Lapatinib
Membrane
Y654
Y1101
SFK
SFK
SFK
SFK Inhibitors
-Dasatinib
PKCε
PKCε
PKCε
PKCε Inhibitors
-Enzastaurin
-Midostaurin
STAT3
E2F1
STAT5
S229
AKT
AKT Inhibitors
-API-1
-MK2206
2. DNA REPAIR
3. DNA REPLICATION
Nucleus
DNAPK
PCNA
Cyclin D1
iNOS
B-Myb
Aurora A
Cox2
Myc
BCRP
STAT1
nEGFR
1. GENE REGULATION

Chapter II

Src Family Kinase Activation of EGFR on Tyrosine 1101 is Critical for its Nuclear Translocation

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ABSTRACT

The epidermal growth factor receptor (EGFR) is a central regulator of tumor progression in human cancers. Cetuximab is an anti-EGFR antibody that has been approved by the FDA for the treatment of patients with head and neck squamous cell carcinoma (HNSCC) and metastatic colorectal cancer (mCRC). Previously we investigated mechanisms of acquired resistance to cetuximab using a model derived from the non-small cell lung cancer line NCI-H226. We demonstrated that cetuximab-resistant clones (Ctx^R) had increased nuclear localization of the EGFR. This process was mediated by Src family kinases (SFK), and nEGFR played a role in resistance to cetuximab. To better understand SFK mediated mechanisms of EGFR nuclear translocation, we aimed to elucidate which SFK member(s) controlled this process as well as the EGFR tyrosine residues that were involved. Analyses of mRNA and protein expression indicated up-regulation of the SFK members Yes and Lyn in all Ctx^R clones. Further, immunoprecipitation analysis revealed that EGFR interacts with Yes and Lyn in Ctx^R clones, but not in cetuximab-sensitive (Ctx^S) parental cells. Using RNAi interference, we found that knockdown of either Yes or Lyn led to loss of EGFR translocation to the nucleus. Conversely, overexpression of Yes or Lyn in low nEGFR expressing Ctx^S parental cells led to increased nEGFR. To elucidate if Yes and Lyn are the predominate SFK members that influence nEGFR translocation, analysis of stable cell lines overexpressing each SFK in MCF-7 cells indicated that all SFKs could promote nEGFR translocation. Chromatin immunoprecipitation (ChIP) assays confirmed nEGFR complexes

associated with the promoter of the known EGFR target genes B-Myb and iNOS. In addition, all Ctx^R clones tested exhibited up-regulation of B-Myb and iNOS at the mRNA and protein levels. siRNAs directed at Yes or Lyn led to decreased binding of EGFR complexes to the B-Myb and iNOS promoters based on ChIP analyses. Further, SFKs have been shown to phosphorylate EGFR on tyrosines 845 and 1101 (Y845 and Y1101) and mutation of Y1101, but not Y845, impaired nuclear entry of the EGFR. Taken together, our findings demonstrate that Yes and Lyn phosphorylate EGFR at Y1101 which influences EGFR nuclear translocation in this model of cetuximab resistance.

INTRODUCTION

Activation of the epidermal growth factor receptor (EGFR), a receptor tyrosine kinase (RTK), provides cells with potent growth and survival signals that enable tumors to manifest[1-3]. Aberrant expression or activity of the EGFR is identified as a major etiological factor in many human epithelial cancers including colorectal cancer (CRC), head and neck squamous cell carcinoma (HNSCC), non-small cell lung cancer (NSCLC) and brain cancer[2,4,5]. In the classical EGFR signaling pathway ligand binding to the EGFR allows for receptor homo- or hetero-dimerization at the plasma membrane. This interaction activates each receptor's tyrosine kinase domain and induces autophosphorylation of each dimer's cytoplasmic tail. The phosphorylated cytoplasmic tail of the EGFR serves as docking sites for numerous proteins that initiates key oncogenic pathways including the RAS/RAF/MEK/ERK and phosphatidylinositol 3-kinase (PI3K)-Akt pathways; however, the activation of src family tyrosine kinases (SFKs), Phospholipase C-gamma (PLCγ), Protein kinase C (PKC) and Signal Transducers and Activators of Transcription (STAT) proteins have also been documented[1,6].

In addition to the classical signaling pathways initiated by the EGFR at the cell surface, there is now an emerging novel signaling pathway influenced by EGFR located in the nucleus. The full-length EGFR can be shuttled from the plasma membrane to the nucleus in a series of well-defined steps[7-9]. These events include receptor internalization to the early endosome followed by COPI-mediated retrograde trafficking to the Golgi and the endoplasmic reticulum (ER)

where the tripartite nuclear localization sequence of the EGFR interacts with importinb1[10,11]. Once in the ER the EGFR-importinb1 complex moves to the outer nuclear membrane where importinb1 interacts with Nup proteins in the nuclear pore channel to shuttle the EGFR-importinb1 complex to the inner nuclear membrane. Here the complex interacts with the Sec61b translocon to be released from the membrane into the nucleus[12,13].

Within the nucleus, EGFR serves as a transcriptional co-activator for a series of tumor promoting genes including cyclin D1, COX2, Aurora Kinase A, c-Myc, inducible nitric oxide synthase (iNOS), B-Myb, Breast Cancer Related Protein (BCRP) and GRP78[14-21]. Additionally, nEGFR can phosphorylate and stabilize the proliferating cell nuclear antigen (PCNA) at the replication fork of the dividing cell[22], and activate DNA-PK to enhance DNA repair [23].

High levels of nuclear EGFR (nEGFR) correlate with poor clinical outcome in breast cancer, oropharyngeal squamous cell cancer, ovarian cancer, and gallbladder cancer [24-28]. NEGFR also contributes to cancer cells resistance to cetuximab [29], gefitinib [20], cisplatin and radiation therapy[30-33]. Taken together these pieces of evidence suggest that nEGFR plays a role in the promotion of cancer and provides a rationale for studying the mechanisms of EGFR nuclear translocation in order to target the nuclear functions of the EGFR.

It is well established that SFKs are necessary for full activation of the EGFR[34,35]. Src kinase is the prototype member of this family of non-RTKs that include Yes, Fyn, Lyn, Lck, Hck Fgr, Blk and Yrk[36,37]. These SFKs mediate mitogenic signals from a variety of RTKs [38,39]. It has been observed that SFKs

can phosphorylate EGFR at both tyrosine 845 (Y845) and tyrosine 1101 (Y1101). EGFR Y845 is located in the activation loop of the kinase domain that is highly conserved among other RTKs. Phosphorylation of EGFR Y845 appears to be critical for EGFR-mediated mitogenesis, and is critical for the phosphorylation and activation of the STAT5b transcription factor[34,40,41]. The second known Src-mediated phosphorylation site is Y1101, which lies within the carboxyl-terminal region of the EGFR; however the function of Y1101 has not been fully elucidated[34]. Oncogenic cooperation between Src and EGFR has been well established in breast cancer[34,42], glioblastoma[43], HNSCC and NSCLC[44-47].

We established six clonal Ctx^R variants of the NCI-H226 NSCLC line[29,48,49]. In previous reports we found that Ctx^R clones had a dramatic increase in nEGFR localization, in addition to having increased SFK activity[29,44]. Further, we reported that the SFK inhibitor dasatinib (BMS-354825, Sprycel™) could 1) block SFK activation, 2) decrease nEGFR translocation, 3) increase plasma membrane levels of the EGFR, and 4) re-sensitize Ctx^R cell lines to cetuximab. Collectively these findings suggest that SFKs play a crucial role in nuclear translocation of the EGFR in this model of cetuximab-resistance. However, the specific SFKs involved in the mediation of EGFR nuclear translocation and how they mediate this process are unknown.

In the current study we demonstrate that Ctx^R clones had increased expression of the Yes and Lyn, and that these specific SFKs were strongly associated with EGFR in Ctx^R clones as compared to the Ctx^S parental cell line. Depletion of either Yes or Lyn kinase decreased EGFR nuclear translocation,

and reduced phosphorylation at Y845 and Y1101 of the EGFR. Reciprocally, overexpression of Yes or Lyn increased EGFR nuclear translocation in Ctx^S parental cell line. To elucidate if Yes and Lyn are the predominate SFK members that influence nEGFR translocation, analysis of stable cell lines overexpressing each SFK indicated that all SFKs could promote nEGFR translocation, suggesting a global role of SFKs in the regulation of nEGFR trafficking. Furthermore, mutation of Y1101 impaired nuclear translocation of EGFR. Collectively these data suggest that Yes, Lyn and EGFR Y1101 are involved in EGFR nuclear translocation in this model of acquired resistance to cetuximab.

MATERIALS AND METHODS

Cell lines

The human NSCLC line NCI-H226, the human breast cancer line MCF-7 and Chinese hamster ovary K1 (CHO-K1) cells were purchased from ATCC (Manassas, VA, USA). The cells were maintained in 10% fetal bovine serum (FBS) in RPMI-1640 for H226, DMEM/F12K for MCF-7 and F12K for CHO-K1 (Mediatech Inc., Manassas, VA, USA) with 1% penicillin and streptomycin. The development of cells with acquired resistance to cetuximab has been previously described[48].

Plasmid constructs and transfection

EGFR wild-type (WT), Y845F and Y1101F mutants, were kindly provided from Dr. Julie Boerner (Wayne State University School of Medicine, Karmanos Cancer Institute, MI). The presence of 845F and 1101F mutations were confirmed by DNA sequencing. For transient transfections, MCF-7 cells were transfected with plasmid DNA for each construct or pcDNA3.1 vector using Lipofectamine LTX and Opti-MEM I (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. Either 24 hr (for RNA) or 48 hr (for protein) after transfection, EGF (100 ng/ml, Millipore, Billerica, MA, USA) was added to the plates for 45 min. Cells were collected, isolated RNA or fractionated and screened for their EGFR expression levels by quantitative PCR (qPCR) or immunoblotting as described below. For siRNAs, Ctx^R cells (HC1, HC4 and HC8) were transiently transfected with siYES (ON-TARGETplus SMARTpool YES1: L-003184-00, Dharmacon, Lafayette, CO, USA) or siLYN (ON-TARGETplus

SMARTpool LYN: L-003153-00) using Lipofectamine RNAiMAX according to the manufacture's instructions (Invitrogen). The non-targeting siRNA (ON-TARGETplus Non-targeting Pool, D-001810-10) was obtained from Dharmacon as a control. Cells were then lysed for analysis of protein knockdown by immunoblotting 72hr after siRNA transfection. Wild-type human YES (source ID: 5260751) and LYN (source ID: 8992174) cDNAs were purchased from Open Biosystems (Lafayette, CO, USA) and cloned into the NOTI/PACI restriction sites of the pQCXIP expression vector (Clontech, Mountain View, CA, USA). YES-PQCXIP, LYN-PQCXIP, or PQXCIP vector were transiently transfected into CHO-K1 cells with using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. Forty-eight hours after transfection, cells were collected and lysed. HP parental cells were transiently transfected with the same constructs using Lipofectamine LTX (Invitrogen) according to the manufacturer's recommendations. Forty-eight hours after transfection, cells were collected and fractionated for nuclear protein. NEGFR expression levels were then detected via immunoblot analysis.

Compounds

Dasatinib (BMS-354825, Sprycel^{TM}) was generously provided by Bristol-Myers Squibb (New York, NY, USA).

Antibodies

All antibodies were purchased from commercial sources as indicated below: EGFR, B-Myb, Actin, Histone H3, HRP-conjugated goat-anti-rabbit IgG, goat-anti-mouse IgG and donkey-anti-goat IgG were obtained from Santa Cruz

Biotechnology Inc., (Santa Cruz, CA, USA). Total SRC, YES, LYN, pSFK (Y419) and normal mouse IgG were obtained from Cell Signaling Technology (Beverly, MA, USA). pEGFR (Y1101) was purchased by Abcam (Cambridge, MA, USA). Anti-mouse EGFR and pEGFR (Y845) were purchased from Invitrogen. Polyclonal iNOS was obtained from BD Biosciences (San Jose, CA, USA). a-tubulin was purchased from Calbiochem (San Diego, CA, USA).

Cellular fractionation and Immunoblotting analysis

Cells were swelled in cytoplasmic lysis buffer (20 mM HEPES, pH 7.0, 10 mM KCl, 2 mM $MgCl_2$, 0.5% NP40, 1 mM Na_3VO_4, 1 mM PMSF, 1mM BGP, 10 μg/ml of leupeptin and aprotinin) for 10 min on ice and homogenized by 20-30 strokes in a tightly fitting dounce homogenizer. The homogenate was centrifuged at 1,500 g for 5 min at 4°C to sediment the nuclei. The supernatant was then centrifuged at 15,000 g for 10 min at 4°C, and the resulting supernatant formed the non-nuclear fraction. The nuclear pellet was washed three times in cytoplasmic lysis buffer and re-suspended in the same buffer containing 0.5 M NaCl to extract nuclear proteins. After sonication and vortex, the extracted sample was centrifuged at 15,000 g for 10 min at 4°C. Whole cell protein lysate was obtained by tween-20 lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 0.1% Tween-20, 10% glycerol, 2.5 mM EGTA, 1 mM EDTA, 1 mM DTT, 1mM Na_3VO_4, 1 mM PMSF, 1mM BGP and 10 μg/ml of leupeptin and aprotinin). Samples were sonicated and then centrifuged at 15,000 g for 10 min at 4°C. Protein concentrations were determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were fractionated by SDS-

PAGE, transferred to a PVDF membrane (Millipore), and analyzed by incubation with the appropriate primary antibody. Proteins were detected via incubation with HRP-conjugated secondary antibodies and ECL chemiluminescence detection system (GE Healthcare Life Sciences, Piscataway, NJ, USA), ECL Western Blotting Substrate (Promega Cooperation, Madison, WI, USA), SuperSignal* West Dura Extended Duration Chemiluminescent Substrate or SuperSignal* West Femto Maximum Sensitivity Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA).

Immunoprecipitation

Cells were lysed with NP-40 lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% Deoxycholic acid, 10% glycerol, 2.5 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1mM BGP and 10 μg/ml of leupeptin and aprotinin). Cell lysates containing 0.5 mg of protein were incubated overnight at 4°C with 1 μg of anti-mouse EGFR antibody (Invitrogen) or normal mouse IgG (Cell Signaling Tecnology). After adding 25 μl of protein A/G agarose beads (Santa Cruz), cell lysates were incubated for another 2 hours at 4°C. The immunoprecipitates were pelleted by centrifugation and washed several times with NP-40 lysis buffer. The captured immunocomplexes were then eluted by boiling the beads in 2x SDS sample buffer for 5 minutes and subjected to immunoblot analysis as described above.

Microarray analysis

Total RNAs extracted from HP, HC1, HC4 and HC8 using an RNeasy kit (Qiagen Inc., Valencia, CA, USA). Gene expression profiling using the HT-HG-

U133 Human Genome Array (Affymetrix, Santa Clara, CA, USA) containing over 22,000 well characterized genes. After RNA normalization, data were analyzed using Partek Discovery Suite (St. Louis, MO, USA) and signature genes were genes increased or decreased > 2-fold expression levels in three Ctx^R clones (HC1, HC4 and HC8) compared to sensitive parental line (HP) with p value <0.05.

cDNA synthesis and quantitative PCR

cDNA from total RNA of HP, HC1, HC4 and HC8 were synthesized using SuperScript III First-Strand Synthesis System (Invitrogen). Quantitative PCR (qPCR) analysis was performed using a Bio-Rad iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories) using the iQ Supermix as recommended by manufacturer. All reactions were performed in triplicate. The sequences of primer sets used for this analysis are as follows: Lyn-F: 5'-GGCTCCAGA AGCAATCAACT-3', Lyn-R: 5'-TCACGTCGGCATTAGTTCTC-3'; Yes-F: 5'-CTAGTAACA AAGGGCCGAGTG-3', Yes-R: 5'-ATCCTGTATCCTCGCTCCAC-3'; Src-F: 5'-GAGGAG CCCATTTACATCGT-3', Src-R: 5'-TGAGAAAGTCCAGCAAACTCC-3', B-Myb-F: 5'-ATG TCCAGTGCCTGGAAGAC-3', B-Myb-R: 5'-AGATGAGGGTCCGAGATGTG-3'. iNOS–F: 5'-CCATAAGGCCAAAGGGATTT-3', iNOS-R: 5'-ATCTGGAGGGGTAGGCTTGT-3'. Fold increases or decreases in gene expression were determined by quantitation of cDNA from target samples (HC1, HC4 and HC8) relative to a calibrator sample (HP). Human b-actin gene (F: 5'-CAGCCATGTACGTTGCTATCCAGG-3', R: 5'-AGGTCCAGA

CGCAGGATGGCATG-3') was used as the endogenous control for normalization of initial RNA levels. To determine this normalized value, $2^{-\Delta\Delta CT}$ values were compared between target and calibrator samples, where the change in crossing threshold (ΔCt)=$Ct_{target\ gene}$ - $Ct_{b\text{-}actin}$ and $\Delta\Delta Ct=\Delta Ct_{HC1,\ HC4\ or\ HC8}$ - ΔCt_{HP}.

Chromatin immunoprecipitation (ChIP) assay

Cells were fixed with formaldehyde at a final concentration of 1 % for 15 min at room temperature, stopped fixation by 1.25 M glycine for 5 min. Subsequently, cells were washed with ice-cold PBS and collected in the tube and centrifuge at 4°C for 5 min. The cell pellets was lysed in cell lysis buffer (5 mM HEPES, pH 8.0, 85 mM KCl, 0.5% NP-40 and 10 mM sodium pyrophosphate) by a dounce homogenizer. After centrifuge, supernatant was removed, and the nuclei pellets were lysed in nuclei lysis buffer (Tris-HCl 50 mM, pH 8.1, 10 mM EDTA, 1% SDS and 10 mM sodium pyrophosphate). The lysate was sonicated on ice to shear DNA, and the supernatant was pre-cleared with protein A/G agarose beads (Santa Cruz) in dilution buffer (16.7 mM Tris-HCl, pH 8.1, 1.2 mM EDTA, 167 mM NaCl, 1.1% Triton X-100, 0.01% SDS and 10 mM sodium pyrophosphate) for 1h at 4°C. The pre-cleared lysates were immunoprecipitated by incubating with protein A/G beads containing 1 ug of anti-EGFR antibody or IgG and rotated at 4°C for overnight. The beads were washed with wash buffer I (25 mM Tris-HCl, pH 8.0, 2 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS and 10 mM sodium pyrophosphate), wash buffer II (25 mM Tris-HCl, pH 8.0, 2 mM EDTA, 500 mM NaCl, 1% Triton X-100, 0.1% SDS and 10 mM sodium pyrophosphate), wash buffer III (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 250 mM

LiCl, 1% NP-40, 1% deoxycholic acid and 10 mM sodium pyrophosphate) and TE buffer (10 mM Tris-HCl, pH8.0, 1 mM EDTA, 10 mM sodium pyrophosphate). The bound protein was eluted twice with elute buffer (100 mM $NaHCO_3$ and 1% SDS). Then, 5 M NaCl was added to the pooled eluent and incubated at 68°C overnight. The DNA was recovered and purified using DNA purification kit (Qiagen). The purified chromatin immunoprecipitated DNA was used as a template for the qPCR of the promoter regions using the following primer pairs: B-Myb-F: 5'-CTGGTCTTAGCTACCCGTGAG TTGA–3' and B-Myb-R: 5'-CAGGAGTATCCCACATAGCGAACAC-3' [15], iNOS-F: 5'-TGATGAACTGCCACCTTGGAG–3' and iNOS-R: 5'-TTCACCCAACCC ACCTCTTTC-3' [16]. The qPCR program was: 95°C for 3min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s for B-Myb or 55°C for 30 s for iNOS. The qPCR was performed using the iQ5 Real-time PCR Detection system (Bio-Rad).

RESULTS

The SFK inhibitor Dasatinib blocks nuclear translocation of the EGFR

We have previously reported that Ctx^R clones have increased nEGFR and activation of SFKs (**Figure 1A, 1B**)[29,48]. Using this model, we determined the effects of the SFK inhibitor dasatinib on the phosphorylation status of the EGFR in three Ctx^R clones (HC1, HC4 and HC8) and the Ctx^S parental clone (HP) after treatment with 100 nM of dasatinib for 24 hours. Dasatinib inhibited the full activation of SFKs as indicated by the loss of phospho-Y419 and decreased the phosphorylation of EGFR at the known SFK phosphorylation sites Y845 and Y1101 (**Figure 1B**). Also, treatment with dasatinib led to modest increases in steady state expression of total SFKs in all Ctx^R clones.

To determine the effects of dasatinib treatment on nuclear translocation of EGFR, we treated the Ctx^R clones and the Ctx^S parental clone with 100 nM of dasatinib for 24 hours followed by nuclear fractionation. As illustrated in **Figure 1C**, dasatinib treatment reduced EGFR nuclear translocation in Ctx^R clones. The Ctx^S parental clone has very low levels of nEGFR and dasatinib treatment had no effect. Thus, the inhibition of SFK activity decreased the phosphorylation of EGFR at Y845 and Y1101 as well as impaired nuclear entry of the EGFR. These results suggested that SFK phosphorylation of EGFR may play a role in inducing its nuclear translocation.

Yes and Lyn are overexpressed and associate with the EGFR in Ctx^R clones

Based on our previous findings with clonal Ctx^R variants of the NCI-H226

NSCLC line we hypothesized that SFK member(s) may regulate EGFR nuclear translocation. To identify the specific SFKs that are necessary for EGFR nuclear translocation, we performed microarray analysis comparing Ctx^S HP parental cells to the three Ctx^R clones (HC1, HC4 and HC8). Microarray analysis demonstrated a ~4-fold up-regulation of Yes and Lyn kinases and ~3-fold down-regulation of Src kinase in all of three Ctx^R cells (data not shown). Other SFK family members did not exhibit significant expression level changes in three Ctx^R clones (HC1, HC4 and HC8) compared to sensitive parental line (HP).

To validate the microarray findings we performed qPCR analysis. These results confirmed the microarray data indicating up-regulation of Lyn (~ 3-fold) and Yes (~1.5 fold), and down-regulation of Src kinase (~2-fold) in all three Ctx^R clones (**Figure 2A**). Next, we examined if these increased mRNA levels reflected total protein levels in Ctx^R when compared to Ctx^S cells. We found that total protein levels of Yes and Lyn were increased 1.3- to 2.1-fold in Ctx^R clones compared to parental cells (**Figure 2B**). Finally, we investigated whether EGFR associated with Yes and Lyn. Immunoprecipitation analysis of EGFR binding partners indicated that EGFR displayed increased association to Yes and Lyn in all three Ctx^R clones as compared to the Ctx^S HP cell line (**Figure 2C**). Collectively, these results indicate that Yes and Lyn are up-regulated in Ctx^R clones and have increased association with the EGFR.

<u>Yes and Lyn are necessary for nuclear translocation of EGFR in Ctx^R cells</u>

To further investigate if Yes and/or Lyn expression altered EGFR

activation and nuclear translocation we performed gene-silencing experiments using siRNA directed against Yes or Lyn in Ctx^R clones (HC1, HC4 and HC8). Ctx^S cells were not included in these siRNA studies because the cells have negligible levels of nEGFR. After treatment with siYES or siLYN in Ctx^R clones for 72 hours we observed decreased phosphorylation of EGFR Y845 (70-99%) and EGFR Y1101 (40-85%) relative to control non-targeting siRNA (NT) (**Figure 3A**). Moreover, siYES and siLYN decreased the nuclear localization of EGFR in Ctx^R clones (**Figure 3B**).

Knockdown studies of Yes or Lyn in cells with high nEGFR expression led to decreased nEGFR levels. Therefore, we corroborated the hypothesis that increased expression of Yes or Lyn in cells with low nEGFR would lead to increased nEGFR translocation. To test this hypothesis we overexpressed Yes or Lyn in Ctx^S cells, which express low-levels of nEGFR. Firstly, Yes and Lyn were cloned into mammalian expression vectors, expressed in CHO-K1 cells and characterized for increased total Yes and Lyn protein expression and activity (**Figure 4A)**. Comparable increases in Yes and Lyn expression and activation were observed in Ctx^S cells after transfection compared to vector only (**Figure 4B**). Consistent with siRNA observations, transient transfection of Yes or Lyn into Ctx^S cells significantly increased (3~3.5 fold) nEGFR translocation (**Figure 4C**). Interestingly, Yes and Lyn were also detected in nucleus of HP cells after transfection. To determine if Ctx^R cells with increased of nEGFR also express more Yes and Lyn in the nucleus compared to Ctx^S cells, we determined nuclear Yes and Lyn levels in Ctx^R clones and Ctx^S cells. As seen with EGFR, increased

levels of both Yes and Lyn were found in the nucleus of Ctx^R clones compared to Ctx^S cells (**Figure 4D**). These siRNA and overexpression results suggest that Yes and Lyn play a role in EGFR nuclear translocation.

SFKs exhibit functional redundancy in their ability to influence nEGFR translocation

While Yes and Lyn are overexpressed in Ctx^R clones, alternative SFKs have been found to be overexpressed in different cancer types[36,37]. To investigate the global role of SFKs in the regulation of nEGFR translocation, stable clones of individual SFKs (Src, Yes, Lyn, Lck, Hck, Fyn, Blk, and Fgr) were engineered in the breast cancer cell line MCF-7. One or two stable clones were chosen for each SFK for comparison to an empty vector stable cell line (**Figure 5A**). The overexpression of each SFK led to the enhanced expression and nuclear translocation of EGFR. All cell lines were stimulated with 5 nM EGF to promote the nuclear translocation of EGFR, however a basal level of nEGFR was detected in non-stimulated SFK stable cells (data not shown). Additionally, the stable overexpression of each SFK led to their increased activation, corresponding to a decrease in expression of the E3 ubiquitin ligase, c-Cbl **(Figure 5B)**. Collectively, these data suggest that SFKs play functional redundant roles in promoting nEGFR translocation.

Depletion of Yes or Lyn decreases binding of nEGFR complexes to B-Myb and iNOS promoter regions in Ctx^R clones

NEGFR and various transcription factor complexes have been shown to bind promoter regions and regulate the transcription of multiple genes including cyclin D1, COX2, Aurora Kinase A, c-Myc, iNOS, B-Myb, BCRP and GRP78[14,15,17-21]. To confirm that nEGFR complexes in Ctx^R clones bound to known EGFR target gene promoters we performed Chromatin immunoprecipitation-quantitative PCR (ChiP-qPCR) analysis of the B-Myb and iNOS promoters. We demonstrated that Ctx^R clones have increased EGFR association with the B-Myb (3-9-fold increase in EGFR binding) and iNOS (6-12-fold increase in EGFR binding) promoter regions as compared to Ctx^S parental cell line (**Figure 6A**). These results indicate that nEGFR complexes bind known EGFR target promoters in Ctx^R clones more strongly than in Ctx^S HP cells.

Given the high binding of nEGFR complexes to the B-Myb and iNOS promoters we performed qPCR to determine whether this binding resulted in increased expression of B-Myb and iNOS genes as previously reported[15,16]. Results in **Figure 6B** indicated that B-Myb mRNA expression was increased approximately 4-fold as compared to Ctx^S parental cells. Whereas, iNOS mRNA expression was increased 4-11-fold in Ctx^R clones when compared to Ctx^S parental cells. Furthermore, B-Myb protein expression was up-regulated around 3-fold and iNOS protein expression was up-regulated 2-14-fold in Ctx^R clones (**Figure 6C**). Silencing of Yes or Lyn using siRNA reduced nEGFR complex formation with the B-Myb and iNOS promoters as detected by ChIP-qPCR (**Figure 6D**). Protein expression of both B-Myb and iNOS were decreased (60-70 %) in HC4 clone after siYES or siLYN transfection compared to control non-

targeting siRNA (**Figure 6D**). Collectively, these data demonstrate that Ctx^R clones with high levels of nEGFR associate more strongly with known EGFR regulated promoter regions, and that these association (demonstrated with B-Myb and iNOS) can be prevented upon depletion of Yes or Lyn.

EGFR Y1101 is involved in nuclear translocation

Analysis of known SFK phosphorylation sites on the EGFR showed increased phosphorylation of Y845 and Y1101 in Ctx^R clones (**Figure 1B**). However, the relevance of these two tyrosine sites for EGFR nuclear translocation is unknown. To determine if phosphorylation of one or both of these tyrosine residues is involved in EGFR nuclear translocation, MCF-7 breast cancer cells (which express very low EGFR levels) were transiently transfected with cDNAs encoding wild-type EGFR (WT) or the following EGFR mutants: EGFR-Y845F or EGFR-Y1101F. Immunoprecipitation of WT and mutant EGFR followed by immunoblotting with antibodies directed against $pEGFR^{Y845}$ or $pEGFR^{Y1101}$ showed reduced phosphorylation of Y845 and Y1101. (**Figure 7A**). To induce nuclear translocation of the EGFR we treated the transfected cells with EGF for 45 minutes prior to cell lysis and nuclear fractionation. The results of this experiment indicated that EGF was able to induce nuclear translocation of EGFR in both EGFR-WT and EGFR-Y845F mutant cells (**Figure 7B and C**). However, EGF-induced EGFR nuclear translocation was diminished ~70% in EGFR-Y1101F mutant cells as compared to EGFR-WT expressing cells (**Figure 7C**). Furthermore qPCR analysis revealed that B-Myb and iNOS mRNA expression

were downregulated in cells transfected with EGFR-Y1101F mutant compared to EGFR-WT transfected cells (**Figure 7D**). Collectively, these data suggest that the phosphorylation of Y1101 is important for the nuclear translocation of EGFR while the phosphorylation of Y845 does not appear to be essential for this process.

DISCUSSION

The nuclear localization of receptor tyrosine kinases (RTKs) have been observed for over 20 years, however only in the last 10 years has research begun to focus on how they translocate from the cell surface to the to the nucleus and what functions they perform there. All four HER family members have been identified in the nucleus of various types of human cancer cells and tumor specimens[9,50-53]. Several target genes of nEGFR have been identified[14-20,22,23], and nEGFR is correlated with resistance to cetuximab, radiation, cisplatin and gefitinib therapies[20,23,29-33]. Collectively, these results suggest an emerging role for the nEGFR signaling network in cancer progression and response to therapeutic modalities.

Several studies have examined how EGFR moves from the plasma membrane to the nucleus of the cell. It has been shown that the full-length EGFR can be shuttled from the plasma membrane to the nucleus[7,10,13]. Despite this mechanism of EGFR nuclear translocation, the early events at the plasma membrane that may serve as critical initiating signals for the movement of the EGFR to the nucleus have yet to be defined and form the basis of the current study.

To further elucidate the molecular requirements for EGFR nuclear transport we utilized a previously established model of acquired resistance to cetuximab in the NCI-H226 NSCLC cell line[48]. In this model, cetuximab-resistant cells were observed to have increased levels of nEGFR as compared to their cetuximab-sensitive parental cells, making it an ideal model for investigating

events involved in nuclear translocation of the EGFR[29]. Additionally, cetuximab-resistant cell lines were shown to have increased expression and activity of SFKs[44]. Further investigation using dasatinib, an inhibitor of SFKs, demonstrated that SFKs activity was necessary for the nuclear transport of EGFR in this model of cetuximab-resistance[29]. In the current study, we identified Yes and Lyn to have increased expression and association with the EGFR (**Figure 2**). This result is consistent with other reports identifying Yes and Lyn interaction and activation of the EGFR[54-57]. In addition, loss of Yes and Lyn expression using siRNA technology, led to reduced phosphorylation of Y845 and Y1101 of the EGFR and more importantly impaired nEGFR accumulation (**Figure 3**). Consistent with this observation, overexpression of Yes and Lyn in Ctx^S cells that express low-levels of nEGFR significantly increased (3~3.5 fold) nEGFR translocation (**Figure 4B**). ChIP assay demonstrated that nEGFR complexes bind to B-Myb and iNOS promoter regions and siYES and siLYN decreased binding to these promoters (**Figure 6**). Mutagenesis studies of Y845 and Y1101 indicated that Y1101, not Y845, might be necessary for nuclear translocation of the EGFR from the membrane to the nucleus (**Figure 7**). Recently, Jaganathan et al. reported that Src and EGFR associate in the nucleus with the transcription factor STAT3 to regulate the expression of the c-Myc gene in pancreatic cancer[19]. Consistent with this report, we found that Yes or Lyn not only increased the levels of nEGFR but also had increased localization within the nucleus, suggesting that they may have been imported into the nucleus with the EGFR. Collectively, these studies

provide evidence for the role of SFKs in mediating nuclear translocation of the EGFR.

SFKs consist of 11 intracellular tyrosine kinases that are differentially expressed in a variety of cancers[58]. In the current study, eight individual SFKs were stably overexpressed, and found to function similarly in their ability to influence 1) the steady state expression of total EGFR, 2) nEGFR translocation, and 3) degradation of c-Cbl **(Figure 5)**. These data suggest that SFKs exhibit functional redundancy in their ability to influence nEGFR translocation, and thus the use of broad-spectrum SFK inhibitors, such as dasatinib, may be highly beneficial in nEGFR positive cancers.

Y845 and Y1101 of the EGFR are phosphorylated by SFKs [34]. Biscardi et al. utilized a GST bound SH2 domain of the c-Src protein to demonstrate its specific binding to the EGFR via affinity chromatography[34]. Subsequently, these investigators identified and validated that EGFR was indeed phosphorylated by c-Src at Y845 and Y1101. Breast cancer cell lines with high levels of Src activity also had increased levels of phospho Y845 and Y1101 of the EGFR, and that phospho-Y845 was necessary for full EGFR activation and EGF-induced DNA synthesis[34]. This study represented a landmark finding by identifying novel Src phosphorylation sites on the EGFR and the role of tyrosine 845 in the complete activation of the EGFR. Further studies looking at the function of EGFR Y845 demonstrated that Y845 mediated EGFR binding to the mitochondrial protein cytochrome c oxidase subunit II at the mitochondria; however, EGFR Y845 was not necessary for its movement to the mitochondria[59]. These findings support

our data that EGFR Y845 may not be required for the intracellular trafficking of the EGFR.

In the current study, we corroborate findings of Biscardi et al. by showing that Y845 and Y1101 are Src specific phosphorylation sites through the use of the SFK inhibitor dasatinib (**Figure 1B**)**.** In addition siRNA directed towards the SFKs, Yes and Lyn (**Figure 3A**), both decreased the phosphorylation of EGFR Y845 and Y1101. Our data further suggests that Y1101 but not Y845 may be a critical molecular determinant in the localization of nEGFR as indicated by site-directed mutagenesis (**Figure 7B**). It should be noted, however, that mutation of Y1101 did not completely block translocation of the EGFR to the nucleus, suggesting other potential sites may be necessary. Recent evidence has identified another key phosphorylation site on the EGFR, serine 229 (S229), as being necessary for EGFR translocation to the nucleus[20]. It was reported that the serine/threonine kinase AKT can influence the nuclear translocation of the EGFR by phosphorylating S229 on the EGFR in a model of gefitinib resistance. In this model, gefitinib-resistant A431/GR cells have both increased AKT activity and increased nEGFR as compared to gefitinib-sensitive A431 cells. Using an antibody that recognizes the phosphorylated consensus motif of AKT substrates and subsequent mass spectrometry, Huang et al. revealed that EGFR was phosphorylated by AKT at S229[20]. Inhibition of AKT kinase activity prevented this phosphorylation event, and decreased the nuclear transport of EGFR providing evidence for the role of alternative kinases and post-translational modifications of the EGFR that indeed affect its nuclear translocation. Collectively, these findings

suggest that the phosphorylation of the EGFR on Y1101 by Yes and Lyn together with AKT phosphorylation of S229 may be critical molecular determinants that influence the nuclear localization of the EGFR.

EGFR is tightly linked to the etiology of HNSCC, NSCLC, CRC, breast and brain cancers. Accordingly, five EGFR inhibitors, three tyrosine kinase inhibitors, and two monoclonal antibodies have been developed for clinical use to inhibit EGFR activation and downstream signaling. Despite the successes of these agents, many tumors do not respond to EGFR inhibition, or eventually become resistant to this therapeutic strategy. Accumulating evidence suggests that nEGFR plays a role in resistance to radiation, cetuximab, cisplatin and gefitinib therapies[20,23,29,32,33]. The mechanisms for how nEGFR leads to this resistance are not clear. However work from our laboratory suggests that nuclear translocation can protect EGFR from the inhibitory effects of cetuximab causing resistance to this therapy[29]. The results presented here provide a potential mechanism for the key molecules involved in nuclear localization of the EGFR providing rational targets to prevent nuclear translocation and thus nuclear function of the EGFR.

In summary, the data presented in the current study has identified the SFKs Yes and Lyn to play a crucial role in nuclear translocation of the EGFR in a model of cetuximab-resistance. While Yes and Lyn were the predominant SFKs overexpressed in this model, alternative SFKs may regulate nEGFR trafficking in other cell types as revealed by the functional redundancy studies in the SFK stable clones. In addition, the SFK phosphorylation site Y1101 of the EGFR

appears to be involved in translocation of the EGFR from the plasma membrane to the nucleus. These findings are of instrumental value in understanding the molecular requirements for nEGFR transport, and for potentially targeting nEGFR in the future.

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Figure 1: SFKs are essential for EGFR translocation to the nucleus. **(A) Ctx^{R} clones (HC1, HC4 and HC8) have increased nEGFR localization as compared to Ctx^{s} cell line (HP).** Cells were harvested for non-nuclear and nuclear protein, and fractionated on SDS-PAGE followed by immunoblotting for indicated proteins. Histone H3 and a-tubulin were used as loading and purity controls for the nuclear and non-nuclear fractions, respectively. **(B) Dasatinib decreased EGFR activity in Ctx^{R} cells.** Ctx^{R} clones (HC1, HC4 and HC8) and Ctx^{S} HP cell line were treated with 100 nM dasatinib for 24 hr. Cells were harvested and protein lysates were fractionated on SDS-PAGE followed by immnoblotting for the indicated proteins. α-tubulin was used as a loading control. Expression was quantitated using ImageJ software. **(C) Dasatinib can inhibit EGFR nuclear translocation in Ctx^{R} clones.** Ctx^{R} clones (HC1, HC4, and HC8) and Ctx^{S} cell line were treated with 100 nM dasatinib for 24 hr. Cells were harvested for non-nuclear and nuclear protein, and fractionated on SDS-PAGE followed by immunoblotting for indicated proteins. Histone H3 and a-tubulin were used as loading and purity controls for the nuclear and non-nuclear fractions, respectively.

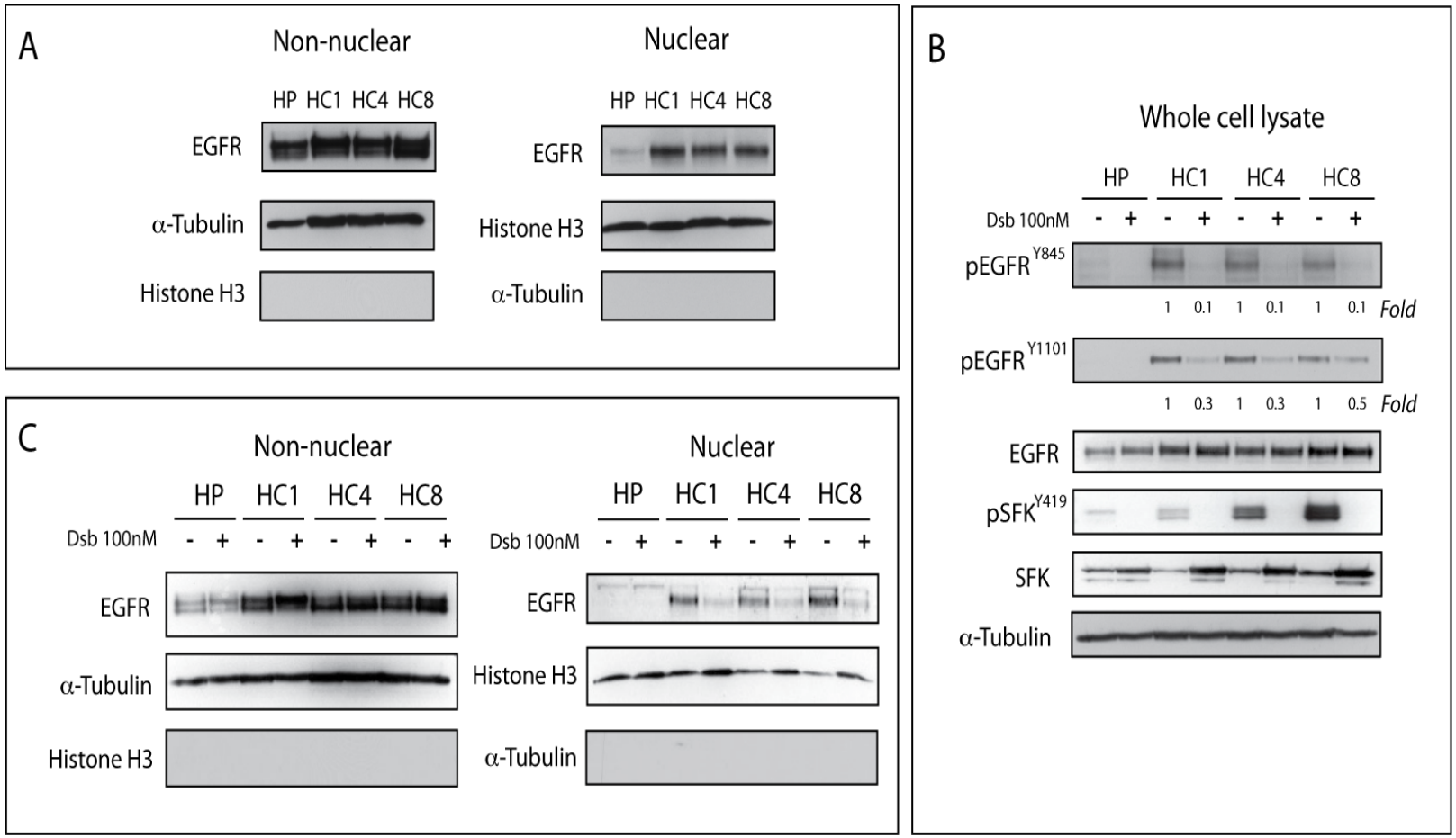

A
Non-nuclear
Nuclear
HP HC1 HC4 HC8
EGFR
α-Tubulin
Histone H3
HP HC1 HC4 HC8
EGFR
Histone H3
α-Tubulin
B
Whole cell lysate
HP HC1 HC4 HC8
Dsb 100nM - + - + - + - +
pEGFR Y845
1 0.1 1 0.1 1 0.1 Fold
pEGFR Y1101
1 0.3 1 0.3 1 0.5 Fold
EGFR
pSFK Y419
SFK
α-Tubulin
C
Non-nuclear
Nuclear
HP HC1 HC4 HC8
Dsb 100nM - + - + - + - +
EGFR
α-Tubulin
Histone H3
HP HC1 HC4 HC8
Dsb 100nM - + - + - + - +
EGFR
Histone H3
α-Tubulin

Figure 2: Yes and Lyn SFKs are overexpressed in Ctx^R cells. (A) Yes and Lyn mRNA is up-regulated in Ctx^R clones. Lyn (3.6~4.5-fold), Yes (~1.5-fold) and Src (-2.3~-3.5-fold) mRNA expression levels were compared to that of the Ctx^S cell line (HP) by qPCR. **(B) Total protein levels of Yes and Lyn were increased (1.3~2.1-fold) in Ctx^R clones (HC1, HC4, and HC8) as compared to the Ctx^S cell line (HP).** Cells were harvested and protein lysates were fractionated on SDS-PAGE followed by immunoblotting for the indicated proteins. α-tubulin was used as a loading control. Protein expression was quantitated using ImageJ software. **(C) EGFR displayed increased binding with Yes and Lyn as compared to the Ctx^S parental cell line.** Cells were harvested and EGFR or IgG were immunoprecipitated with anti-mouse EGFR antibody or normal mouse IgG. The immunoprecipitate complexes were fractionated on SDS-PAGE followed by immunoblotting for indicated proteins.

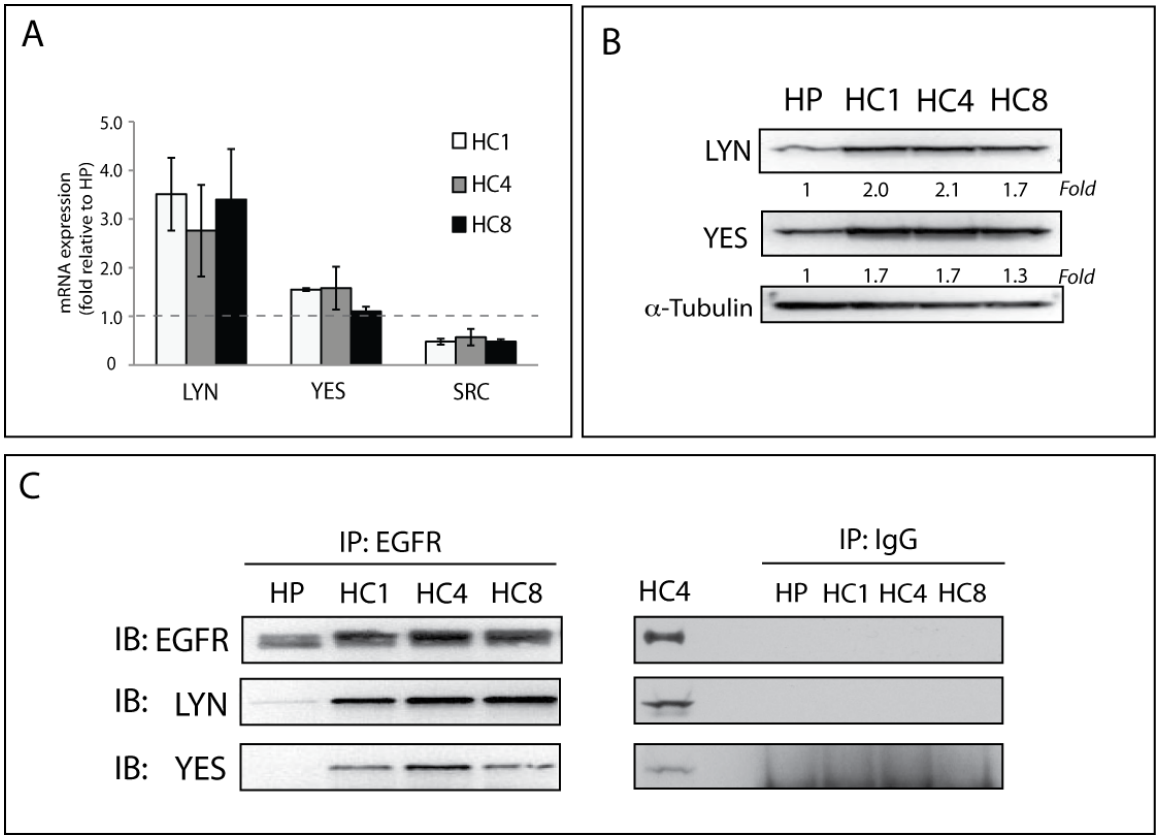
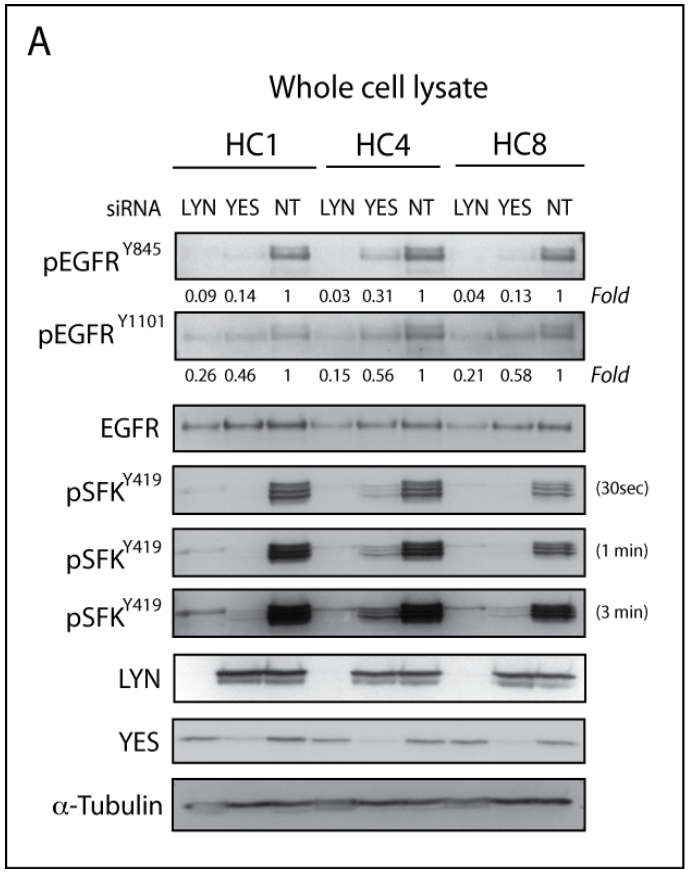

A
mRNA expression (fold relative to HP)
5.0
4.0
3.0
2.0
1.0
0
HC1
HC4
HC8
LYN
YES
SRC
B
HP HC1 HC4 HC8
LYN
1 2.0 2.1 1.7 Fold
YES
1 1.7 1.7 1.3 Fold
α-Tubulin
C
IP: EGFR
HP HC1 HC4 HC8
IB: EGFR
IB: LYN
IB: YES
IP: IgG
HC4
HP HC1 HC4 HC8

Figure 3: siYES and siLYN can reduce the nuclear localization of the EGFR (A) siYES and siLYN decreased phosphorylation of EGFR tyrosine 845 (Y845), tyrosine 1101 (Y1101) and SFK tyrosine 419. Cells were harvested 72 hr after treatment with either siYES or siLYN, and protein lysates were fractionated on SDS-PAGE followed by immunoblotting for the indicated proteins. The non-targeting siRNA (NT) was used as a control. α-tubulin was used as a loading control. Protein expression was quantitated using ImageJ software. (**B) siYES and siLYN can reduce the nuclear localization of the EGFR.** Cells were harvested for non-nuclear and nuclear protein, and fractionated on SDS-PAGE followed by immunoblotting for indicated proteins after 72hr treatment with either siYES or siLYN. The non-targeting siRNA (NT) was used as a control. Histone H3 and a-tubulin were used as loading and purity controls for the nuclear and non-nuclear fractions, respectively. Protein expression was quantitated using ImageJ software.


A
Whole cell lysate
HC1
HC4
HC8
siRNA
LYN YES NT LYN YES NT LYN YES NT
pEGFR Y845
0.09 0.14 1 0.03 0.31 1 0.04 0.13 1 Fold
pEGFR Y1101
0.26 0.46 1 0.15 0.56 1 0.21 0.58 1 Fold
EGFR
pSFK Y419
(30sec)
pSFK Y419
(1 min)
pSFK Y419
(3 min)
LYN
YES
α-Tubulin

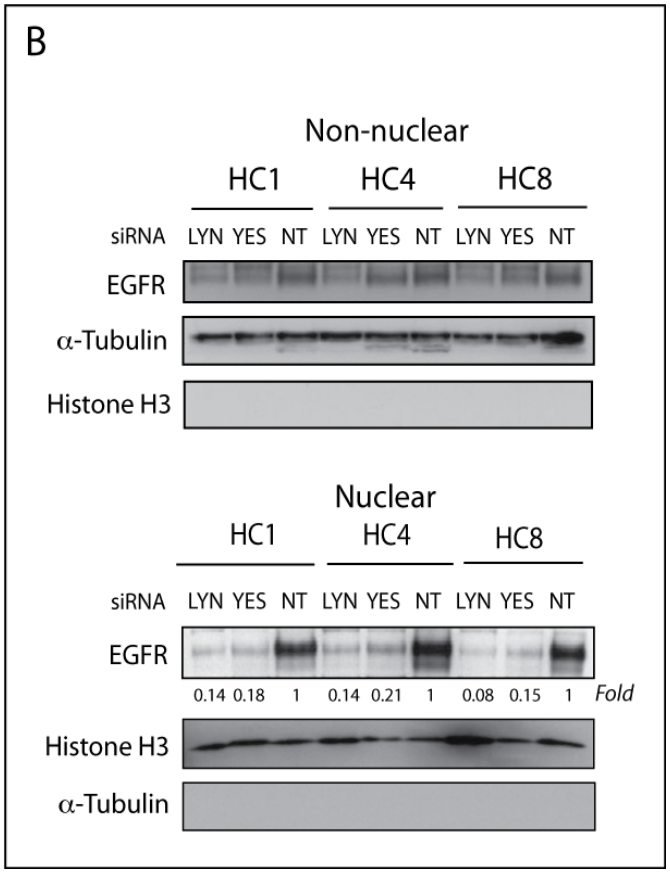

B
Non-nuclear
HC1
HC4
HC8
siRNA
LYN YES NT LYN YES NT LYN YES NT
EGFR
α-Tubulin
Histone H3
Nuclear
HC1
HC4
HC8
siRNA
LYN YES NT LYN YES NT LYN YES NT
EGFR
0.14 0.18 1 0.14 0.21 1 0.08 0.15 1 Fold
Histone H3
α-Tubulin

Figure 4: YES and LYN overexprssion can induce the nuclear localization of the EGFR. (A) Increased levels of total protein expression of Yes and Lyn were detected in Chinese hamster ovary (CHO-K1) cells. **(B)** Increased Yes and Lyn expression as well as increased levels of pSFK Y419 were observed in Ctx^S cells after transfection compared to vector only. **(C) nEGFR translocation was increased by overexpression of Yes and Lyn.** Cells were transfected overexpressed Yes or Lyn and harvested for non-nuclear and nuclear protein. Each protein was fractionated on SDS-PAGE followed by immunoblotting. Histone H3 and a-tubulin were used as loading and purity controls for the nuclear and non-nuclear fractions, respectively. (**D) Ctx^R clones increased nuclear Yes and Lyn localization as compared to Ctx^s cell line (HP).** Cells were harvested for non-nuclear and nuclear protein, and fractionated on SDS-PAGE followed by immunoblotting for indicated proteins. Histone H3 and a-tubulin were used as loading and purity controls for the nuclear and non-nuclear fractions, respectively.

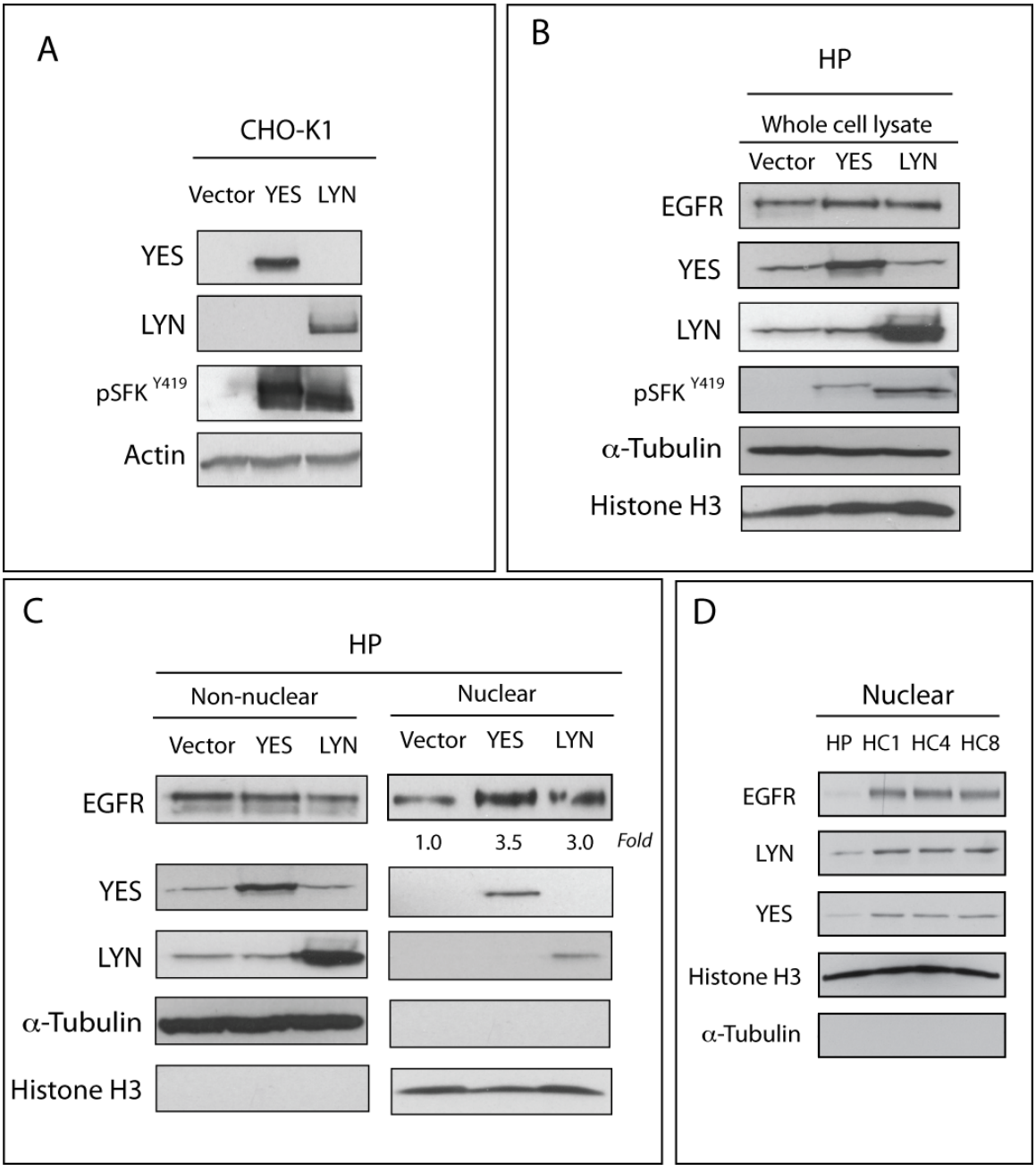

A
CHO-K1
Vector YES LYN
YES
LYN
pSFK Y419
Actin
B
HP
Whole cell lysate
Vector YES LYN
EGFR
YES
LYN
pSFK Y419
α-Tubulin
Histone H3
C
HP
Non-nuclear
Nuclear
Vector YES LYN
Vector YES LYN
EGFR
1.0 3.5 3.0 Fold
YES
LYN
α-Tubulin
Histone H3
D
Nuclear
HP HC1 HC4 HC8
EGFR
LYN
YES
Histone H3
α-Tubulin

Figure 5: SFKs exhibit functional redundancy in their ability to influence nEGFR translocation. (A) The stable overexpression of SFKs increase nEGFR expression. Eight different SFK's were stably overexpressed in the breast cancer cell line MCF-7. SFK stable clones or an empty vector stable cell line were stimulated with EGF (5 nM, 45 min) to induce nEGFR translocation, prior to harvesting non-nuclear and nuclear proteins. GAPDH and Histone H3 were used as loading and purity controls, respectively. **(B) The stable overexpression of SFKs downregulate c-Cbl.** Whole cell lysate was harvested from SFK stable clones or an empty vector stable cell line. GAPDH was used as loading control.

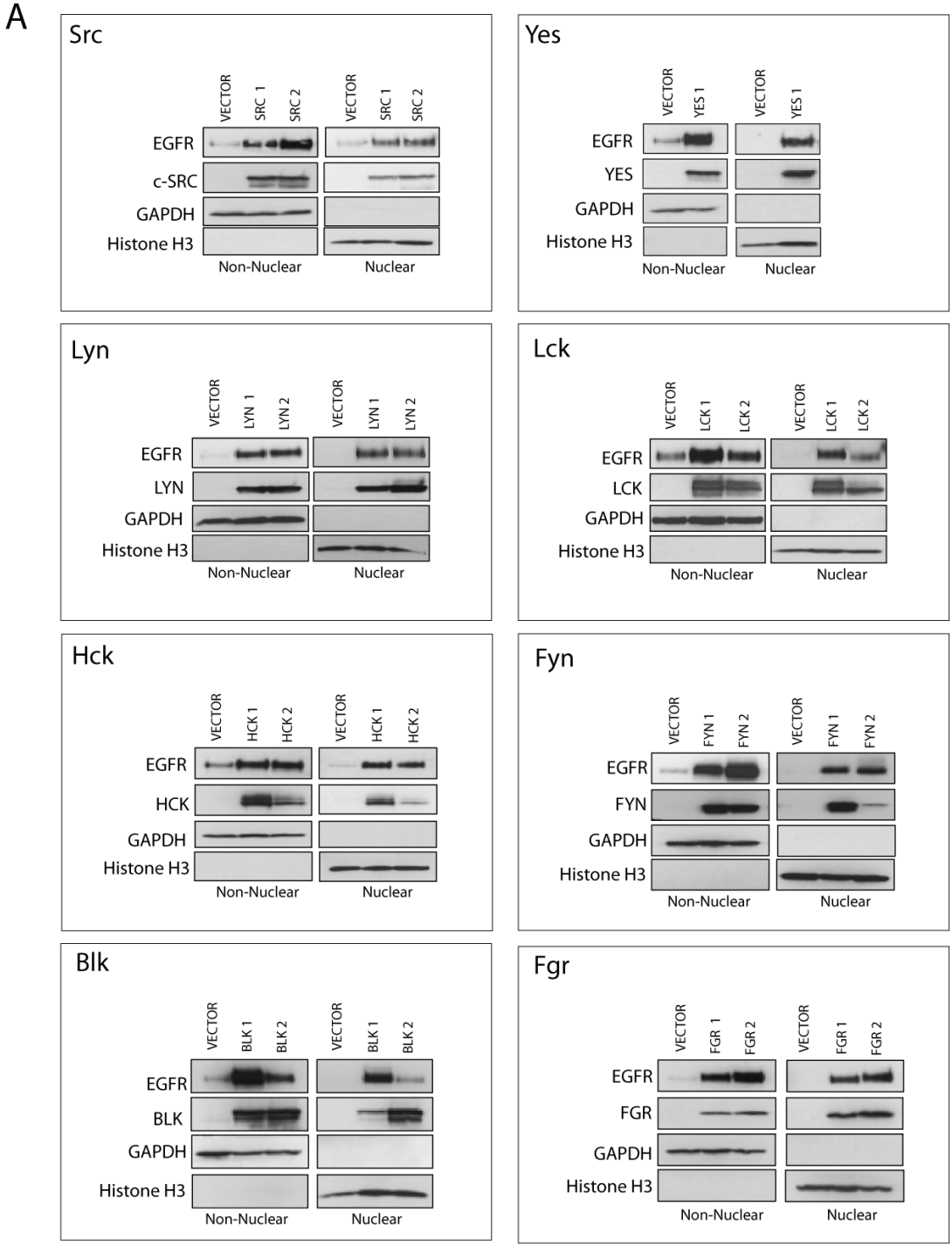

A
Src
VECTOR
SRC 1
SRC 2
EGFR
c-SRC
GAPDH
Histone H3
Non-Nuclear
Nuclear
Yes
YES 1
YES
Lyn
LYN 1
LYN 2
LYN
Lck
LCK 1
LCK 2
LCK
Hck
HCK 1
HCK 2
HCK
Fyn
FYN 1
FYN 2
FYN
Blk
BLK 1
BLK 2
BLK
Fgr
FGR 1
FGR 2
FGR
B
pSFK Y419
c-Cbl
GAPDH

Figure 6: Yes and Lyn influence the binding of EGFR complexes to the B-Myb and iNOS promoters. (A) EGFR regulated gene promoter regions are more strongly associated with EGFR in Ctx^R clones (HC1, HC4, and HC8) as compared to Ctx^S cells (HP). EGFR-ChIP and subsequent qPCR for detection of B-Myb and iNOS promoter sequences. Data points are represented as mean ± SEM (n = 3). *p< 0.05. qPCR specificity for the B-Myb and iNOS promoter regions was also confirmed by agarose gel electrophoresis of semi-quantitative PCR products. **(B) B-Myb and iNOS mRNA levels were significantly up-regulated in Ctx^R cells (HC1, HC4, and HC8) as compared to the Ctx^S cell line (HP) by qPCR.** The mRNA expression of B-Myb and iNOS in HP, HC1, HC4 and HC8 were determined by qPCR. Data points are represented as mean ± SEM (n = 3). **(C) B-Myb and iNOS protein levels were increased in Ctx^R cells (HC1, HC4, and HC8) as compared to the Ctx^S cell line (HP) by immunoblot analysis.** Cells were harvested and protein lysates were fractionated on SDS-PAGE followed by immunoblotting for the indicated proteins. α-tubulin was used as a loading control. **(D) Loss of Yes or Lyn prevents EGFR association with B-Myb and iNOS.** EGFR-ChIP and subsequent qPCR from the ChIP sample for the presence of B-Myb and iNOS promoter sequences. The non-targeting siRNA (NT) was used as a control. B-Myb and iNOS protein levels were decreased in HC4 after siYES or siLYN treatment by immunoblot analysis. Cells were harvested after treatment with siLYN or siYES for 72 hr and protein lysates were fractionated on SDS-PAGE followed by immunoblotting for the indicated proteins. α-tubulin was used as a loading control.

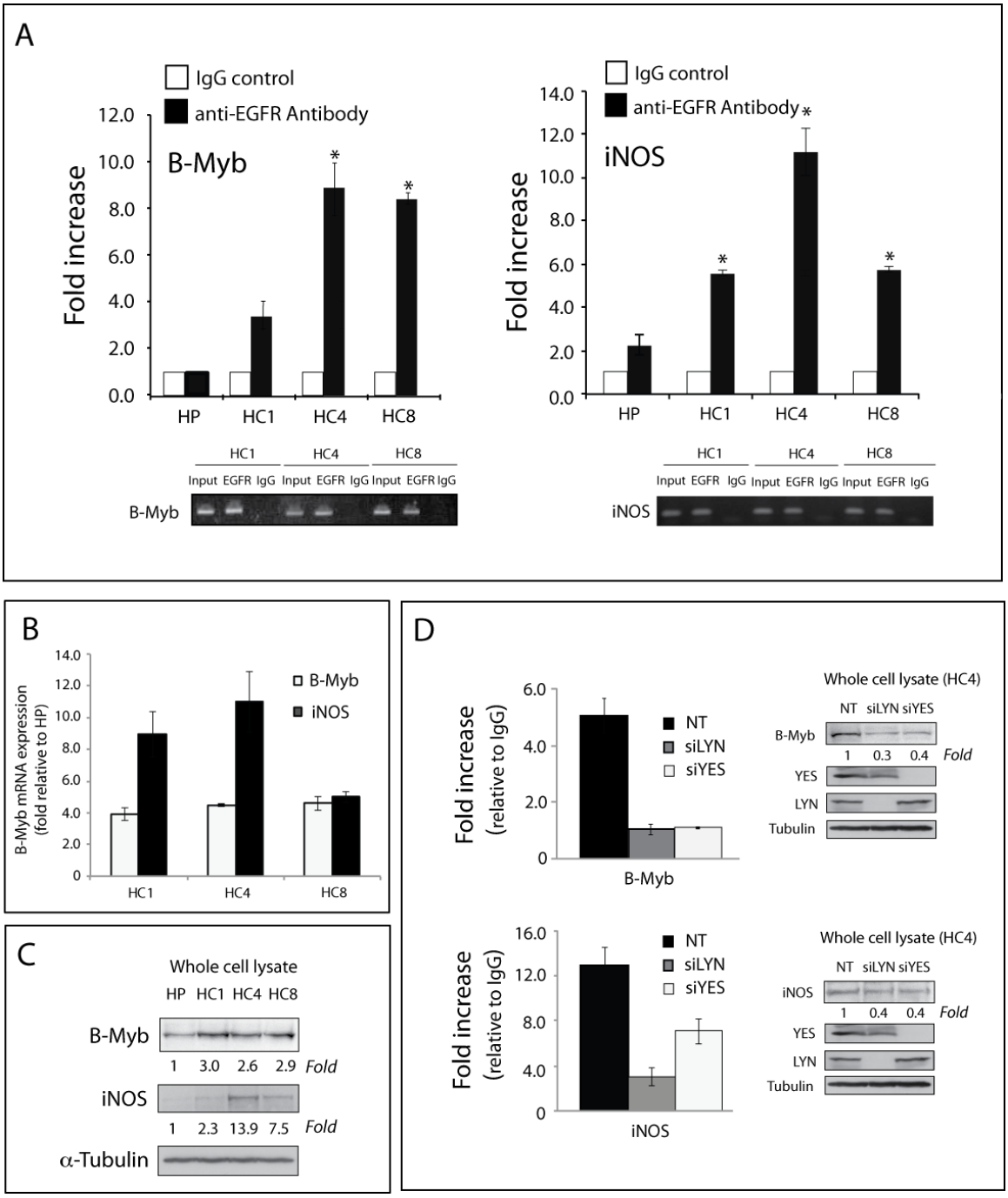
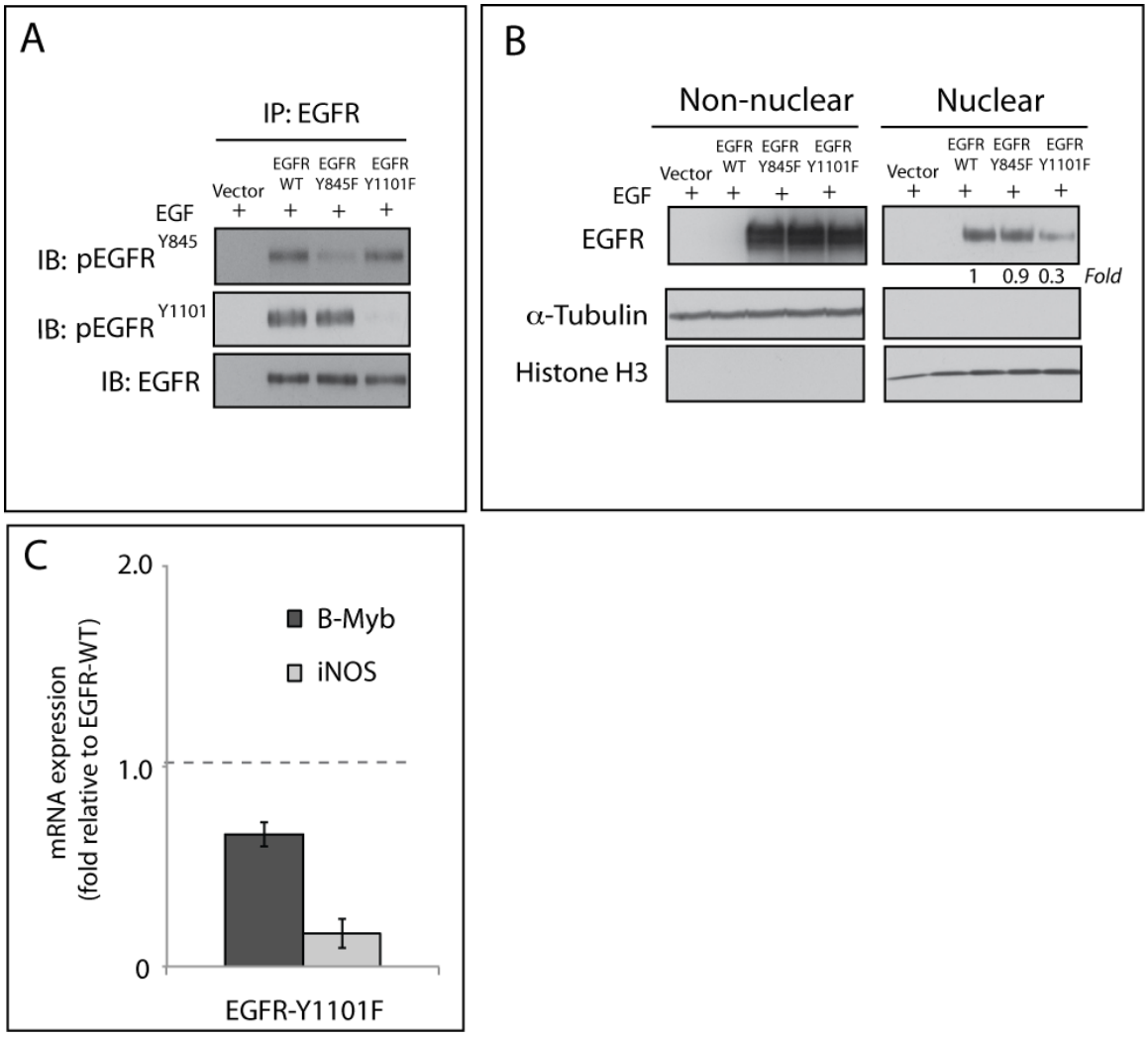

A
IgG control
anti-EGFR Antibody
B-Myb
Fold increase
12.0
10.0
8.0
6.0
4.0
2.0
0.0
HP
HC1
HC4
HC8
HC1
HC4
HC8
Input EGFR IgG Input EGFR IgG Input EGFR IgG
B-Myb
IgG control
anti-EGFR Antibody
iNOS
Fold increase
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
HP
HC1
HC4
HC8
HC1
HC4
HC8
Input EGFR IgG Input EGFR IgG Input EGFR IgG
iNOS
B
B-Myb mRNA expression (fold relative to HP)
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
B-Myb
iNOS
HC1
HC4
HC8
C
Whole cell lysate
HP HC1 HC4 HC8
B-Myb
1 3.0 2.6 2.9 Fold
iNOS
1 2.3 13.9 7.5 Fold
α-Tubulin
D
Fold increase (relative to IgG)
6.0
4.0
2.0
0
NT
siLYN
siYES
B-Myb
Whole cell lysate (HC4)
NT siLYN siYES
B-Myb
1 0.3 0.4 Fold
YES
LYN
Tubulin
Fold increase (relative to IgG)
16.0
12.0
8.0
4.0
0
NT
siLYN
siYES
iNOS
Whole cell lysate (HC4)
NT siLYN siYES
iNOS
1 0.4 0.4 Fold
YES
LYN
Tubulin

Figure 7: Phosphorylation of EGFR at tyrosine 1101 is involved in the nuclear translocation of EGFR. (A) Immunoprecipitation of total EGFR followed by immunoblotting with anti-pEGFR Y845, anti-pEGFR Y1101 or pan-EGFR antibodies. MCF7 Cells overexpressing EGFR-WT, EGFR-Y845F or EGFR-Y1101F were harvested after stimulation with 100 ng/mL of EGF for 45 min. A total of 500 ug of cell lysate was immunoprecipitated with pan-EGFR antibody. The immunoprecipitates were fractionated on SDS-PAGE followed by immunoblotting for the indicated proteins. **(B) Mutation of Y1101, but not Y845 reduces the nuclear localization of EGFR in MCF-7 breast cancer cells.** Cells were transiently transfected with plasmids encoding the EGFR wild-type (WT), EGFR-Y845F, EGFR-Y1101F or vector only. Forty-eight hr post-transfection the cells were incubated with EGF (100 ng/ml) for 45min, harvested for whole cell lysate, non-nuclear, and nuclear protein, and fractionated on SDS-PAGE followed by immunoblotting for indicated proteins. Histone H3 and a-tubulin were used as loading and purity controls for the nuclear and non-nuclear fractions, respectively. **(C) B-Myb and iNOS mRNA levels were down-regulated in cells transfected with EGFR-Y1101F mutant compared to EGFR-WT transfected cells by qPCR**. Cells were transiently transfected with plasmids encoding the EGFR wild-type (WT) or EGFR-Y1101F. Twenty four hr post-transfection the cells were treated with EGF (100 ng/ml) for 45min, harvested for RNA isolation. The mRNA expression of B-Myb and iNOS in each cells were determined by qPCR.


A
IP: EGFR
Vector
EGFR WT
EGFR Y845F
EGFR Y1101F
EGF
IB: pEGFR Y845
IB: pEGFR Y1101
IB: EGFR
B
Non-nuclear
Nuclear
Vector
EGFR WT
EGFR Y845F
EGFR Y1101F
EGF
EGFR
1 0.9 0.3 Fold
α-Tubulin
Histone H3
C
2.0
1.0
0
mRNA expression
(fold relative to EGFR-WT)
B-Myb
iNOS
EGFR-Y1101F

Chapter III

Nuclear Epidermal Growth Factor Receptor is a Functional Molecular Target in Triple-Negative Breast Cancer

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ABSTRACT

Triple-negative breast cancer (TNBC) is a subclass of breast cancers (i.e. estrogen receptor negative, progesterone receptor negative, and HER2 negative) that have poor prognosis and very few identified molecular targets. Strikingly, a high percentage of TNBC's overexpress the epidermal growth factor receptor (EGFR), yet EGFR inhibition has yielded little clinical benefit. Over the last decade, advances in EGFR biology have established that EGFR functions in two distinct signaling pathways: 1) classical membrane-bound signaling, and 2) nuclear signaling. Previous studies have demonstrated that nuclear EGFR (nEGFR) can enhance resistance to anti-EGFR therapies and is correlated with poor overall survival in breast cancer. Based on these findings we hypothesized that nEGFR may promote intrinsic resistance to cetuximab in TNBC. To examine this question, a battery of TNBC cell lines and human tumors were screened and found to express nEGFR. Knockdown of EGFR expression demonstrated that TNBC cell lines retained dependency on EGFR for proliferation, yet all cell lines were resistant to cetuximab. Further, Src Family Kinases (SFKs) influenced nEGFR translocation in TNBC cell lines and *in vivo* tumor models, where inhibition of SFK activity led to potent reductions in nEGFR expression. Inhibition of nEGFR translocation led to a subsequent accumulation of EGFR on the plasma membrane, which greatly enhanced sensitivity of TNBC cells to cetuximab. Collectively, these data suggest that targeting both the nEGFR signaling pathway, through the inhibition of its nuclear transport, and the classical EGFR signaling pathway with cetuximab may be a viable approach for the treatment of TNBC patients.

INTRODUCTION

Approximately 15-20% of all breast cancers lack expression of the estrogen receptor, progesterone receptor, and epidermal growth factor receptor 2 (HER2), and are thus considered to be triple-negative breast cancers (TNBCs)[1,2]. While a high percentage of TNBC patients initially respond to conventional chemotherapy, they tend to have a higher rate of relapse and worse prognosis as compared to other breast cancer subtypes[1,2]. In efforts to identify new molecular targets in TNBC, various groups have performed gene expression profiling studies and identified that the epidermal growth factor receptor (EGFR) is commonly overexpressed[3-6]. While inhibition of EGFR activity has yielded modest clinical success in TNBC, substantial gains in clinical response rates have not been achieved[7,8]. Thus, improving the efficacy of anti-EGFR therapy in TNBC is imperative.

Classically, EGFR functions as a plasma membrane-bound receptor tyrosine kinase (RTK) that initiates growth and survival signals[9]. However, studies over the last 15 years have identified that EGFR can be localized and function from intracellular organelles, one of which includes the nucleus[10,11]. Within the nucleus, EGFR can function as a co-transcription factor to regulate genes involved in tumor progression[10,11], in addition to functioning as a nuclear kinase to enhance DNA replication and repair[12-14]. These nuclear functions have been linked to three parameters of tumor biology: **1)** inverse correlation with overall survival in numerous cancers[15-20], **2)** resistance to therapeutic agents including radiation[12,21-24], chemotherapy[12,13,24], and anti-EGFR therapies gefitinib[25] and cetuximab[26], and **3)** enhanced tumor growth[27,28]. These findings suggest that tumors rely on two distinct compartments of EGFR signaling to

sustain their oncogenic phenotype: **1)** classical membrane-bound EGFR signaling, and **2)** nEGFR signaling.

Previous work from our laboratory has identified that non-small cell lung cancer (NSCLC) cells that have acquired resistance to cetuximab express increased nEGFR and Src Family Kinase (SFK) activity[26,29]. SFK inhibition blocked nEGFR translocation in cetuximab resistant cells, and led to an increase in plasma membrane EGFR expression and enhanced sensitivity to cetuximab[26,30]. Further, the SFK dependent phosphorylation site on EGFR, tyrosine 1101 (Y1101), was identified to play a critical role in initiating EGFR's nuclear transport[30]. These studies suggest that nEGFR is a critical molecular determinant for cetuximab resistance and that SFK's play an important role in regulating nEGFR translocation.

Based on these previous studies, we hypothesized that nEGFR may promote intrinsic resistance to cetuximab in TNBC. To examine this question, a battery of TNBC cell lines and human tumors were screened and found to express nEGFR. While TNBC cell lines were notably resistant to cetuximab therapy, all lines retained dependency on EGFR for proliferation. Further, SFKs influenced nEGFR transport in TNBC, where the overexpression of a negative regulator of Src decreased EGFR activity at tyrosine 1101 and inhibited nEGFR translocation. Treatment of TNBC cell lines and xenograft tumors with the anti-SFK therapeutic dasatinib inhibited nEGFR translocation, and enhanced surface level EGFR accumulation. Importantly, pre-treatment of TNBC cell lines with dasatinib greatly enhanced the sensitivity of cetuximab-resistant TNBC cell lines to cetuximab. Collectively, our data suggest that abrogating nEGFR translocation with SFK inhibitors may greatly enhance the efficacy of cetuximab in TNBC.

MATERIALS AND METHODS

Cell Lines

The human breast cancer cell lines SKBr3, BT474, BT549, MDAMB231 and MDAMB468, MCF-7, and the Chinese hamster ovary cell line CHOK1 were purchased from ATCC in November 2010 (Manassas, VA, USA). SUM149, SUM229, and SUM159 were purchased from Asterand in November 2010 (Detroit, MI, USA). All cell lines were authenticated by the indicated source and not by our laboratory. All cell lines were maintained in their respective media (Mediatech Inc., VA, USA) with 1% penicillin and streptomycin; SKBr3, BT549, and MDAMB231, Dulbecco's modified Eagle's medium with 10% fetal bovine serum (FBS); BT474, RPMI 1640 with 10% FBS; SUM149, SUM229, and SUM159, F12K medium with 5% FBS, 1 μg/mL hydrocortisone and 5 μg/mL insulin; MDAMB468 and MCF-7, DMEM/F12K medium with 10% FBS; CHOK1 F12K medium with 10% FBS.

Antibodies, Compounds and TMAs

All antibodies were obtained from the following sources: EGFR (SC-03), pEGFR-1173 (SC-10168), HER2 (SC-284), SLAP (SC-1215), Histone H3 (SC-8654), HRP-conjugated goat-anti-rabbit IgG, goat-anti-mouse IgG, donkey-anti-goat IgG, EGFR blocking peptide (SC-03 P) purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). SFK (CS2123), pSFK-Y419 (CS2101), pEGFR-Y1045 (CS2237), pEGFR-Y1068 (CS3777), pHER2-Y1221/1222 (CS2243), c-Cbl (CS2747), GAPDH (CS2118), calnexin (CS2679), and anti-Flag (CS8146) purchased from Cell Signaling Technology (Beverly, MA, USA). pEGFR-Y1101 (ab76195) and EGFR (ab52894) purchased from Abcam (Cambridge, MA, USA). α-Tubulin purchased from Calbiochem (San Diego, CA,

USA). Dasatinib (BMS-354825, Sprycel) was purchased from LC Laboratories (Wobum, MA, USA) and cetuximab (C225, Erbitux™) was purchased from University of Wisconsin Pharmacy. EGF was purchased from Millipore (Billerica, MA, USA). Two human TNBC TMAs (#695711112B and #69572306) were purchased from TriStar Technology Group (Rockville, MD, USA).

Cellular Fractionation and Immunoblotting Analysis

Cellular fractionation and whole cell lysis were performed and quantitated as previously described[26,31]. ECL chemiluminescence detection system was used to visualize proteins. α-Tubulin, calnexin, and Histone H3 were used as loading and purity controls, respectively.

Immunoprecipitation

Cells were processed for IP as previously described[31]. 250 µg of protein and 2 µg of SLAP primary antibody were used for IP.

Plasmids Construction, Transfection, and siRNA Technology

The following vectors were kindly supplied: pcDNA3.0-caSrc, -wtSRC and –EGFR wild type (WT) and –EGFRY1101F, Dr. J.Boerner (Wayne State University School of Medicine, Karmanos Cancer Institute, MI, USA); pcDNA3-SLAP, Dr. S. Roche (Centre de Recherche de Biochimie Macromoléculaire, Montpellier France); pTRE2pur-HA-Fyn, -Hck, and -Lck, Dr. P.S. Mischel (University of California, San Diego). WT human pDONR223-FGR (Plasmid 23877) pDONR223-Blk (Plasmid 23940) were purchased from Addgene (Cambridge, MA, USA). pQCXIP-YES, and –LYN as previously described [30]. All SFK's were sub-cloned into the PAC1/AGEI restriction sites of the pQCXIP expression vector (Clontech, Mountain View, CA, USA). Both transient and

stable transfections were performed using Lipofectamine LTX and Opti-MEM I (Life Technology, Grand Island, NY). Stable transfection commenced 48 hr post transfection via addition of 500 ng/ml puromycin to the growth media. Single cell clones were chosen for expansion and validation for specific SFK expression.

For siRNAs, cells were transfected with 30 nM siEGFR (ON-TARGETplus, SMART pool #L-003114-00, Dharmacon, Lafayette, CO, USA) or siNon-targeting (NT) (ON-TARGETplus Non-targeting Pool, D-001810, Dharmacon) using Lipofectamine RNAiMAX (Life Technology) according to the manufacturer's instructions. Vehicle (Veh) treated cells were treated with RNAiMAX only.

Cell Proliferation Assay

Crystal violet assay and Cell Counting Kit-8 (Dojindo Molecular Technologies, Rockville, MD, USA) were performed as previously described[26,32]. Cellular proliferation was measured 72-96 hr post siRNA and 96 hour post drug treatment.

Transmission Electron Microscopy

Cells were plated on glass cover slips at ~ 90% confluency. The pre-embedding labeling method was used for processing as previously described[33]. Specifically, 0.8% Triton-X-100 was used for permeabilization and 7 ug/mL of EGFR primary antibody was used (SC-03, Santa Cruz). Cells were silver enhanced for 1.5 hr. Cells were sectioned onto copper grids at ~90 nm slices.

Immunofluorescence

Cells were processed for IF staining of EGFR as previously described[31]. Primary antibody: EGFR-SC-03, 1:100. Secondary antibody (Life technologies): Alex Fluor 546 at 1:600 for 30 min-1 hr. All cells were mounted with ProLong® Gold Antifade Reagent

with DAPI (Life Technologies). Confocal IF microscopy was performed using an A1 Nikon confocal microscope (600X). Z-slices were taken at 150 nm slices.

Nuance Imaging Analysis

For image analysis, EGFR (ab52894, 1:50) and anti-E Cadherin antibody (NCH-38, Dako at 1:100 dilution) were used for IF staining. Images were acquired on the Nuance Multispectral Imaging System (Caliper Life Sciences, 200X). A spectral library comprised of the fluorescent spectrum of each fluorophore was constructed from vehicle treated cells stained with each fluorophore individually. Images were analyzed on the inForm Image Analysis Software (Caliper Life Sciences) as previously described [34] by pathologist D.Y. Relative expression of EGFR in each compartment was expressed as a ratio of proportion of counts in the high intensity bins (bins 6 to 10) divided by the proportion of counts in the low intensity bins (bins 1 to 5).

Immunohistochemistry

Cells were processed for IHC as previously described[32]. EGFR antibody (SC-03) was used at a 1:100 dilution. The nEGFR staining pattern was scored by pathologist (D.Y) analysis at 5% increments by visual estimation at 20X magnification. Cases with at least one replicate core containing at least 5% of tumor cells demonstrating strong nuclear EGFR IHC protein expression were scored as nEGFR positive.

Flow Cytometry

Cells were processed as previously described[26]. Cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Propidium iodide was added to each sample at a final concentration of 5mg/ml. Histogram analysis was performed using FlowJo software (Tree Star Inc. Ashland, OR, USA).

Mouse Xenograft Model and Tumor Collection

Athymic nude mice (4-6 week-old females) were obtained from Harlan Laboratories (Indianapolis, IN, USA). All animal procedures and maintenance were conducted in accordance with the institutional guidelines of the University of Wisconsin. 12 mice were injected in the dorsal flank with $2x10^6$ MDAMB468 cells. Once tumors reached 100 mm^3, mice were randomized into treatment groups: vehicle (sodium citrate monobasic buffer) or dasatinib (50 mg/kg/day). Mice were treated once daily for 4 days via oral gavage. Tumor volume measurements were evaluated by digital calipers and calculated by the formula (p)/6 x (large diameter) x (small diameter)^2. Tumors were collected, processed, and stained as previously described[32,35].

Statistical Analysis

Student t-tests were used to evaluate the significance in proliferation rate between vehicle and siEGFR or drug treated cells. Student t-tests were also used to evaluate significance in nEGFR expression levels by Nuance imaging analysis between vehicle and dasatinib treated cells. Differences were considered statistically significant if $*P<0.05$. Pearson's correlation coefficient and Manders' overlap coefficient for co-localization were calculated using Nikon NIS-Elements software. Significance of strong interaction is considered for values ≥ 0.5[36].

RESULTS

TNBC cell lines and human tumors express nuclear localized EGFR

Six established TNBC cell lines were evaluated for EGFR expression (**Figure 1A**). All cell lines expressed total and activated forms of EGFR, in which the autophosphorylation status of EGFR at tyrosine 1068, 1173, and 1045 as well as the SFK specific phosphorylation site, tyrosine 1101, were evaluated. All TNBC cell lines expressed activated SFKs, as observed in previous studies[37,38] (**Figure 1A**). Total and activated HER2 expression levels were low in all TNBC cell lines compared to HER2 positive cell lines SKBr3 and BT474.

Since TNBC cell lines expressed EGFR, we hypothesized that some cell lines may also express nEGFR. Variant levels of nEGFR expression were observed in TNBC cell lines by nuclear fractionation analysis (**Figure 1B**). The harvested nuclear lysate was free from contaminating cytoplasmic and ER associated proteins, as indicated by lack of α-Tubulin and calnexin. The nuclear protein Histone H3 was used as a loading and nuclear protein purity control. In addition, confocal immunofluorescent (IF) microscopy indicated strong nEGFR immunofluorescent staining in MDAMB468, SUM229, and SUM149 cells (**Figure 1B**) by merging DAPI and Alexa Fluor 546 labeled EGFR (white arrows, magnified image). Statistical significance of co-localization was analyzed by Pearson's and Manders' correlation coefficients (significance of a strong interaction is ≥0.5)[36]. For MDAMB468, SUM229, and SUM149 the Pearson's coefficients were 0.52±0.04, 0.58±0.01, and 0.65±0.02, and the Manders' overlap coefficients were 0.70±0.02, 0.78±0.03, and 0.84±0.01, (n=50 cells). While homogenous nEGFR staining was observed in SUM149 and SUM229 cells by IF, nEGFR staining in

MDAMB468 cells was more heterogeneous. Knockdown of EGFR using siRNA or pre-incubation of primary antibody with blocking peptides led to dramatic decreases in EGFR signal. There was no signal detected from cells incubated with secondary antibody only (data not shown). We further validated nEGFR expression using transmission electron microscopy (EM) (**Figure 1C**). EGFR labeled with immunogold conjugated secondary antibodies indicated that EGFR was indeed localized in the nucleus, with localization in the nucleolus and around the nuclear envelope.

Given that nEGFR was expressed in established TNBC cell lines, we probed a human tissue microarray (TMA) containing 74 TNBC patient tumors for EGFR expression and localization. Pathologist analysis of tumors stained for EGFR via immunohistochemistry (IHC) indicated that 19% of the tumors expressed nEGFR (**Figure 1D**). Interestingly, nEGFR was highly localized to the nucleolus in greater than 5% of nEGFR positive tumors. Additionally, some tumor sections contained concentrated nEGFR, while other areas of the same tumor lacked nEGFR expression. There was no signal detected from cores stained with secondary antibody only (data not shown). Collectively, these data demonstrate that TNBC cell lines and human tumors express nEGFR.

TNBC cells are resistant to cetuximab therapy, but dependent on EGFR for proliferation

To determine the role of EGFR in TNBC proliferation, studies were performed to knock down EGFR expression in various TNBC cell lines using an EGFR directed siRNA pool. Loss of EGFR expression led to a 23-50% reduction in cell proliferation as compared to cells treated with vehicle or non-targeting (NT) siRNA (**Figure 2**). Each

cell line challenged with increasing doses of cetuximab (from 0.01nM to 100nM) demonstrated only minor reductions in proliferation. The cell lines MDAMB231 (**Figure 2B**) and MDAMB468 (**Figure 2D**) demonstrated a 15% reduction in proliferation upon treatment with 100nM of cetuximab, whereas the SUM159 (**Figure 2A**), SUM229 (**Figure 2C**) and SUM149 (**Figure 2E**) were unaffected at this dose. Additionally, TNBC cell lines treated with increasing doses of dasatinib (0.01nM-100nM) were relatively resistant to growth inhibition. These results indicate that TNBC cell lines depend on EGFR for proliferation but are relatively resistant to cetuximab.

SFKs mediate the nuclear translocation of EGFR in TNBC

Previous studies from our laboratory indicate that SFKs influence nEGFR translocation in lung cancer[26,30]. To investigate if SFKs influence EGFR translocation from the plasma membrane to the nucleus in TNBC, a constitutively active Src (caSrc) was overexpressed in SUM159, BT549, and MDAMB231 cells. The overexpression of caSrc, indicated by enhanced pSFK-Y419, led to increases in nEGFR expression (**Figure 3A**). Next, a negative regulator of Src, Src-like adaptor protein (SLAP) [39], was overexpressed in SUM149, SUM229, and MDAMB468 cells. The overexpression of SLAP, indicated by the expression of the Flag tag, led to decreases in nEGFR levels (**Figure 3B**). These studies indicate that modulation of SFK activity can influence nEGFR expression in TNBC cell lines.

Previous studies elucidating the functions of SLAP have identified that SLAP functions as an antagonist for Src induced mitogenesis partly through the binding of Src substrates and effector molecules[39]. Overexpression of SLAP resulted in the

association with EGFR in three TNBC cell lines by co-immunoprecipitation (IP) analysis (**Figure 3C**). IP with an IgG control yielded no signal (data not shown). Since EGFR deficient in tyrosine 1101 (Y1101) phosphorylation is hindered in nuclear translocation (**Figure 3C, Inset 1**)[30], we probed for phosphorylated EGFR at Y1101 post SLAP transfection. Indeed, TNBC cell lines overexpressing SLAP had decreased phosphorylation of EGFR at Y1101 (**Figure 3C**), which correlated with decreased nEGFR **(Figure 3B)**. These data demonstrate that SFK phosphorylation of EGFR at Y1101 can influence nEGFR translocation in TNBC.

Therapeutic inhibition of SFKs can block nEGFR translocation *in vitro* and *in vivo* TNBC tumor models

Since the modulation of SFK activity influenced nEGFR, the SFK inhibitor dasatinib was utilized to determine if it could abrogate EGFR translocation from the membrane to nucleus. Treatment of TNBC cells with dasatinib led to potent decreases in nEGFR levels (at 24 and 72 hours in SUM149 and SUM229, and at 72 hours in MDAMB468 cells) (**Figure 4A**). Analysis of whole cell lysate indicated that EGFR activity on Y1101 was inhibited by dasatinib at both time points. Additionally, dasatinib treatment led to subsequent increases in non-nEGFR levels (**Figure 4A**). Nuance imaging and Inform software was further used to analyze nEGFR levels post dasatinib treatment (**Figure 4B**). Cells were stained for EGFR, E-Cadherin and DAPI; E-Cadherin and DAPI were used to create a spectral library that segmented each cell into cytoplasm and nucleus as previously described [34]. InForm software analysis of each cell line (n=2) demonstrated that dasatinib treated cells trended towards less nEGFR

staining as compared to vehicle treated cells (p=0.08 at 48 hr).

To further characterize the effect of dasatinib on non-nEGFR expression, surface level EGFR was analyzed by flow cytometry. TNBC cells treated with dasatinib for 24 hours contained 30-42% more plasma membrane-bound EGFR as compared to vehicle treated cells (**Figure 4C**). There was no additional increase in EGFR surface expression 72 hours post treatment (data not shown). Together, these data suggest that EGFR accumulates on the plasma membrane when nEGFR translocation is blocked by dasatinib.

To investigate if therapeutic inhibition of SFKs can abrogate nEGFR translocation *in vivo*, MDAMB468 cells were established as xenograft tumors in female athymic nude mice. Mice were randomized into two groups receiving 50 mg/kg of dasatinib or vehicle once daily for 4 days. **Figure 4D** represents confocal IF analyses of representative tumor sections harvested from either vehicle or dasatinib treated mice stained for EGFR. EGFR was highly nuclear localized in tumors from vehicle treated mice. However, tumors harvested from dasatinib treated mice harbored much less nEGFR, with a noticeable increase in surface EGFR expression. Immunoblot analysis of harvested tumors validated that dasatinib inhibited SFK activity; one dasatinib treated tumor (#5) contained less total EGFR expression. The inhibition of nEGFR translocation was also visualized by IHC staining of tumors harvested from dasatinib treated mice (**Figure 4E**). Interestingly, we found that dasatinib treatment of mice harboring colorectal tumors also contained less nEGFR expression within the tumor (**Figure 5**), suggesting that SFKs may influence nEGFR translocation in different tumor types. Collectively, these data indicate that SFK inhibition prevents nEGFR translocation and

enhances membrane accumulation of EGFR *in vivo*.

SFK inhibition can sensitize TNBC cells to cetuximab growth inhibition

Since SFK inhibition enhanced plasma membrane-bound EGFR expression, we hypothesized that TNBC cells may become more sensitive to cetuximab upon pre-treatment with dasatinib. To investigate this, we performed proliferation assays after pre-treating TNBC cells with dasatinib or vehicle for 24 hours, the time point at which an increase in surface level EGFR was detected, and subsequently treating cells with increasing doses of cetuximab for an additional 72 hours (**Figure 6**). All cell lines pre-treated with vehicle and subsequently treated with increasing doses of cetuximab demonstrated minor reductions in proliferation, consistent with data in **Figure 2**. Additionally, cells treated with 25 nM dasatinib monotherapy did not exhibit significant inhibition of proliferation. However, TNBC cell lines that received dasatinib for 24 hours prior to cetuximab treatment demonstrated significant reductions in proliferation over a wide range of cetuximab doses (1 nM to 100 nM). SUM149 and SUM229 cells demonstrated significant reductions in proliferation at low doses of cetuximab (1 nM), while MDAMB468 and MDAMB231 cells exhibited proliferation inhibition at higher doses of cetuximab (10 nM and 100 nM). Collectively, these data suggest that the blockade of nEGFR translocation via SFK inhibition can increase TNBC cell sensitivity to cetuximab.

DISCUSSION

TNBC is a subset of breast cancers that commonly overexpress the EGFR[3-6]. Unfortunately, clinical trials targeting EGFR with cetuximab have yielded minimal benefit in TNBC[7,8], even with the addition of platinum based chemotherapies[1,5,36]. Thus, understanding why TNBCs are intrinsically resistant to cetuximab has become an important clinical question. Over the last decade, numerous studies have identified a role for nEGFR in resistance to anti-EGFR agents[25,26]. Previous studies from our laboratory demonstrated that NSCLC cell lines that had acquired resistance to cetuximab relied on nEGFR signaling to maintain their resistant phenotype [26]. Based on these studies, we hypothesized that nEGFR may be a critical molecular determinant for cetuximab resistance in TNBC.

In the current study, nEGFR was detected in a panel of established TNBC cell lines and human tumors (**Figure 1)**. In prior studies, 38% of a 130-breast cancer patient cohort[15] and 40% of a 113–breast cancer patient cohort[19] stained positive for nEGFR, which was further correlated with worse overall survival. The heterogeneity observed in nEGFR expression in the current study of TNBC tumors highlights the importance of simultaneously targeting both nEGFR and non-nEGFR cell populations. Another interesting observation lies in the localization of EGFR in the nucleolus, functions that have yet to be investigated and may be playing important roles in TNBC pathogenesis. Collectively, the preclinical data presented in the current study suggest that nEGFR may be indicative of cetuximab resistant tumors warranting further investigation for its role as a predictive marker for cetuximab response in TNBC.

Recent work from our laboratory has found that SFK dependent phosphorylation

of EGFR on Y1101 is a necessary and early event for EGFR translocation from the plasma membrane to the nucleus[30]. The current study aimed to identify if this mechanism of nuclear translocation was present in TNBC. We found that three TNBC cell lines (MDAMB468, SUM149 and SUM229) with the highest levels of phosphorylated Y1101 also expressed the highest levels of nEGFR (**Figure 1A and 1B**). Additionally, inhibition of SFK activity led to decreased phosphorylation of EGFR on Y1101 and reduced nEGFR levels (**Figure 3C and 4A, 4B**). Interestingly, **Figure 4C** indicates that surface level EGFR was enhanced within 24 hours of dasatinib treatment, even though a decrease in nEGFR expression was more prominent at later time points post treatment (**Figure 4A and 4B**); this suggests that the rate of nEGFR export, via its nuclear export sequence[28], varies between cell lines. Collectively, these data suggest that SFK phosphorylation of EGFR on Y1101 may be a critical step for EGFR nuclear translocation in TNBC.

In the current study, SFK inhibition of nEGFR translocation led to an accumulation of plasma membrane-bound EGFR and sensitization to cetuximab therapy (**Figure 4-6**). Recent studies support our findings, where anti-tumorigenic effects of both cetuximab and dasatinib dual treatment with chemotherapy[40] and the use of non-competitive monoclonal antibodies degrading the EGFR[41] have been documented in TNBC. Additionally, a recent report demonstrated that targeting PCNA, a nEGFR substrate, could delay TNBC tumor growth [42]. In the current study, sensitization to cetuximab was observed after pre-treatment of TNBC cells with dasatinib for 24 hours, the time point at which EGFR accumulation was detected on the plasma membrane due to the inhibition of nEGFR translocation. We speculate that the inhibition

of nEGFR translocation drives TNBC cells to rely solely on classical membrane-bound EGFR signaling for sustained proliferation and survival signals; thus, TNBC cells become sensitized to cetuximab because cetuximab can abrogate classical EGFR signaling pathways (**Figure 7**). Previous studies in EGFR expressing NSCLC and HNSCC cell lines support this, where cell lines that lacked nEGFR expression were found to be more sensitive to cetuximab monotherapy[26,30]. Currently, the growth inhibitory effect of cetuximab and dasatinib therapy is being accessed in vivo TNBC models in our laboratory, a critical step for the movement of this proposed drug combination into clinical trials. Collectively, the data presented herein indicates that the dual targeting of both nEGFR and plasma membrane-bound EGFR is necessary for the complete inhibition of EGFR's oncogenic functions, a therapeutic strategy that can be readily translated for the treatment of nEGFR expressing TNBC patients.

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Figure 1: TNBC cell lines and human tumors express nuclear localized EGFR. (A) TNBC cells express total and activated forms of EGFR. Whole cell lysate was harvested from six TNBC cell lines and two HER2 positive cell lines. α-Tubulin was used as a loading control. **(B) TNBC cells express nEGFR.** Cell lines were harvested for nuclear proteins. α-Tubulin, calnexin, and Histone H3 were used as loading and purity controls, respectively. Confocal IF microscopy depicts nEGFR expression. Merged images were magnified to depict nEGFR (Confocal zoom, white arrows). A single Z-Slice image depicts overlap between blue and red signal (white dashed-line boxes). Magnification 600X. EGFR primary antibody specificity was validated with siEGFR and blocking peptides. **(C) Immunogold labeling of nEGFR.** TNBC cells were fixed and processed for transmission EM. CY, cytoplasm; NE, nuclear envelope; NUC, nucleus; NOS, nucleolus. Images were digitally zoomed to highlight gold particles in the nucleus (black arrows). **(D) Human TNBC tumors express nEGFR.** IHC staining for EGFR was performed on a total of 74 TNBC patient tumor sections. Representative cases demonstrating nEGFR expression are depicted (black arrows).

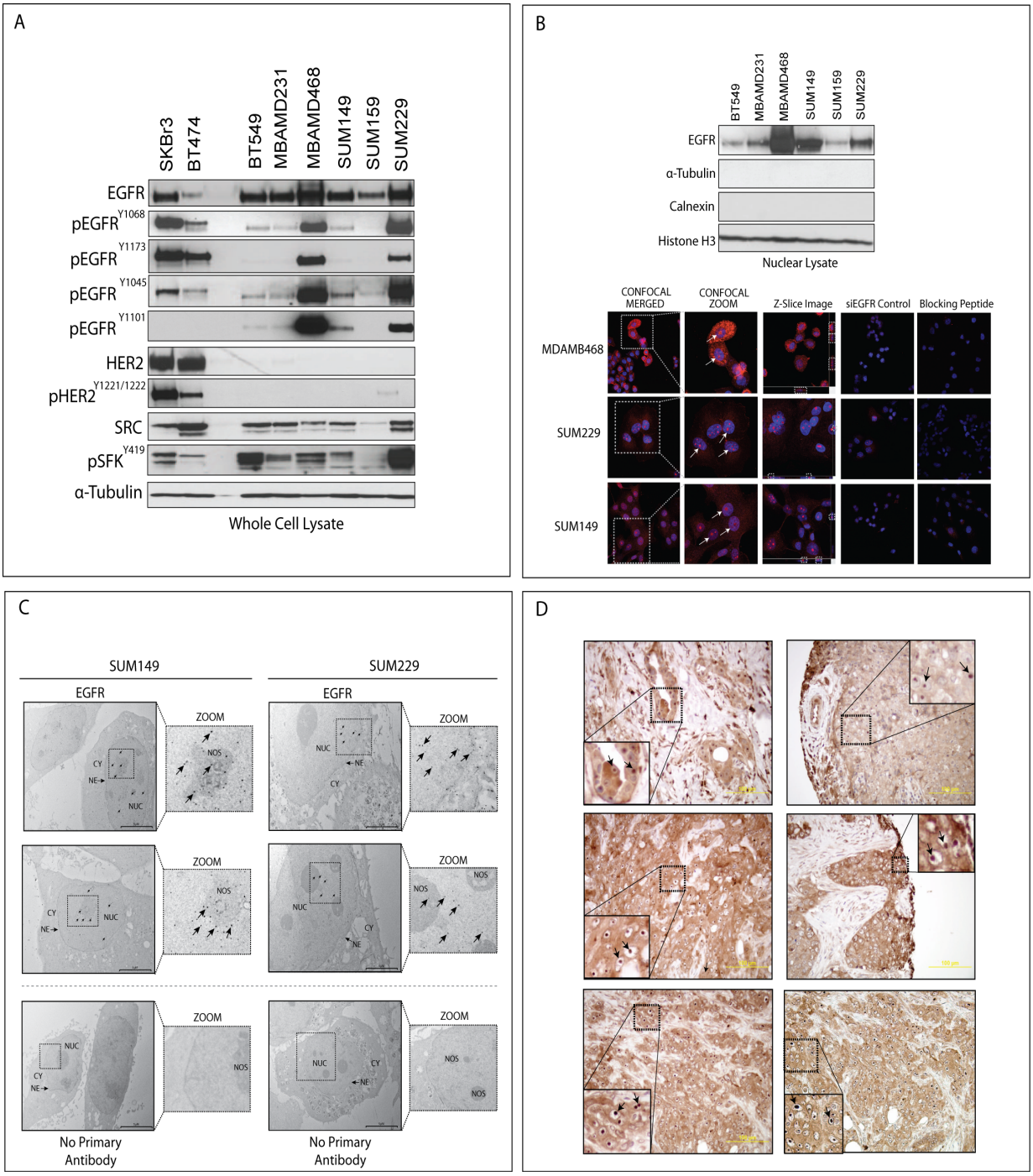

A
SKBr3
BT474
BT549
MBAMD231
MBAMD468
SUM149
SUM159
SUM229
EGFR
pEGFR Y1068
pEGFR Y1173
pEGFR Y1045
pEGFR Y1101
HER2
pHER2 Y1221/1222
SRC
pSFK Y419
α-Tubulin
Whole Cell Lysate
B
BT549
MBAMD231
MBAMD468
SUM149
SUM159
SUM229
EGFR
α-Tubulin
Calnexin
Histone H3
Nuclear Lysate
CONFOCAL MERGED
CONFOCAL ZOOM
Z-Slice Image
siEGFR Control
Blocking Peptide
MDAMB468
SUM229
SUM149
C
SUM149
SUM229
EGFR
ZOOM
CY
NE
NUC
NOS
No Primary Antibody
D

Figure 2: TNBC cell lines are dependent on EGFR for proliferation, but are intrinsically resistant to cetuximab and dasatinib. Cell lines were incubated with siEGFR, non-targeting (NT) siRNA, or vehicle for 72-96 hr prior to performing proliferation assays (A-E). Cells were treated with cetuximab or dasatinib at indicated doses for the same time course. Proliferation is plotted as a percentage of growth relative to vehicle treated cells (n=3). Whole cell lysate was harvested from all cell lines at the same time point to confirm knockdown of EGFR. Data points are represented as mean±s.e.m. **p<0.01.

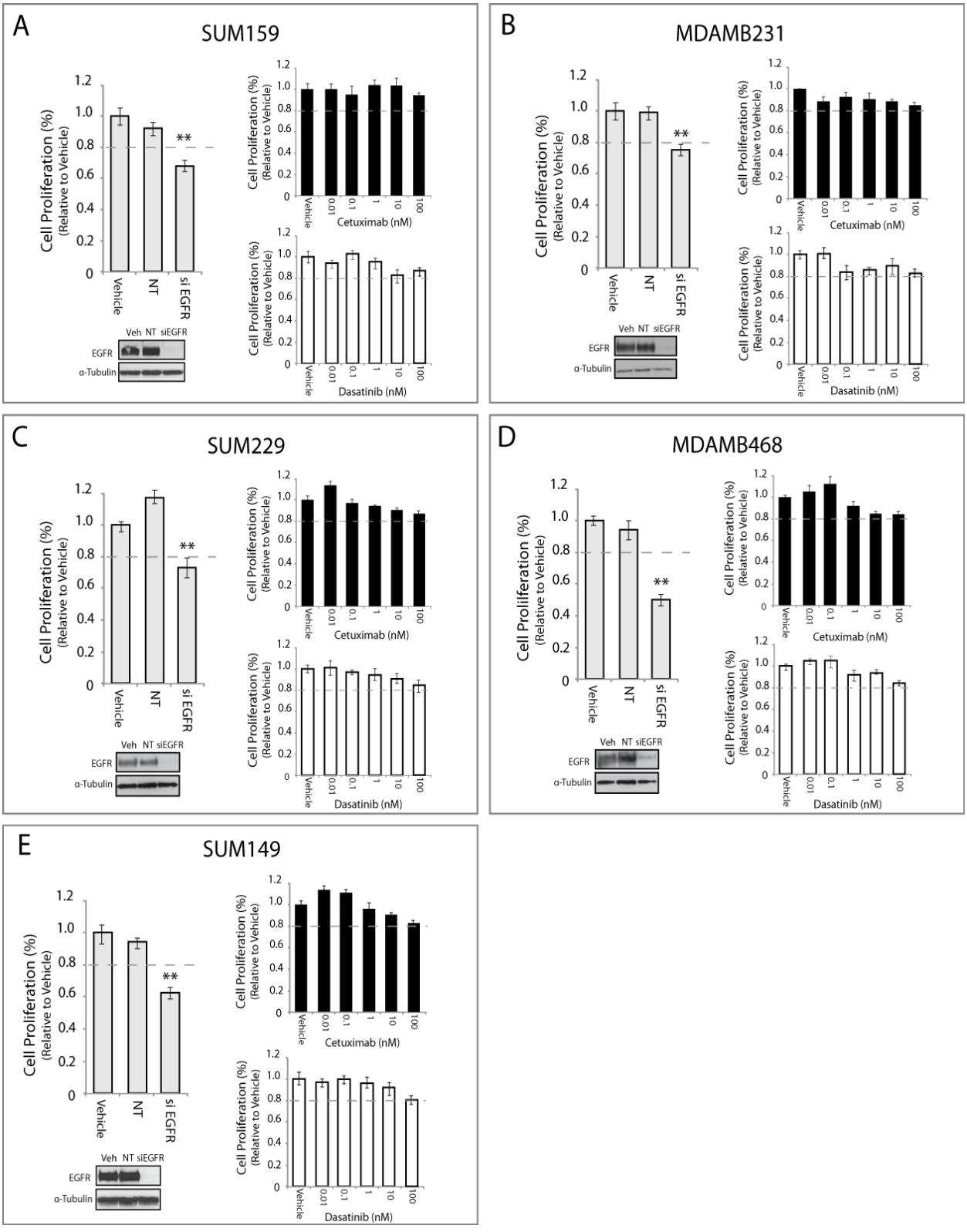

A
SUM159
Cell Proliferation (%)
(Relative to Vehicle)
Vehicle
NT
si EGFR
**
Veh NT siEGFR
EGFR
α-Tubulin
Cetuximab (nM)
Dasatinib (nM)
B
MDAMB231
C
SUM229
D
MDAMB468
E
SUM149

Figure 3. Src Family Kinases mediate nEGFR translocation in TNBC. (A) Constitutively active Src (caSrc) enhances nEGFR translocation in TNBC cell lines. Cells were transfected with caSrc or an empty vector control for 48 hr prior to stimulation with EGF (5 nM, 45 min) to induce nEGFR translocation. Non-nuclear and nuclear proteins were harvested. nEGFR expression was quantitated using ImageJ software **(B) A negative regulator of Src, src-like adaptor protein (SLAP), blocks nEGFR translocation in TNBC cell lines.** Cells were transfected with SLAP-FLAG or an empty vector control for 48 hr prior to harvesting non-nuclear and nuclear proteins. nEGFR expression was analyzed. **(C) SLAP can interact with EGFR and decrease EGFR activation at Tyrosine 1101.** Cells were transfected with SLAP-FLAG or an empty vector control for 48 hr prior to harvesting whole cell lysate. 250 ug of cell lysate was immunoprecipitated with an anti-SLAP antibody. The same lysate was subjected to immunoblot analysis for activation of EGFR at Tyrosine 1101. pEGFR-Y1101 activity was quantitated using ImageJ software. **Inset 1: EGFR mutated at Tyrosine 1101 is deficient in nuclear localization.** Vector, EGFR-WT, and EGFR-Y1101F were transfected into CHOK1 cells for 48 hr prior to stimulation with EGF (5 nM, 45 min). Non-nuclear and nuclear proteins were harvested, and nEGFR expression was analyzed.

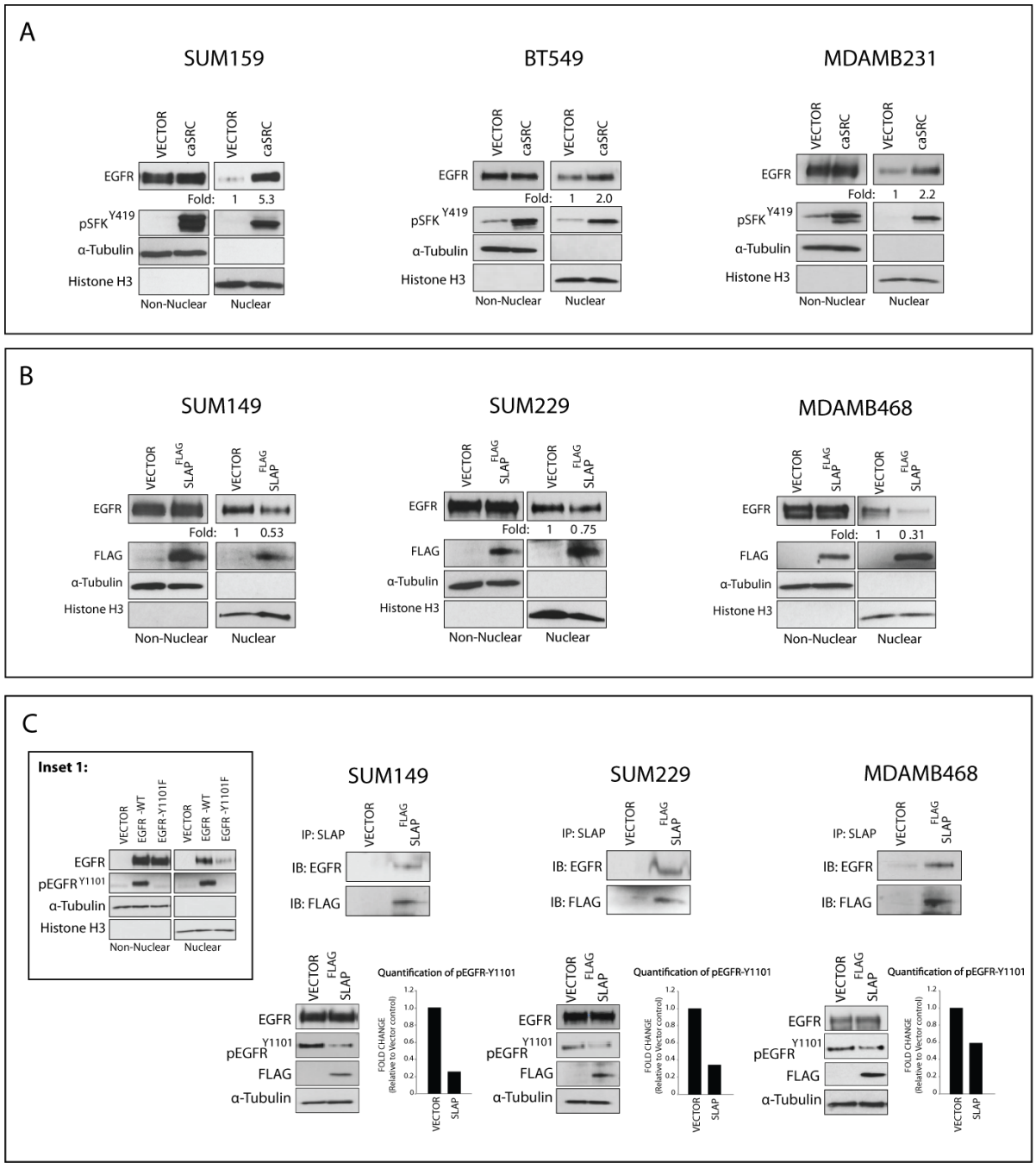

A
SUM159
BT549
MDAMB231
VECTOR
caSRC
EGFR
Fold: 1 5.3
Fold: 1 2.0
Fold: 1 2.2
pSFK Y419
α-Tubulin
Histone H3
Non-Nuclear
Nuclear
B
SUM149
SUM229
MDAMB468
FLAG SLAP
Fold: 1 0.53
Fold: 1 0.75
Fold: 1 0.31
FLAG
C
Inset 1:
EGFR -WT
EGFR-Y1101F
pEGFR Y1101
IP: SLAP
IB: EGFR
IB: FLAG
Quantification of pEGFR-Y1101
FOLD CHANGE (Relative to Vector control)

Figure 4. Therapeutic inhibition of SFKs can block nEGFR translocation in TNBC cell lines and tumor models. (A) Dasatinib can inhibit nEGFR translocation and enhance non-nuclear EGFR levels. Cells were treated with vehicle or dasatinib (25 nM) for 24 and 72 hr prior to harvesting whole cell, non-nuclear and nuclear proteins. **(B) Dasatinib can block nEGFR translocation measured by Nuance imaging analysis.** Cells were treated with vehicle or dasatinib (50 nM) for 24 and 48 hr prior to staining for EGFR, E-Cadherin, and DAPI. Nuclear EGFR fluorescence detected from dasatinib treated cells was normalized to nEGFR fluorescence detected from vehicle treated cells using InForm software (n=2). **(C) Dasatinib can enhance plasma membrane-bound EGFR levels measured by flow cytometry.** Cells were treated with dasatinib (25 nM) for 24 hr prior to EGFR surface level analysis. Surface level EGFR expression of dasatinib treated cells was normalized to vehicle treated cells (n=3). Shaded histogram= vehicle treated cells, Non-shaded histograms= dasatinib treated cells. IgG treated cells are used as a control (dotted line). **(D, E) Dasatinib can block nEGFR translocation in MDAMB468 xenograft tumors.** Mice with established MDAMB468 tumors were treated with 50 mg/kg of dasatinib or vehicle once a day for 4 days. Tumors were analyzed by confocal IF **(D)** and IHC **(E)** for EGFR expression. IF: merged images were magnified to depict nEGFR (arrows) and non-nEGFR (triangle). Magnification 600X for IF and 400X for IHC. Four tumors from vehicle (tumor # 1-4) or dasatinib treated mice (tumor # 5-8) were harvested for protein and analyzed for the indicated proteins. Data points are represented as mean±s.e.m. **p<0.01.

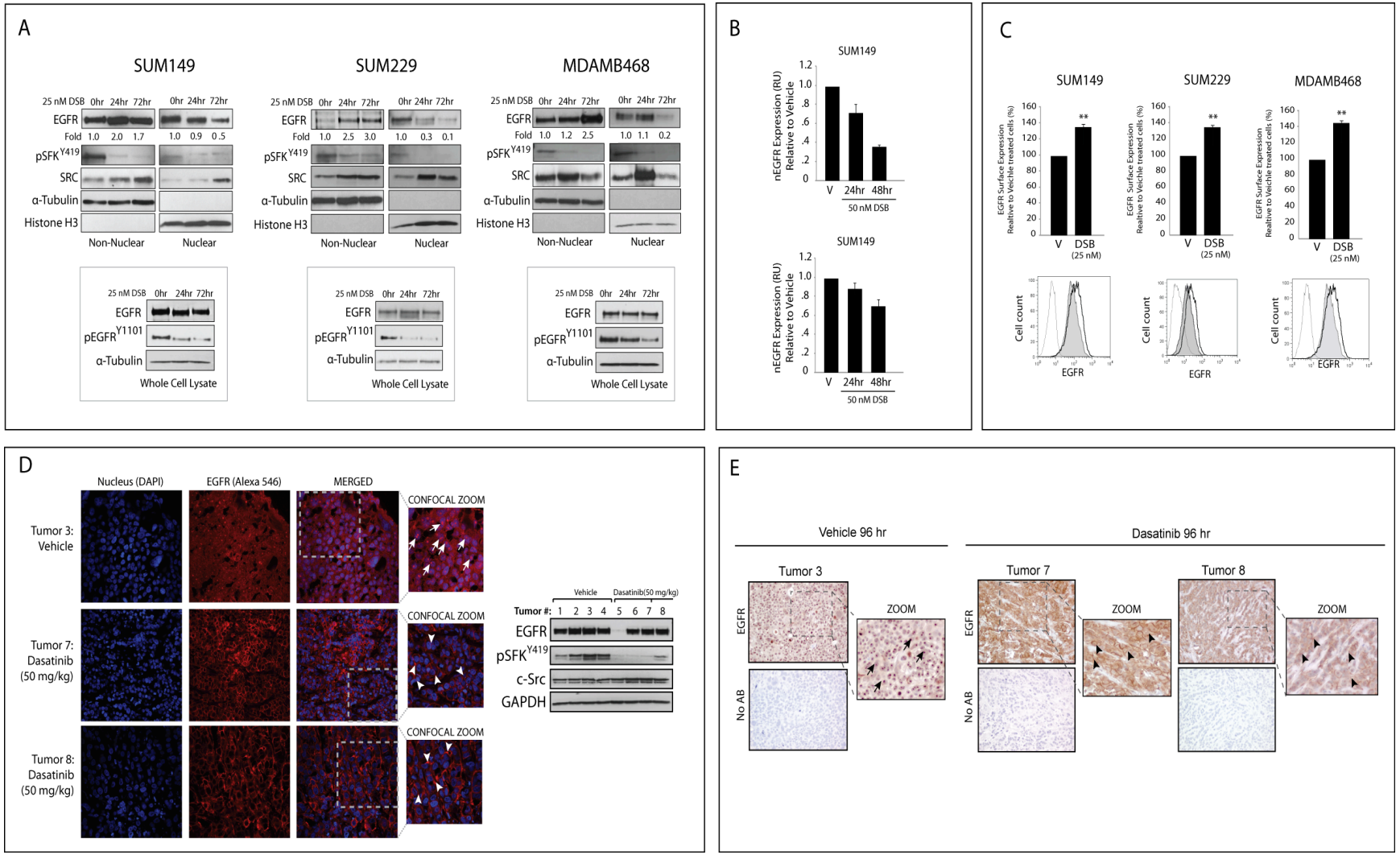

A
SUM149
SUM229
MDAMB468
25 nM DSB 0hr 24hr 72hr
EGFR
Fold 1.0 2.0 1.7 1.0 0.9 0.5
pSFK Y419
SRC
α-Tubulin
Histone H3
Non-Nuclear
Nuclear
Fold 1.0 2.5 3.0 1.0 0.3 0.1
Fold 1.0 1.2 2.5 1.0 1.1 0.2
pEGFR Y1101
Whole Cell Lysate
B
SUM149
nEGFR Expression (RU) Relative to Vehicle
V 24hr 48hr
50 nM DSB
C
EGFR Surface Expression Realtive to Veichle treated cells (%)
V DSB (25 nM)
Cell count
EGFR
D
Nucleus (DAPI)
EGFR (Alexa 546)
MERGED
CONFOCAL ZOOM
Tumor 3: Vehicle
Tumor 7: Dasatinib (50 mg/kg)
Tumor 8: Dasatinib (50 mg/kg)
Vehicle
Dasatinib(50 mg/kg)
Tumor #: 1 2 3 4 5 6 7 8
c-Src
GAPDH
E
Vehicle 96 hr
Dasatinib 96 hr
Tumor 3
Tumor 7
Tumor 8
ZOOM
No AB

Figure 5. Therapeutic inhibition of SFKs can block nEGFR translocation in colorectal tumor models. Established colorectal tumors were treated with 50 mg/kg of dasatinib or vehicle once a day for 5 days. Tumors were collected from mice and analyzed for EGFR localization via IHC. Nuclear EGFR staining is highlighted in tumors harvested from vehicle treated mice (black arrows in dashed-line box). Magnification 400X. **Inset 1:** Colorectal cells were harvested for non-nuclear and nuclear proteins and analyzed for nEGFR expression.

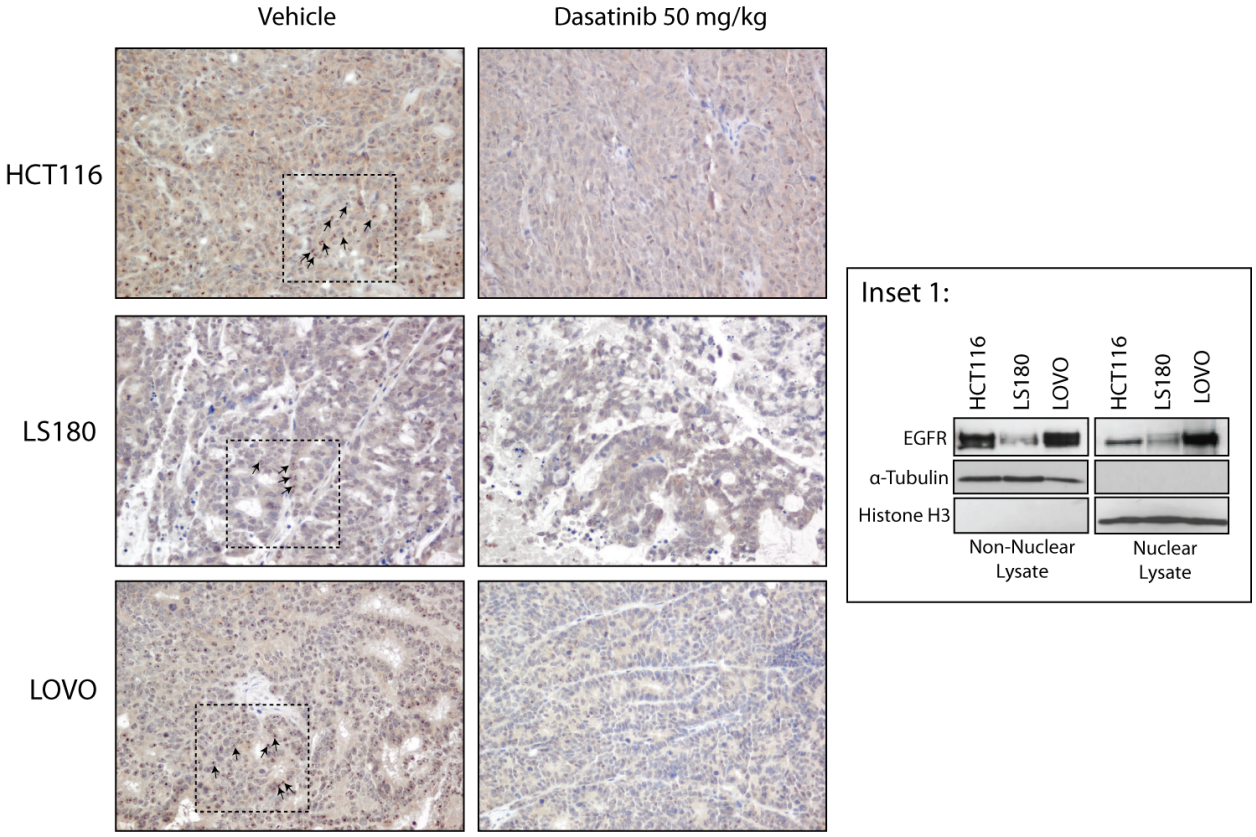

Vehicle
Dasatinib 50 mg/kg
HCT116
LS180
LOVO
Inset 1:
HCT116
LS180
LOVO
HCT116
LS180
LOVO
EGFR
α-Tubulin
Histone H3
Non-Nuclear Lysate
Nuclear Lysate

Figure 6. Therapeutic inhibition of SFK activity can sensitize TNBC cells to cetuximab. Cells were pretreated with vehicle or dasatinib (25 nM) for 24 hr prior to adding cetuximab to the growth medium at the indicated doses (1 nM, 10 nM, and 100 nM) for an additional 72 hr. Proliferation assays were performed and plotted as a percentage of growth relative to vehicle treated cells (n=3). Data points are represented as mean±s.e.m. **p<0.01.

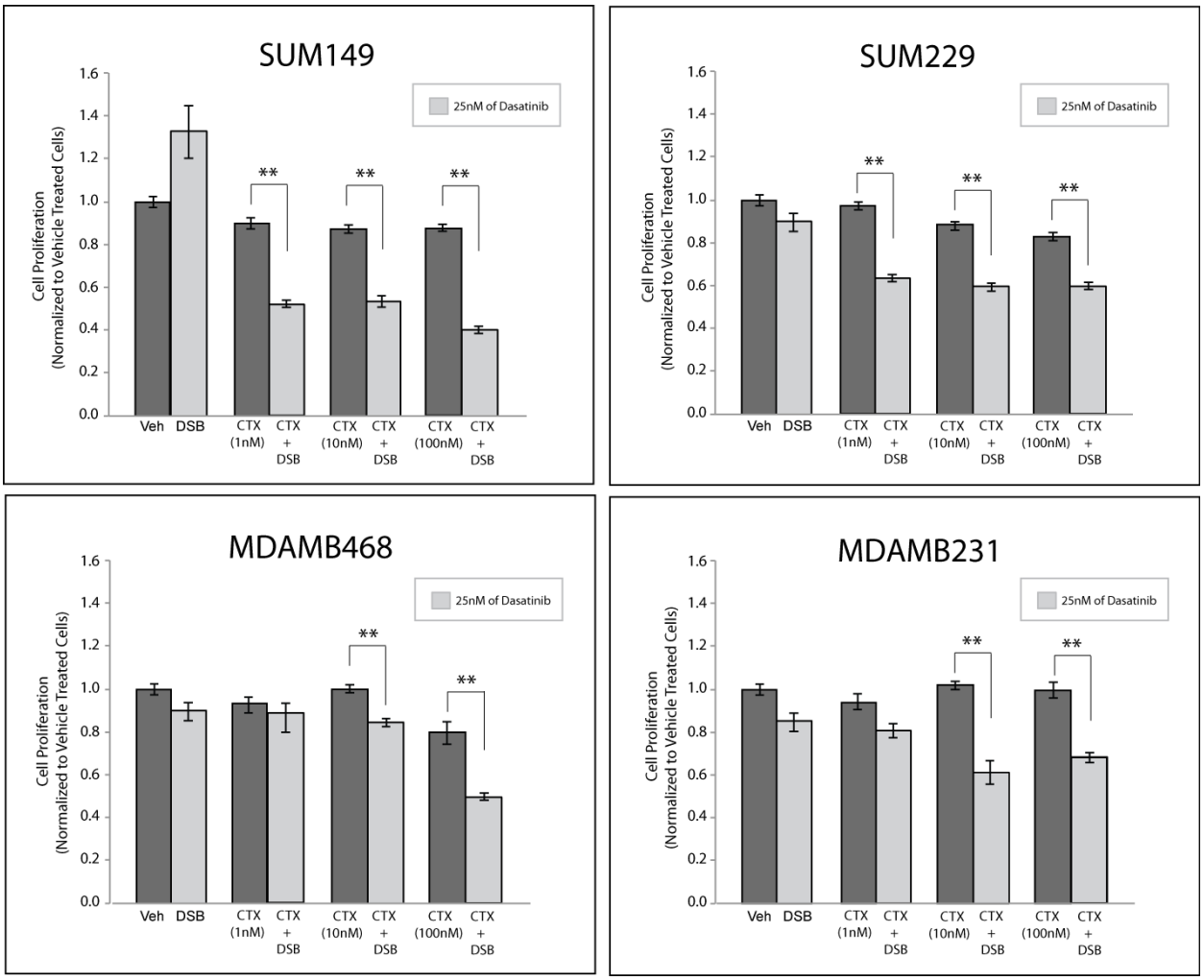
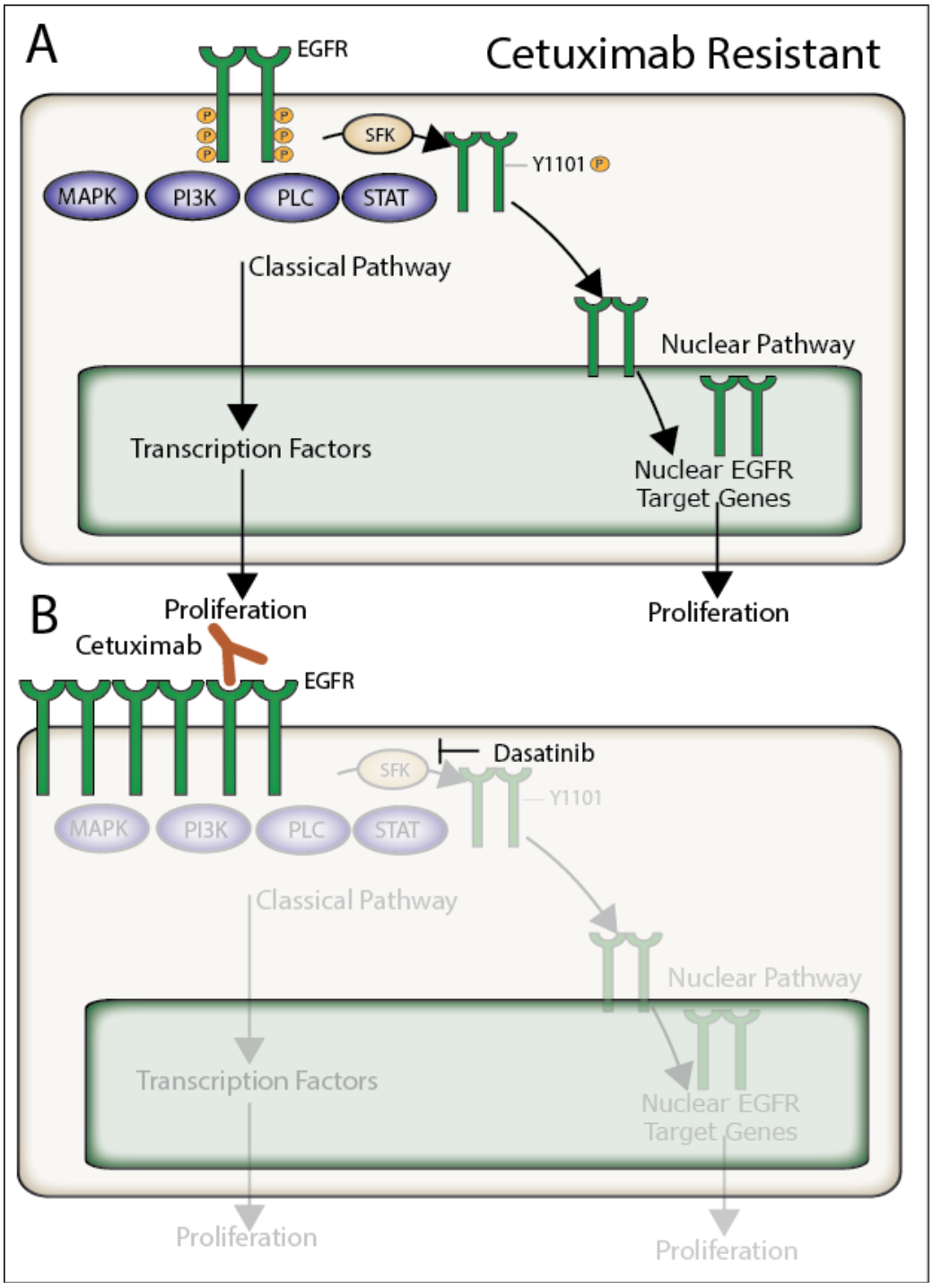

SUM149
SUM229
MDAMB468
MDAMB231
25nM of Dasatinib
Cell Proliferation
(Normalized to Vehicle Treated Cells)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
Veh
DSB
CTX (1nM)
CTX + DSB
CTX (10nM)
CTX + DSB
CTX (100nM)
CTX + DSB

Figure 7. The dual targeting of both nEGFR and plasma membrane-bound EGFR is necessary for the complete inhibition of EGFR's oncogenic functions in TNBC. TNBC cells that are intrinsically resistant to cetuximab therapy are characterized by expressing both membrane-bound EGFR and nEGFR receptors, both of which can signal down cell proliferation pathways influencing cetuximab resistance **(A)**. Inhibition of nEGFR signaling, through the abrogation of its trafficking to the nucleus with dasatinib, in addition to targeting is plasma membrane-bound signaling component with cetuximab is necessary to inhibit both signaling components of the EGFR and thereby an anti-proliferative response is observed in TNBC cells **(B).**


A
EGFR
Cetuximab Resistant
SFK
Y1101
MAPK
PI3K
PLC
STAT
Classical Pathway
Nuclear Pathway
Transcription Factors
Nuclear EGFR
Target Genes
Proliferation
Proliferation
B
Proliferation
Cetuximab
EGFR
Dasatinib
SFK
Y1101
MAPK
PI3K
PLC
STAT
Classical Pathway
Nuclear Pathway
Transcription Factors
Nuclear EGFR
Target Genes
Proliferation
Proliferation

Chapter IV

Mapping C-terminal transactivation domains of the nuclear HER family receptor tyrosine kinase HER3

Toni M. Brand and Deric L. Wheeler

TM Brand, M Iida, N Luthar, M Wleklinski, MM Starr, and DL Wheeler, PLoS ONE, 8(8): e71518 (2013)

ABSTRACT

Nuclear localized HER family receptor tyrosine kinases (RTKs) have been observed in primary tumor specimens and cancer cell lines for nearly two decades. Inside the nucleus, HER family members (EGFR, HER2, and HER3) have been shown to function as co-transcriptional activators for various cancer-promoting genes. However, the regions of each receptor that confer transcriptional potential remain poorly defined. The current study aimed to map the putative transactivation domains (TADs) of the HER3 receptor. To accomplish this goal, various intracellular regions of HER3 were fused to the DNA binding domain of the yeast transcription factor Gal4 (Gal4DBD) and tested for their ability to transactivate Gal4 UAS-luciferase. Results from these analyses demonstrated that the C-terminal domain of HER3 (CTD, amino acids distal to the tyrosine kinase domain) contained potent transactivation potential. Next, nine HER3-CTD truncation mutants were constructed to map minimal regions of transactivation potential using the Gal4 UAS-luciferase based system. These analyses identified a bipartite region of 34 (B_1) and 27 (B_2) amino acids in length that conferred the majority of HER3's transactivation potential. Next, we identified full-length nuclear HER3 association and regulation of a 122 bp region of the cyclin D1 promoter. To understand how the B_1 and B_2 regions influenced the transcriptional functions of nuclear HER3, we performed cyclin D1 promoter-luciferase assays in which HER3 deleted of the B_1 and B_2 regions was severely hindered in regulating this promoter. Further, the overexpression of HER3 enhanced cyclin D1 mRNA expression, while HER3 deleted of its identified TADs was hindered at doing so. Thus, the ability for HER3 to function as a transcriptional co-activator may be dependent on specific C-terminal TADs.

INTRODUCTION

The ErbB/HER family of receptor tyrosine kinases (RTKs) consists of four family members: the epidermal growth factor receptor (EGFR/ErbB1), HER2 (ErbB2/Neu), HER3 (ErbB3), and HER4 (ErbB4). This family of RTKs has been highly implicated in the formation and progression of various cancers via aberrant overexpression, kinase activation, and mutation[1,2]. Classically, HER family members function from the cell surface, where binding to cognate ligands can induce receptor homo- or hetero-dimerization with other HER family receptors[3]. The HER2 receptor does not bind to any known ligands, however, its dimerization arm is innately positioned in an open conformation. This process leads to the activation of each receptors' tyrosine kinase and the subsequent phosphorylation of tyrosine residues located on their C-terminal tails. Phosphorylated tyrosine residues recruit various intracellular adaptor and effector molecules that result in the propagation of growth promoting signal transduction cascades[1,2]. Membrane-bound HER receptors activate numerous tumor promoting signaling cascades via this mechanism, including the PI3K/AKT, Ras/Raf/Mek/Erk, PLCγ/PKC, and signal transducer and activator of transcription (STAT) pathways[1,2].

While the classical membrane-bound functions of HER family RTKs have been extensively studied, accumulating data suggest that these receptors can be found in the cell's nucleus where they can function as co-transcriptional activators[4,5]. To date, EGFR, HER2, and a nuclear variant of HER3 have been shown to function as co-transcriptional activators for cyclin D1[6-8]. Clinically, nuclear EGFR has been correlated with poor overall survival in breast [9,10], ovarian[11], oropharyngeal[9,12], and gallbladder[13] cancers. Nuclear EGFR has also been shown to play a role in resistance to numerous

cancer therapies, including radiation[14-18], cisplatin[17-19], and the anti-EGFR therapies cetuximab[20] and gefitinib[21]. Collectively, these pivotal studies suggest that nuclear HER family receptors may enhance the tumorigenic phenotype of cancer cells, and therefore their nuclear roles must be further elucidated.

The HER3 receptor has recently come to the forefront as playing a key role in HER family driven cancers[22,23]. HER3 is a unique HER family member in that it has diminished tyrosine kinase activity due to the lack of specific amino acids within its kinase domain[24,25]. However, HER3 plays a crucial role as an allosteric activator of other HER family members in addition to functioning as a signaling substrate through the direct recruitment and activation of PI3K[26,27]. With the discovery of the various functions of nuclear EGFR and HER2, recent interest has prompted the investigation of nuclear HER3. HER3 was first identified to be nuclear localized in normal and malignant mammary epithelial cells in 2002[28]. HER3 was also shown to be prominently nuclear localized in malignant prostate cancer tissues, where it was correlated with risk of disease progression[29]. Most recently, two nuclear C-terminal splice variants of HER3 were identified and shown to function as co-transcriptional activators[8,30]. Thus, the functions of nuclear HER3 are just beginning to unfold.

In the current study we focused on identifying the amino acid regions on the C-terminal tail of HER3 that function as transactivation domains (TADs). First, numerous cancer cell lines were characterized for the expression of HER3, which was prominently nuclear localized in its full-length form. Next, various HER3 intracellular cytoplasmic domain (ICD) regions fused to the Gal4 DNA binding domain (Gal4DBD) were analyzed, and the C-terminal domain (CTD, amino acids distal to the tyrosine kinase

domain) of HER3 was shown to activate the Gal4 upstream activation sequence (UAS) fused to luciferase. To identify specific C-terminal TADs, we created nine HER3-CTD truncation mutants and identified a bipartite region of 34 and 27 amino acids in length (denoted as B_1 and B_2) that contained the majority of HER3's transactivation potential. To determine if the identified B_1 and B_2 TADs could augment nuclear HER3's transcriptional function in cells, we first identified that full-length nuclear HER3 could associate and activate a 122 bp region of the cyclin D1 promoter when overexpressed. Importantly, the overexpression of HER3 lacking the B_1 and B_2 regions was severely hindered both in activating the 122 bp region of the cyclin D1 promoter and in enhancing transcription from the endogenous cyclin D1 gene. Collectively, these data suggest that this bipartite region of HER3 functions as a prominent TAD to mediate HER3's nuclear functions.

MATERIALS AND METHODS

Cell Lines

The human breast cancer cell line MCF-7, human colorectal cancer (CRC) cell lines LoVo, CaCO2, and CHOK1 cells were purchased from ATCC (Manassas, VA, USA). The human NSCLC line NCI-H226^{R} was developed as a cetuximab resistant cell line previously described[31,32]. The human breast cancer cell lines SKBr3, BT474, HCC1954, and BT549 were kindly supplied by Dr. J. Boerner (Wayne State University School of Medicine, Karmanos Cancer Institute, MI, USA)[33], the human NSCLC cell line H522 was kindly supplied by Dr. R. Salgia (The University of Chicago Medical Center, IL, USA)[34], and human HNSCC cell lines SCC-1 and SCC-6 were kindly supplied by Dr. T. Carey (University of Michigan, MI, USA)[35]. All cell lines were maintained in their respective media with 10% fetal bovine serum and 1% penicillin and streptomycin; SKBr3, BT549, SCC-1, and SCC-6 were maintained in Dulbecco's modified Eagle's medium (Mediatech Inc., Manassas, VA, USA); BT474, HCC1954, and H226^{R} were maintained in RPMI 1640 (Mediatech Inc.); CaCO2 was maintained in minimum essential medium eagle (Mediatech Inc.); LoVo and CHOK1 cells were maintained in F12K medium (Mediatech Inc.); MCF-7 cells were maintained in DMEM/F12K medium (Mediatech Inc.).

Antibodies and Compounds

All antibodies were obtained from the following sources: HER3-C- TERM (SC-285), HER3-N-TERM (SC-292557), Histone H3 (SC-28654), EGFR (SC-03), HER2 (SC-284), Gal4DBD (SC-510), HRP-conjugated goat-anti-rabbit IgG, and HRP-conjugated goat-anti mouse IgG were purchased from Santa Cruz Biotechnology Inc.

(Santa Cruz, CA, USA). EGFR (1902-1, EP38Y) was purchased from Epitomics/Abcam (Cambridge, MA, USA). pHER3-Y1222 (#4784), pHER3-Y1289 (#4791), and GAPDH (#2118) were purchased from Cell Signaling Technology (Beverly, MA, USA). α-tubulin was purchased from Calbiochem (San Diego, CA, USA). Neuregulin-1 (NRG) was purchased from R&D Systems (Minneapolis, MN, USA).

Cellular Fractionation and Immunoblotting Analysis

Cellular fractionation was performed as previously described[6,36]. Cells were plated in either 10 cm or 15 cm dishes. At ~80-90% confluency, cells were scraped in PBS and swelled in cytoplasmic lysis buffer (20 mM HEPES, pH 7.0, 10 mM KCl, 2 mM $MgCl_2$, 0.5% NP40, 1 mM Na_3VO_4, 1 mM PMSF, 1 mM β-glycerophosphate (BGP), 10 ug/ml of leupeptin and aprotinin) for 15 min on ice. Cells were then homogenized by 30-40 strokes in a tightly fitting Dounce homogenizer and checked under microscope for intact nuclei. The homogenate was centrifuged at 1,500 g for 5 min at 4°C to sediment the nuclei. The nuclear pellet was washed 5 times in cytoplasmic lysis buffer to ensure complete removal of cytosolic membranes. After washes, the nuclear pellet was lysed in the same buffer with the addition of 0.5 M NaCl. Nuclear pellets were sonicated for 10 sec, and vortexed for 30 sec 3 times. The extracted nuclear lysate was centrifuged at 15,000 g for 10 min at 4°C, and the supernatants were collected as nuclear lysate. Whole cell protein lysate was obtained through lysis with RIPA buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, .1% Tween-20, 10% glycerol, 2.5 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM Na_3VO_4, 1 mM PMSF, 1 mM BGP, and 10 ug/ml of leupeptin and aprotinin). Samples were sonicated for 10 sec, and then centrifuged at 15,000 g for 10 min at 4°C. All protein lysates were quantified by Bradford assay (Bio-Rad Laboratories, Hercules,

CA, USA). Equal amounts of protein (~20 ug) were fractionated by SDA-PAGE, transferred to a PVDF membrane (Millipore, Billerica, MA, USA), and analyzed by incubation with the appropriate primary antibody overnight at 4°C. Membranes were then subjected to incubation with HRP-conjugated secondary antibodies for 1 hr at room temperature. ECL chemiluminescence detection system was used to visual proteins with either of the following reagents: ECL Western Blotting Substrate (Promega Cooperation, Madison, WI, USA) or SuperSignal West Dura Extended Duration Chemiluminescent Substrate (Thermo Fisher Scientific, Walham, MA, USA).

Immunoprecipitation of HER3

Cells were lysed with NP-40 lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% Deoxycholic acid, 10% glycerol, 2.5 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 mM BGP, 1 mM Na_3VO_4, and 10 ug/ml of leupeptin and aprotinin). Lysates were sheered via syringe 3 times, and then centrifuged at 15,000 g for 10 min at 4°C. 250 ug of protein was incubated overnight at 4°C with 2 ug of primary C-TERM or N-TERM HER3 antibody. Normal human IgG (Sigma, St. Louis, MO) was used as a negative control. Next day, 25 ul of protein A/G agarose beads (Santa Cruz Biotechnology) were added for 3 hr at 4°C. The immunoprecipitates were pelleted by centrifugation and washed 5 times with NP-40 lysis buffer. The captured immunocomplexes were eluted by boiling in 2xSDS sample buffer for 5 min and subjected to immunoblot analysis as described above.

Immunofluorescent Staining of HER3

Approximately 3 x 10^3 cells/well were seeded on a four-well glass chamber slide (Millipore). 24 hr later, cells were washed briefly with PBS, and fixed with 4% methanol-

free formaldehyde for 15 min at room temperature. Cells were rinsed with PBS again, and permeabilized with PBS containing 0.2% TritonX-100 for 15 min. Cells were blocked in 5% normal goal serum diluted in PBS containing 0.3% Triton X-100 for 1 hr at room temperature, and primary C-TERM or N-TERM HER3 antibody was incubated overnight at 4°C. The next day, cells were rinsed with PBS 3 times, and incubated with Alexa Fluor-546 rabbit secondary antibody for 30 min at room temperature (Life Technologies, Carlsbad, CA, USA). Cells were rinsed with PBS again and mounted with ProLong gold with DAPI antifade mounting solution (Life Technologies). Fluorescence microscopy and photography were performed using a Nikon 80i upright confocal microscope.

Plasmid Construction and Transfection

Wild-type human HER3-pSPORT6 vector was kindly provided to us by Dr. P. Bertics (University of Wisconsin-Madison, WI, USA). HER3 was subsequently amplified via polymerase chain reaction and subcloned into KpnI/NotI restriction sites of the pcDNA6/V5-HisA vector (Life Technologies). PCDNA3.0-EGFRWT was kindly supplied by Dr. J. Boerner (Wayne State University School of Medicine, Karmanos Cancer Institute, MI, USA). EGFR-ICD and CTD, and HER3-ICD, JKD, and CTD were cloned into the pM-Gal4DBD expression vector using the SmaI/XbaI restriction sites (Clontech, Mountain View, CA, USA). Gal4DBD-EGFR, Gal4DBD-HER3, pcDNA6-HER3, pcDNA6-HER3ΔB_1B_2, pcDNA6-HER3WT-ICD, and pcDNA6-HER3ΔB_1B_2-ICD constructs were generated using the Phusion Site-Directed Mutagenesis Kit (Finnzymes, Keilaranta, Finland). pSPORT6-HER3-Y1222F/Y1289F construct was created via QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA,

USA) following the manufacturer's instructions. All mutations were verified for correct orientation and integrity via sequencing. The pGL4.35[luc2P/9XGal4UAS/Hygro], pGL3-Basic-Luciferase, and pRL-Tk-*Renilla* vectors were purchased from Promega. The 122 bp region of the cyclin D1 promoter (-33- +89) was subcloned into the pGL3-Basic promoterless vector using the KpnI/HindIII restriction sites. The sequences for this primer set are the following: CyD1 FWD 5'-CGGGGTACCCCGGGCTT GATCTTTGCT-3', CyD1 REV 5'-CCCAAGCTTGACTCTGCTGCTCGCTGCTA-3'.

All plasmid transfections were performed using Lipofectamine LTX and Opti-MEM I (Life Technologies) according to the manufacturer's instructions. Cells were analyzed 24-72 hr post transfection for protein, luciferase activity, and mRNA. For siRNAs, cells were transfected with siHER3 (ON-TARGETplus SMARTpool HER3, L-003127 Dharmacon, Lafayette, CO, USA) or siNon-targeting (NT) (ON-TARGETplus Non-targeting Pool, D-001810, Dharmacon) using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer's instructions. Vehicle (V) treated cells were treated with RNAiMAX only. For luciferase studies post siRNA, cells were transfected 24 hr later with cyclin D1-luciferase and *Renilla* plasmids, and analyzed for luciferase activity 48 hr later. For protein analysis, cells were lysed 72 hr post transfection and analyzed via western blot for HER3 knockdown.

Luciferase Assay

Cells were plated in 6-well plates at approximate 70% confluence. For the Gal4 UAS-luciferase assay, cells were transiently transfected with 100 ng plasmid DNA, 1 ug of pGL4.35 Gal4 UAS-luciferase vector (Promega), and 100 ng of pRL-Tk-*Renilla* vector (Promega). For the cyclin D1 promoter luciferase assay, 2 ug of HER3 expression

vectors, 1 ug of the 122 bp cyclin D1 promoter-pGL3 luciferase construct, and 10-100 ng of pRL-Tk-*Renilla* vector (Promega) were used. 48 hr post transfection, cells were collected for analysis via the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. Both *Firefly* and *Renilla* luciferase reporters were detected in a 96 well format using a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA). Transfection efficiencies were normalized against DNA content, protein content, and the expression of *Renilla* Luciferase.

Chromatin Immunoprecipitation (ChIP)

Cells were plated in 15 cm plates at ~80% confluency in quadruplicate. Each plate was fixed with formaldehyde at a final concentration of 1% for 15 min at room temperature. Fixation was terminated via 1.25 M glycine for 5 min, and cells were subsequently scraped in ice-cold PBS with 1 mM of PMSF. The cells were pelleted by centrifugation at 1500 rpm for 5 min at 4°C and then lysed in cell lysis buffer (5 mM HEPES, pH 8.0, 85 mM KCl, 0.5% NP-40 and 10 mM sodium pyrophosphate). 10 min later cells were further homogenized via Dounce homogenizer (30 strokes), and centrifuged for 1500 g for 5 min at 4°C. The supernatant was removed and nuclei pellets were lysed in nuclei lysis buffer (Tris-HCl 50 mM, pH 8.1, 10 mM EDTA, 1% SDS and 10 mM sodium pyrophosphate). The lysate was sonicated on ice for varied time points to achieve ~500 bp fragments of DNA (sonication was optimized via running sheared DNA on 1% agarose gels). The supernatant was pre-cleared with protein A/G agarose beads (Santa Cruz Biotechnology) in dilution buffer (16.7 mM Tris-HCl, pH 8.1, 1.2 mM EDTA, 167 mM NaCl, 1.1% Triton X-100, 0.01% SDS and 10 mM sodium pyrophosphate) for 1 hr at 4°C. The pre-cleared lysates were immunoprecipated by

incubating with protein A/G beads containing 5 ug of the N-TERM anti-HER3 antibody or human IgG (Sigma) rotating overnight at 4°C. The next day immunocomplexes were centrifuged at 1500 rpm for 5 min, and the beads were washed via light vortex for 15 min time intervals with the following wash buffers: wash buffer I (25 mM Tris-HCl, pH 8.0, 2 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS and 10mM sodium pyrophosphate), wash buffer II (25 mM Tris-HCl, pH 8.0, 2 mM EDTA, 500 mM NaCl, 1% Triton X-100, 0.1% SDS and 10 mM sodium pyrophosphate), wash buffer III (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 250 mM LiCl, 1% NP-40, 1% deoxycholic acid and 10 mM sodium pyrophosphate), and TE buffer (10 mM Tris-HCl, pH8.0, 1 mM EDTA, 10 mM sodium pyrophosphate). The bound DNA was eluted twice with elution buffer (10 mM $NaHCO_3$ and 1% SDS). 5 mM NaCl was then added to the pooled DNA and incubated at 68°C overnight. The DNA was recovered and purified using a DNA purification kit (Qiagen, Valencia, CA, USA). The purified chromatin-immunoprecipitated DNA was used as template for qPCR with primers flanking the 122 bp region (-33- +89) of the cyclin D1 promoter: Cyclin D1 FWD 5'-CCGGGCTTGATCTTTGCT-3', Cyclin D1 REV 5'-GACTCTGCTGCTCGCTGCTA-3'. The qPCR program was: 95°C for 3 min, followed by 40 cycles of 95°C for 15 sec and 55.5°C for 30 sec. The qPCR was performed using the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories)

DNA Affinity Pull Down Assay (DAPA)

DAPA was performed as previously described[37]. The nucleotide sequences of the 5' biotinylated oligonucleotides corresponding to the 122 bp cyclin D1 promoter (-33- +89) are the following: Sense 5'- CCGGGCTTGATCTTTGCTTAACA ACAGTAACGTCACACGGACTACAGGGGAGTTTTGTTGAAGTTGCAAAGTCCTGGA

GCCTCCAGAGGGCTGTCGGCGCAGTAGCAGCGAGCAGCAGAGTC-3', and Antisense 5'GACTCTGCTGCTCGCTGCTACTGCGCCGACAGCCCTCTGGA GGCTCCAGGACTTTGCAACTTCAACAAAACTCCCCTGTAGTCCGTGTGACGTTACT GTTGTTAAGCAAAGATCAAGCCCGG-3'. The probes were purchased from Integrated DNA Technologies (Coralville, IA, USA). Following annealing of Sense and Anti-sense oligonucleotdies for 1 hr at 95°C, 4 ug of annealed biotinylated probe was incubated with 500 ug of nuclear cell lysate, and 40 ul of streptavidin-agarose bead suspension (Sigma) in PBS diluted with 1 mM Na_3VO_4, 1 mM PMSF, 1 mM BGP, 10 ug/ml of leupeptin and aprotinin for 2-3 hr rocking at room temperature. The streptavidin-agarose beads were pelleted by centrifugation and washed 5 times with PBS. The captured probe binding proteins were eluted from the streptavidin-agarose beads by boiling in 2xSDS sample buffer for 5 min and subjected to immunoblot analysis for HER3 binding.

cDNA synthesis and quantitative

PCR Total RNA from cells was prepared using an RNeasy Mini kit (Qiagen, Inc., Valencia, CA). cDNA from total RNA of cells was synthesized using qScript cDNA SuperMix (Quanta BioSciences, Inc., Gaithersburg, MD, USA) according to manufacturer's protocol. qPCR analysis was performed using a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories). The primer sets used for this analysis were purchased from Life Technologies TaqMan® Gene Expression Assay: Cyclin D1 (Hs00765553_m1) and b-actin (Hs99999903_m1). For amplification cDNA was combined with primers and TaqMan universal PCR Master Mix (Life Technologies) according to the manufacturer's instructions. All reactions were performed in triplicate. To determine the normalized value, $2^{-\Delta\Delta Ct}$ values were compared between cyclin D1 and

endogenous control (β-actin) samples, where the change in crossing threshold (DCt) = $Ct_{Cyclin\ D1}$-$Ct_{\beta\text{-}actin}$ and DDCt=$DCt_{(HER3WT\ or\ HER3\Delta B1\Delta B2)}$-$DCt_{(Vector)}$.

Statistical analysis

Student t-tests were used to evaluate the significance of changes in all reporter and expression assays as compared to vector only or non-targeting controls. Statistical analysis comparing antibody pull downs in all ChIP assays were also evaluated via Student t-test. Differences were considered statistically significant if $P \leq 0.05$.

RESULTS

The HER3 receptor is localized to the nucleus in its full-length form

The overexpression of the HER3 receptor has been observed in cancers of the breast[38], non-small cell lung (NSCLC)[39], head and neck (HNSCC)[40], and colon (CRC)[41]. Therefore various cell lines from each cancer type were probed for HER3 expression, including the breast cancer cell lines SKBr3, MCF-7, BT474, HCC1954, and BT549, NSCLC cell lines H522 and $H226^R$ (a cell line previously developed to be resistant to the anti-EGFR monoclonal antibody cetuximab[31,32]), HNSCC lines SCC6 and SCC1, and the CRC lines LoVo and CaCO2 (**Figure 1A)**. Results from this experiment indicated that HER3 was expressed broadly across a variety of tumor types. Further, isolation of nuclei from $H226^R$, SKBr3 and MCF-7 cells indicated that full-length HER3 (~185kDa) could be detected in the nucleus**,** whereas nuclear HER3 was undetectable in the HER3 low-expressing breast cancer cell line BT549 (**Figure 1B)**. The plasma membrane associated proteins clathrin and dynamin, as well as the endoplasmic reticulum (ER) associated protein calnexin, were used to demonstrate nuclear fraction (Nuc) purity from other membrane-bound proteins and ER proteins. Additionally, the cytoplasmic protein α-tubulin and the nuclear protein Histone H3 were used as non-nuclear fraction (NN) and Nuc purity controls.

To validate the specificity of the nuclear HER3 signal detected in **Figure 1B,** we used siRNA to knockdown HER3 expression and subsequently performed nuclear fractionation in $H226^R$ and SKBr3 cell lines. The knockdown of HER3 led to a loss of the 185kDa band in both the NN and Nuc fractions, whereas vehicle (V) and non-targeting

(NT) siRNA did not affect HER3 expression (**Figure 1C)**; this demonstrates that the nuclear 185kDa band detected is specific for HER3.

To further show that nuclear HER3 is full-length within the nucleus, we performed IP analysis using an antibody that recognizes the extracellular N-terminal region of HER3 (N-TERM) followed by immunoblot analysis with an antibody that recognizes the C-terminal region of HER3 (C-TERM) in the presence of HER3 siRNA. HER3 could be effectively immunoprecipitated with an N-TERM antibody from whole cell lysate (WC) in both $H226^R$ and SKBr3 cells, which was prevented upon knockdown of HER3 with siRNA **(Figure 1D)**. IP analysis was further performed from NN and Nuc lysate harvested from $H226^R$ and SKBr3 cells using the same N-TERM HER3 antibody (**Figure 1D**), demonstrating that full-length HER3 can be extracted from the Nuc fraction.

Confocal immunofluorescent (IF) microscopy was used to visualize HER3 localization in $H226^R$ and SKBr3 cells using both C-TERM and N-TERM HER3 antibodies (**Figure 2**). HER3 was detected using an Alexa Fluor-546 labeled secondary antibody (visualized in red). Merging DAPI and 546 labeled HER3 yielded distinct nuclear HER3 signals in both cell lines with little bleed through from the 405 laser (used to visualize DAPI). Additionally, we detected minimal HER3 staining in the HER3 low expressing cell line BT549, demonstrating specificity for the HER3 primary antibodies. These data demonstrate that full-length HER3 is localized to the nucleus in various cancer cell lines.

<u>The C-terminus of HER3 contains a strong transactivation domain</u>

Recent studies have demonstrated that EGFR, HER2, and small splice variants of HER3 can function as co-transcriptional activators for various gene targets [4,5,8,30]. The weak tyrosine kinase activity of HER3 and its role in resistance to numerous therapeutic agents promoted our investigation into the non-kinase functions of HER3, including its transactivation potential. To investigate this, we utilized the Gal4/luciferase assay system, where the Gal4 DNA binding domain (Gal4DBD) of the yeast transcription factor Gal4 was fused to the intracellular domain (ICD), juxtamembrane and tyrosine kinase domain (JKD), and the C-terminal domain (CTD) of HER3. As a control for this assay, the EGFR-ICD and CTD were fused to the Gal4DBD as performed by Lin et al [6] (**Figure 3A**). While the EGFR-CTD strongly activated Gal4 UAS-luciferase, the EGFR-ICD was less capable of transactivating UAS-luciferase (**Figure 3B**) recapitulating previous findings [6]. The overexpression of the HER3-CTD led to strong transactivation of Gal4 UAS-luciferase (~8.7 fold) in CHOK1 cells, while the HER3-ICD and HER3-JKD constructs resulted in minimal luciferase activity that were not statistically different from luciferase detected from the Gal4DBD vector control (**Figure 3C**). To broaden the scope of this finding we transfected two other cell lines and observed similar results (**Figure 3D**). Overall, these data demonstrate that the HER3-CTD contains strong transactivation potential.

<u>Minimal mapping of the HER3 C-terminus demonstrates the existence of a bipartite transactivation domain</u>

To further understand the capability of HER3 to function as a transcription factor, we sought to map the minimal C-terminal TADs. Previous reports suggest that TADs

often contain proline-rich regions[42,43]; therefore, we analyzed the CTD of HER3 for proline-rich sequences. Two C-terminal proline-rich regions were identified, one between amino acids 1,142-1,150, and a second between amino acids 1,208-1,214. To investigate if these proline-rich sequences function as TADs both regions were deleted from the HER3-CTD (**Figure 4A**). The ability for HER3-CTD deleted of proline-rich region 1 ($CTD\Delta P_1$), proline-rich region 2 ($CTD\Delta P_2$), or both proline-rich regions ($CTD\Delta P_1\Delta P_2$) to transactivate Gal4 UAS-luciferase was not statistically different from that of the HER3-CTD (**Figure 4B)**. These data suggest that HER3's C-terminal proline-rich regions do not confer strong transactivation potential and therefore may not function as prominent TADs.

To identify HER3 TADs we next constructed a series of nine Gal4DBD HER3-CTD mapping mutants, where small regions of the HER3-CTD were sequentially added back to each construct until the entire HER3-CTD was restored (**Figure 4C**). The transactivation capability of each mapping mutant (CTD_1-CTD_9) was then tested via Gal4 UAS-luciferase assays in CHOK1 cells (**Figure 4D**). The CTD_1 and CTD_2 constructs had minimal transactivation activity (similar activity to the Gal4DBD vector control), while $CTD_{3\text{-}5}$ expression yielded slightly more transactivation activity. However, CTD_6 demonstrated a 5.0 fold increase in Gal4 UAS-luciferase activation (~3.0 fold higher than CTD_5, $p<0.001$). The CTD_7 construct demonstrated similar transactivation activity to CTD_6, while CTD_8 displayed a 7.5 fold increase in Gal4 UAS-luciferase activation (~2.5 fold higher than CTD_7, $p<0.01$), which recapitulated the luciferase activity detected from the full-length HER3-CTD. The CTD_9 construct exhibited similar transactivation activity to CTD_8 and the full-length HER3-CTD. These data suggest that

the HER3-CTD contains two prominent TADs, one between CTD_5 and CTD_6, and a second between CTD_7 and CTD_8.

To validate the bipartite TAD identified in **Figure 4D**, both regions were deleted from the HER3-CTD (**Figure 4E**). The first deletion mutant consisted of the region located between CTD_5 and CTD_6 (amino acids 1,215-1,248) denoted as $CTD\Delta B_1$ and the second deletion mutant consisted of the region located between CTD_7 and CTD_8 (amino acids 1,281-1,302) denoted as $CTD\Delta B_2$. The double bipartite deletion mutant was also constructed, denoted as $CTD\Delta B_1\Delta B_2$. To ensure that the B_1 and B_2 regions are specific TADs, we also deleted one region in the HER3-CTD of approximately the same size as B_1 and B_2 denoted as $CTD\Delta R_1$ (**Figure 4E**). While the HER3-CTD transactivated Gal4 UAS-luciferase 7.4 fold higher than the Gal4DBD vector control, $CTD\Delta B_1$ and $CTD\Delta B_2$ led to statistically significant reductions in UAS-luciferase activity (3.2 and 2.0 fold decreases), while the $CTD\Delta B_1\Delta B_2$ demonstrated the greatest reduction (4.9 fold decrease) (**Figure 4F**). The $CTD\Delta R_1$ was not hindered in its ability to transactivate UAS-luciferase, demonstrating specificity of the B_1 and B_2 regions. Collectively, these data demonstrate that the B_1 and B_2 regions contain the majority of HER3's transactivation potential.

Nuclear HER3 can associate with a 122 bp region of the cyclin D1 promoter

To understand how the identified bipartite TAD influences nuclear HER3's transcriptional functions we sought to identify a nuclear HER3 gene target. Since full-length nuclear EGFR and HER2 and a nuclear variant of HER3 have been shown to associate with the cyclin D1 promoter [6-8], we hypothesized that full-length nuclear HER3

would be similarly associated. As a control, we first validated via chromatin immunoprecipitation (ChIP) analysis that EGFR and HER2 can associate with a 122 bp region of the cyclin D1 promoter (-33 - +89) approximately 6.8 fold (EGFR) and 9.4 fold (HER2) higher than an IgG control **(Figure 5A)**. Additionally, DNA affinity precipitation assays (DAPA) were performed using a biotinylated 122 bp cyclin D1 promoter probe. Both EGFR and HER2 associated with the cyclin D1 promoter probe while lysate incubated with beads only lacked association.

Next, ChIP was performed using an N-TERM HER3 antibody in $H226^R$, SKBr3, and BT549 cells. Full-length HER3 associated with the 122 bp region of the cyclin D1 promoter in both $H226^R$ (3.2 fold) and SKBr3 (5.6 fold) cells while HER3 association with cyclin D1 could not be detected in the HER3 low expressing cell line BT549 (**Figure 5B**). Additionally, HER3 associated with the cyclin D1 promoter probe in a DAPA assay (**Figure 5C)**. Detection of HER3 in this assay was specific because siHER3 knockdown studies demonstrated loss of probe binding (**Figure 5D**). Collectively, these data demonstrate that nuclear HER3 can associate with a 122 bp region of the cyclin D1 promoter.

<u>Nuclear HER3 can regulate the transcription of a 122 bp region of the cyclin D1 promoter via its bipartite transactivation domain</u>

Since full-length nuclear HER3 can associate with a 122 bp region of the cyclin D1 promoter, we sought to understand its ability to regulate this region. To do this, the 122 bp region of the cyclin D1 promoter was cloned into a luciferase reporter construct. In **Figure 6A,** the knockdown of HER3 expression using siRNA decreased cyclin D1-

promoter luciferase 24% in $H226^R$, 35% in SKBr3, and 27% in MCF-7 cells compared to cells transfected with NT siRNA. Lysate from the luciferase assay was used to confirm the knockdown of HER3 expression. In **Figure 6B,** HER3 was overexpressed in SCC6, HCC1954, SKBr3, and BT474 cells, where cyclin D1 promoter-luciferase activity was significantly increased (90%, 50%, 35%, and 65% respectively). Nuclear lysate was obtained to validate the presence of nuclear HER3 upon overexpression. Overall, these experiments demonstrate that HER3 can influence the activation of a 122 bp region of the cyclin D1 promoter in numerous cell lines.

Next, we measured the ability for full-length HER3 deleted of both the B_1 and B_2 regions (denoted as HER3$\Delta B_1 \Delta B_2$) to transactivate cyclin D1 promoter-luciferase. While the overexpression of wild-type HER3 (HER3WT) led to increases in cyclin D1 promoter-luciferase activity in SCC6, BT474 and HCC1954 cells (97%, 63% and 30% respectively), the overexpression of HER3$\Delta B_1 \Delta B_2$ was hindered in its ability to transactivate cyclin D1 in all cell lines (10%, 14% and 0% respectively) (**Figure 6C**). Analysis of the B_1 and B_2 regions yielded the presence of two tyrosine residues: tyrosine 1222 located in B_1, and tyrosine 1289 located in B_2. To verify that the loss of luciferase activity by HER3$\Delta B_1 \Delta B_2$ was not due to altered signaling dependent on these tyrosines, we tested the ability for a double tyrosine mutant (HER3 Y1222F/Y1289F denoted as HER3DM) to transactivate cyclin D1 promoter-luciferase (**Figure 6C**). The overexpression of HER3DM in all cell lines demonstrated a minor reduction in functionality as compared to HER3WT in this assay (85%, 55%, and 27% respectively); however, the luciferase values between HER3WT and HER3DM were not statistically different from one another in each cell line (**Figure 6C**). Additionally, both HER3WT and

HER3ΔB$_1$ΔB$_2$ were effectively nuclear localized (**Inset 1, Figure 6C**) and the activation of the downstream signaling molecule AKT was not affected in all mutants (**Inset 2, Figure 6C**). These data suggest that the alterations in cyclin D1 luciferase observed upon overexpression of HER3ΔB$_1$ΔB$_2$ were not due to a loss of nuclear HER3 localization or changes in AKT activity.

To further understand if the B$_1$ and B$_2$ TADs are required for HER3 induced cyclin D1 expression, we performed quantitative PCR (qPCR) to measure cyclin D1 transcripts after treatment with siHER3 (**Figure 6D**) or overexpression of HER3WT and HER3ΔB$_1$ΔB$_2$ (**Figure 6E**). siRNA knockdown of HER3 in H226^R and MCF-7 cells led to significant decreases in cyclin D1 expression (39% and 50% downregulation respectively) as compared to NT treated cells (**Figure 6D**). Importantly, the overexpression of HER3WT vs. HER3ΔB$_1$ΔB$_2$ in SCC6 and BT474 cells demonstrated that HER3WT could significantly enhance cyclin D1 expression (92% and 35% upregulation respectively), while the HER3ΔB$_1$ΔB$_2$ mutant was severely hindered at doing so (8% and 1% upregulation respectively) (**Figure 6E**). The minor increases in cyclin D1 transcripts detected upon HER3ΔB$_1$ΔB$_2$ overexpression were not statistically different from vector transfected cells. Collectively, these data suggest that nuclear HER3 can regulate cyclin D1 expression, in part, through its B$_1$ and B$_2$ TADs.

<u>An intracellular domain mutant of HER3 can regulate the transcription of cyclin D1 via its bipartite transactivation domain</u>

To further ensure that nuclear HER3, rather than classical signaling emanating from membrane-bound HER3, functions in the regulation of cyclin D1 we created

intracellular domain (ICD) truncation mutants of HER3 that lack both the N-terminus and the transmembrane domain (**Inset 1, Figure 7A for illustration**). HER3WT-ICD (denoted as WT-ICD) and HER3$\Delta B_1\Delta B_2$-ICD (denoted as $\Delta B_1\Delta B_2$-ICD) were overexpressed in SCC6, BT474, and HCC1954 cells alongside the 122bp cyclin D1 promoter-luciferase construct. While the overexpression of WT-ICD led to increases in cyclin D1 promoter-luciferase activity (60%, 25% and 94% respectively), the overexpression of $\Delta B_1\Delta B_2$-ICD was hindered in its ability to transactivate cyclin D1 in all cell lines (12%, 8% and 11% respectively) (**Figure 7A**). Additionally, both WT-ICD and $\Delta B_1\Delta B_2$-ICD were effectively nuclear localized (**Inset 2, Figure 7A**). Next, qPCR was performed to assess the ability for each ICD mutant to augment cyclin D1 expression (**Figure 7B**). The overexpression of WT-ICD in SCC6 and BT474 cells significantly enhanced cyclin D1 expression (200% and 40% upregulation respectively), while $\Delta B_1\Delta B_2$ –ICD was hindered at doing so (5% and 12% upregulation respectively). The minor increases in cyclin D1 promoter-luciferase and transcripts detected upon HER3$\Delta B_1\Delta B_2$ overexpression were not statistically different from vector transfected cells. Collectively, these data indicate that HER3 depleted of its membrane-bound functions can effectively regulate the cyclin D1 promoter through its bipartite TAD.

DISCUSSION

To date, all HER family RTKs have been shown to be nuclear localized in primary tumor tissues and cancer cell lines where they can function as co-transcription factors[4,5]. The nuclear expression of both EGFR and HER3 have been correlated with worse disease prognosis in specific cancers[9-11,13,29], and nuclear EGFR has been shown to enhance resistance to various therapeutic agents[4,5]. These findings highlight the need to better understand nuclear HER family function. In the current study, we sought to identify the regions on the HER3 receptor that function as TADs to enhance the understanding of HER3's transcriptional functions. To identify HER3 C-terminal TADs, various regions of the HER3 CTD were fused to the Gal4DBD and tested for their ability transactivate Gal4 UAS-luciferase. Two regions of prominent transactivation potential were identified (B_1 and B_2) via this method and were further shown to impact nuclear HER3's ability to regulate both the cyclin D1 promoter and mRNA expression.

To date, several investigators have identified full-length nuclear EGFR and HER2 in different tumor types[5,44]. In the current report, we detected nuclear HER3 in its full-length form with both N- and C- terminal HER3 antibodies (**Figure 1 and 2)**. Additionally ChIP analysis was performed using an N-terminal HER3 antibody (**Figure 5**). In 2002, Offterdinger et al. observed full-length nuclear HER3 in human breast cancer MTSV1-7 cells[28], which was validated by Koumakpayi et al. in multiple prostate cancer cell lines[29]. Additionally, using both fluorescently labeled HER3 and cell fractionation techniques, Tao et al. demonstrated that full-length HER3 homodimers were highly localized to the nucleus in various cell models[45]. Recently, alternative splice variants of HER3 have been discovered to be nuclear localized, including an 80-kDa fragment in H358 lung

cancer cells[8], and a 50-kDa fragment in rat Schwann cells[30]. In the present study, an 80-kDa fragment of HER3 was detected in whole cell lysate from some cell lines, while a 50-kDa fragment was undetectable (data not shown). Thus, in the current study the predominant form of nuclear HER3 detected was that of the full-length receptor.

In 2001, a pioneering study by Lin et al. demonstrated that the C-terminus of EGFR (EGFR-CTD) contained strong transactivation potential through the use of a Gal4 UAS-chloramphenicol transferase reporter assay[6], while the EGFR-ICD and EGFR-JKD constructs elicited minimal activation. In the current study we identified that the HER3-CTD also contained strong transactivation potential, similar to that of the EGFR-CTD, while the HER3-ICD and HER3–JKD constructs had minimal activity (**Figure 3**). Lin et al. hypothesized that the EGFR-JKD may contain negative regulatory sites that hinder the EGFR-CTD from functioning as a transcriptional co-activator[6]. The HER3-JKD may also contain negative regulatory sites, and therefore may inhibit the HER3-CTD from functioning in a Gal4 UAS-luciferase assay. Further experimentation is needed to identify these putative negative regulatory sites and their influence on HER3-CTD function.

TADs of transcription factors often contain proline and acidic amino acid rich regions that help recruit proteins necessary for transcription[38,43]. To define HER3's TADs, we first identified two prominent C-terminal proline-rich sequences (**Figure 4A**). When the proline-rich sequences were deleted from the HER3-CTD a significant change in Gal4 UAS-luciferase activity was not observed, suggesting that these regions do not function as prominent TADs (**Figure 4B**). Through sequential mapping studies we found two independent regions (B_1 and B_2) on the HER3-CTD that most prominently

activated Gal4 UAS-luciferase (**Figure 4C-4F**). These regions demonstrated specificity because deletion of another region (R_1) of similar size did not affect Gal4 UAS-luciferase activity (**Figure 4F**). Motif analysis of this bipartite region did not yield the presence of any known TAD sequence, however various transcription factors contain bipartite TADs, examples including p53 and CREB[46,47]. Research over the last two decades has demonstrated that TADs are not necessarily rich in any particular sequence, and that TADs have high diversity of sequence and domain structure, unlike the uniform sequence and structure of DBDs[43,48,49]. While the bipartite region discovered accounted for ~66% of HER3's transactivation potential in a Gal4 UAS-luciferase assay, it did not recapitulate the full activity detected from the entire HER3-CTD, and therefore other CTD regions may function as less prominent TADs.

To identify how B_1 and B_2 influence nuclear HER3 function, we first demonstrated that HER3 can bind to a 122bp region of the cyclin D1 promoter, a region that was originally found to associate with nuclear EGFR[6]. Interestingly, EGFR, HER2, and HER3 all associated with this relatively small promoter region in SKBr3 cells (**Figure 5**). Whether HER family dimers exist in the nucleus and function together as co-transcription factors has yet to be determined. We further demonstrate that HER3 lacking the B_1 and B_2 regions (HER3ΔB_1B_2) had either reduced ability or an inability to transactivate cyclin D1 promoter-luciferase and cyclin D1 expression in multiple cell lines (**Figure 6**), suggesting that these regions function as prominent TADs. SCC6 and BT474 cells express endogenous HER3 (See **Figure 1A**) and therefore the slight increases in reporter activity detected upon overexpression of HER3$\Delta B_1\Delta B_2$ may have been due to activation by endogenous nuclear HER3. Additionally, both HER3WT and

HER3$\Delta B_1\Delta B_2$ were effectively localized to the nucleus (**Inset 1, Figure 6C**). Therefore, the lack of cyclin D1 promoter-luciferase detected upon HER3$\Delta B_1\Delta B_2$ overexpression was likely due to its inability to function in the nucleus rather than impairment in nuclear translocation. The specific transcription factors that associate with nuclear HER3 is under current investigation, but we speculate that HER3$\Delta B_1\Delta B_2$ is deficient in the proper association and/or recruitment of these factors. Collectively, these data suggest that the B_1 and B_2 regions of HER3 function as TADs.

One of the major hurdles in the study of nuclear RTKs is to experimentally isolate their nuclear functions from plasma membrane-bound functions. To ensure that the loss of cyclin D1 promoter- luciferase activity detected upon overexpression of HER3$\Delta B_1\Delta B_2$ was due to deficiency in nuclear HER3 functions the tyrosine residues located in the B_1 and B_2 regions known to play a role in activating signaling cascades were mutated. HER3 mutated at both tyrosine 1222 and 1289 (HER3DM) was only slightly hindered in the activation of the cyclin D1 promoter, unlike HER3$\Delta B_1\Delta B_2$, and both HER3DM and HER3$\Delta B_1\Delta B_2$ were still capable of activating HER3's downstream effector kinase AKT (**Inset 2, Figure 6C**). To further validate that the regulation of cyclin D1 was not a result of classical membrane-bound functions of HER3, ICD mutants of HER3 were created in which HER3WT and HER3$\Delta B_1\Delta B_2$ were deleted of both the N-terminus and transmembrane domain (**Figure 7**). The WT-ICD, which cannot be localized at the plasma membrane or serve as a dimerization partner to activate classical signaling pathways, was still capable of regulating cyclin D1 luciferase activity and mRNA expression. This finding falls in line with the identified HER3 C-terminal splice variants that have been shown to function as co-transcription factors[8,30]. Importantly, $\Delta B_1\Delta B_2$-

ICD was hindered in cyclin D1 regulation, further supporting the role of these TADs in influencing nuclear HER3 transcriptional function. We speculate that the minor increases in luciferase and mRNA expression observed upon $\Delta B_1\Delta B_2$-ICD overexpression may emanate from endogenous HER3 in SCC6 and BT474 cells and/or the residual transcriptional activity remaining on the C-terminus of the $\Delta B_1\Delta B_2$-ICD. Collectively, these data suggest that the loss of cyclin D1 promoter-luciferase activity and mRNA expression was not due to modulation of signaling pathways emanating from membrane-bound HER3, but likely due to an inability for HER3$\Delta B_1\Delta B_2$ to function as a co-transcription factor.

To date, various functions of nuclear localized HER family receptors have been identified. The present study is the first to map specific TADs on a nuclear HER family member, and further TAD mapping studies of both HER2 and EGFR are underway. These studies are important because it is becoming increasingly apparent that nuclear HER family transcriptional functions play a role in cancer biology and that these functions may be independent of their kinase activity[4,5]. The current study supports these findings because 1) HER3 lacks high levels of kinase activity, and 2) nuclear HER3 regulation of cyclin D1 was independent of plasma membrane-bound HER3 functions and AKT signaling. Since the kinase functions of HER family members may not be solely responsible for the tumorigenic properties of these receptors, the identification of HER family TADs may serve as a map to better target nuclear HER functions in the future.

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FIGURE 1. The HER3 receptor is localized to the nucleus in its full-length form. (**A) HER3 is expressed in numerous cancer cell lines.** Whole cell protein lysates were isolated from various breast, lung, HNSCC, and colon cancer cell lines. Lysate was fractionated on SDS-PAGE followed by immunoblotting for HER3. α-tubulin was used as a loading control. (**B) HER3 is localized to the nucleus in cancer cell lines.** $H226^R$, SKBr3, MCF-7, and BT549 cells were harvested for whole cell (WC), non-nuclear (NN) and nuclear (Nuc) protein, fractionated on SDS-PAGE followed by immunoblotting for HER3. Clathrin, dynamin, calnexin, α-tubulin and Histone H3 were used as loading and purity controls for the NN and Nuc fractions, respectively. (**C) Specificity of nuclear HER3 by siRNA.** $H226^R$ and SKBr3 cells were harvested for NN and Nuc protein 48 hr post treatment with siHER3 or non-targeting (NT) siRNA. Experimental procedure as in 1B. α-tubulin and Histone H3 were used as loading and purity controls for the NN and Nuc fractions, respectively. (**D) Full-length HER3 is localized to the nucleus.** $H226^R$ and SKBr3 cells were harvested for WC, NN, and Nuc lysate**.** WC lysates were harvested 48 hr post treatment with siHER3. 250 ug of cell lysate was immunoprecipitated with an N-TERM HER3 antibody or human IgG control. The immunoprecipitates were fractionated on SDS-PAGE followed by immunoblotting for HER3 with a C-TERM antibody.

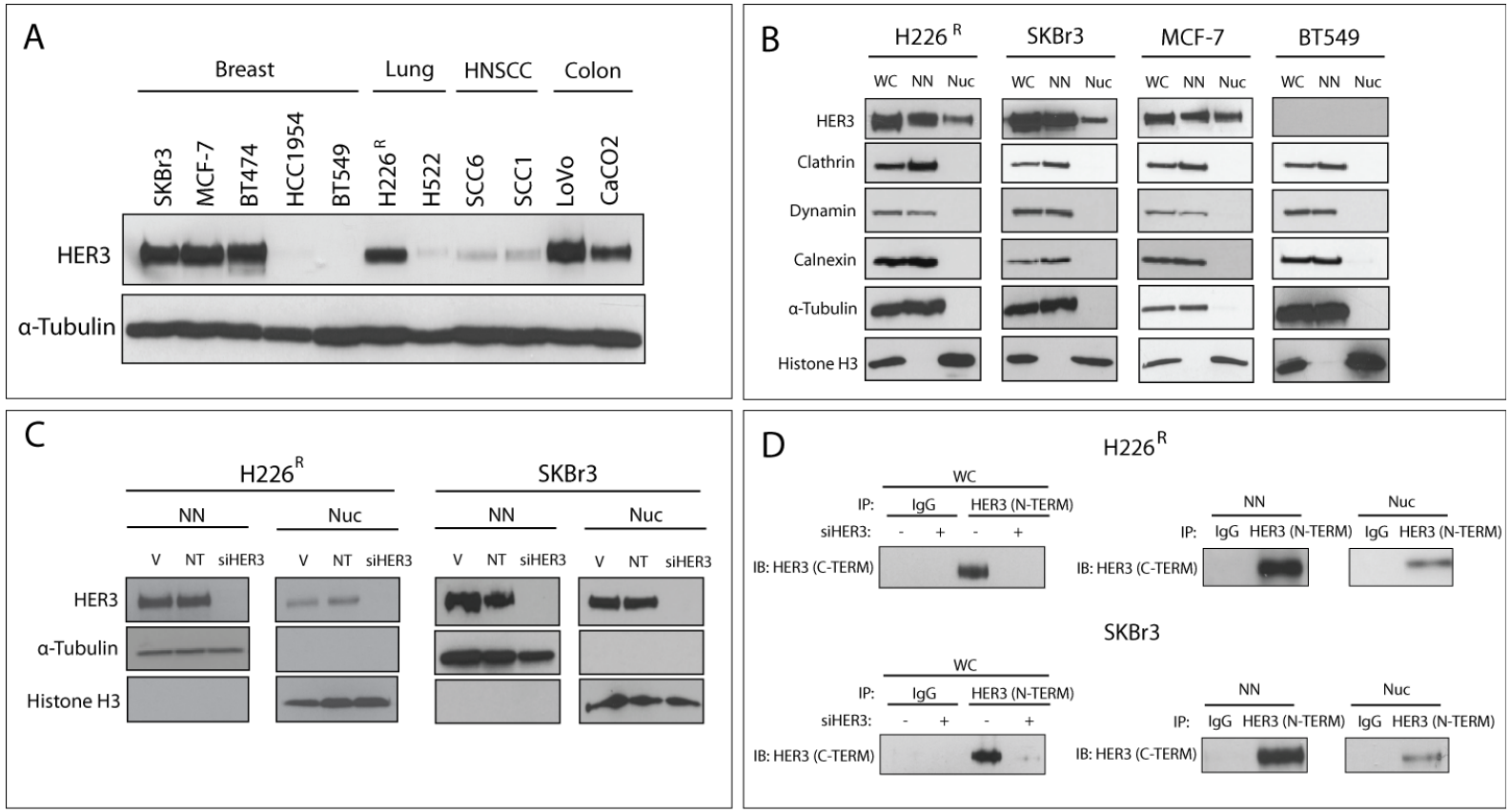

A
Breast
Lung
HNSCC
Colon
SKBr3
MCF-7
BT474
HCC1954
BT549
H226 R
H522
SCC6
SCC1
LoVo
CaCO2
HER3
α-Tubulin
B
H226 R
SKBr3
MCF-7
BT549
WC NN Nuc
HER3
Clathrin
Dynamin
Calnexin
α-Tubulin
Histone H3
C
H226R
SKBr3
NN
Nuc
V NT siHER3
HER3
α-Tubulin
Histone H3
D
H226R
WC
IP: IgG HER3 (N-TERM)
siHER3: - + - +
IB: HER3 (C-TERM)
NN
Nuc
SKBr3

FIGURE 2: Confocal Immunofluorescent staining of nuclear HER3. C-TERM and N-TERM HER3 antibodies were used to visualize HER3 localization in $H226^{R}$, SKBr3, and BT549 cells. Alexa Fluor-546 secondary antibody was used to visualize HER3 (RED) and DAPI was used to visualize the nucleus (BLUE). Merged images were magnified to depict the nuclear localization of HER3 (see white arrows). Magnification 600X.

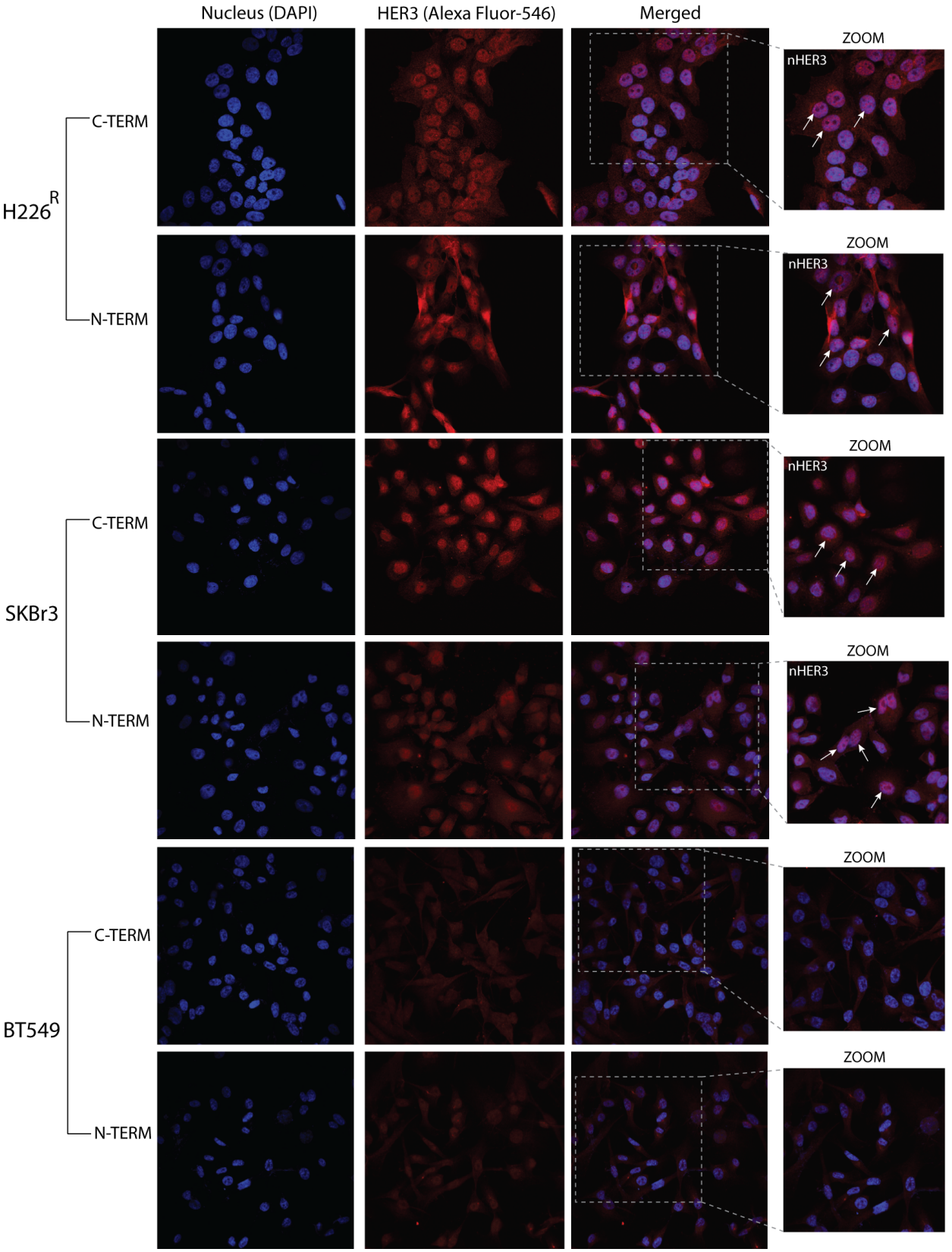
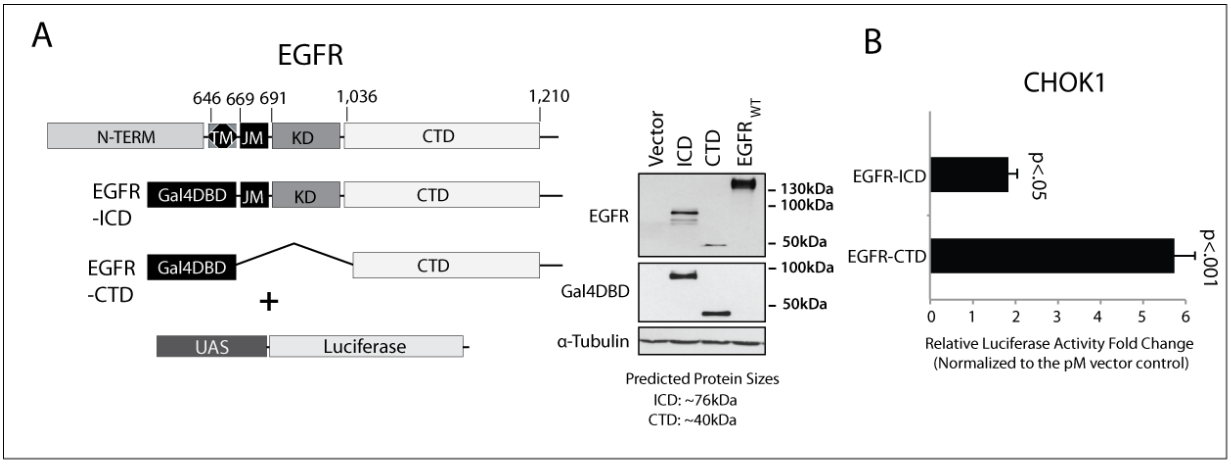

Nucleus (DAPI)
HER3 (Alexa Fluor-546)
Merged
ZOOM
nHER3
H226R
C-TERM
N-TERM
SKBr3
C-TERM
N-TERM
BT549
C-TERM
N-TERM

FIGURE 3: The C-terminus of HER3 contains a strong transactivation domain. (**A) EGFR intracellular domain (ICD) map and plasmid validation.** The intracellular domain (ICD) and C-terminal domain (CTD) of EGFR were fused to the Gal4 DNA binding domain (Gal4DBD). CHOK1 cells were transfected with each construct for 48 hr prior to harvesting WC lysate and fractionation on SDS-PAGE followed by immunoblotting for EGFR and Gal4DBD. EGFRWT vector was transfected into CHOK1 cells as a positive control. α-tubulin was used as a loading control. (**B) EGFR-CTD contains strong transactivation potential.** CHOK1 cells were transfected with EGFR-ICD or EGFR-CTD constructs, UAS-luciferase and Tk-*Renilla* reporter plasmids for 48 hr prior to quantification by dual luciferase assay (n=3). The graphs are representative of four independent experiments. (**C) HER3-ICD map and plasmid validation.** The HER3- ICD, juxtamembrane and tyrosine kinase domain (JKD), and CTD were fused to the Gal4DBD. Transfection was performed the same as in 1A and immunoblot analysis was performed for HER3 and Gal4DBD. HER3WT was transfected into CHOK1 cells as a positive control. α-tubulin was used as a loading control. (**D) HER3-CTD contains strong transactivation potential.** CHOK1, $H226^{R}$, and SKBr3 cells were transfected with HER3-ICD, HER3-JKD, and HER3-CTD constructs, UAS-luciferase and Tk-*Renilla* reporter plasmids for 48 hr prior to quantification by dual luciferase assay (n=3). The graphs are representative of four independent experiments. Luciferase activity was normalized to DNA content, protein content, and the expression of *Renilla* luciferase in all assays. Luciferase activity detected for each construct was normalized to the Gal4DBD vector control. Data points are represented as mean+/-s.e.m. $p<0.05$. TM (transmembrane); JM (juxtamembrane); KD (kinase domain).


A
EGFR
646 669 691
1,036
1,210
N-TERM
TM
JM
KD
CTD
EGFR -ICD
Gal4DBD
EGFR -CTD
UAS
Luciferase
Vector
ICD
CTD
EGFR WT
EGFR
Gal4DBD
α-Tubulin
130kDa
100kDa
50kDa
Predicted Protein Sizes
ICD: ~76kDa
CTD: ~40kDa
B
CHOK1
EGFR-ICD
EGFR-CTD
p<.05
p<.001
Relative Luciferase Activity Fold Change
(Normalized to the pM vector control)

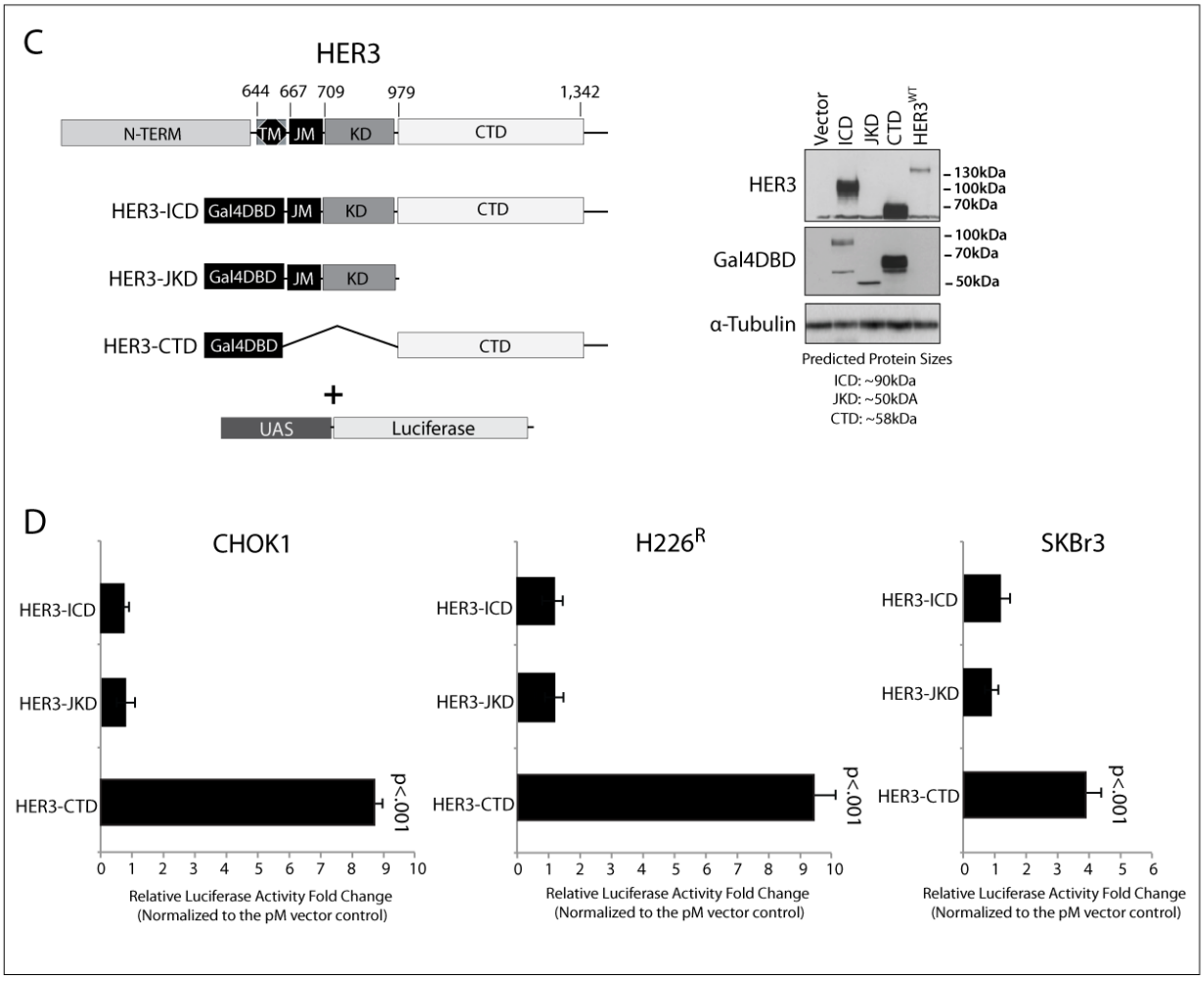
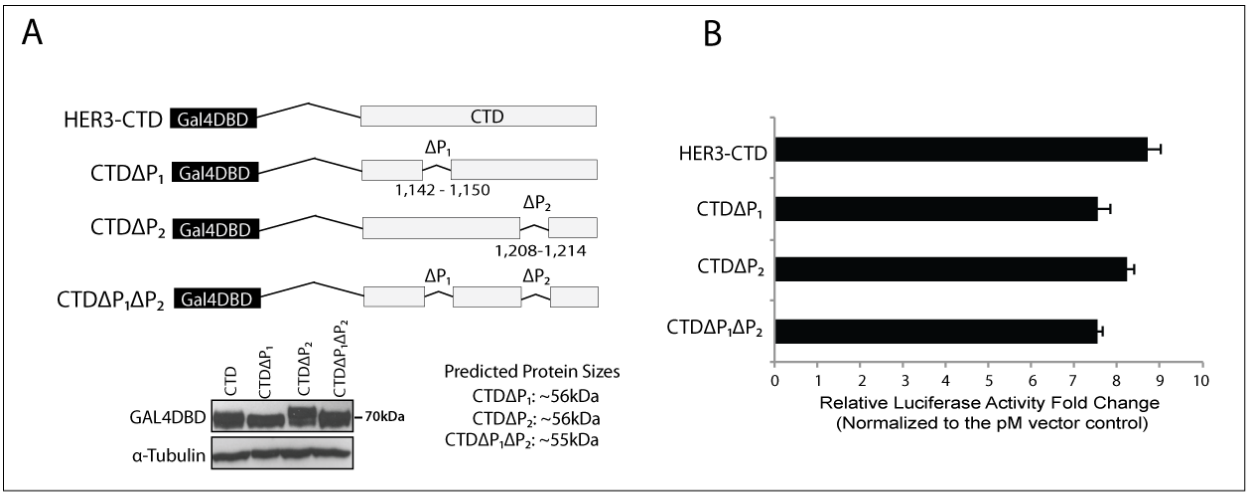
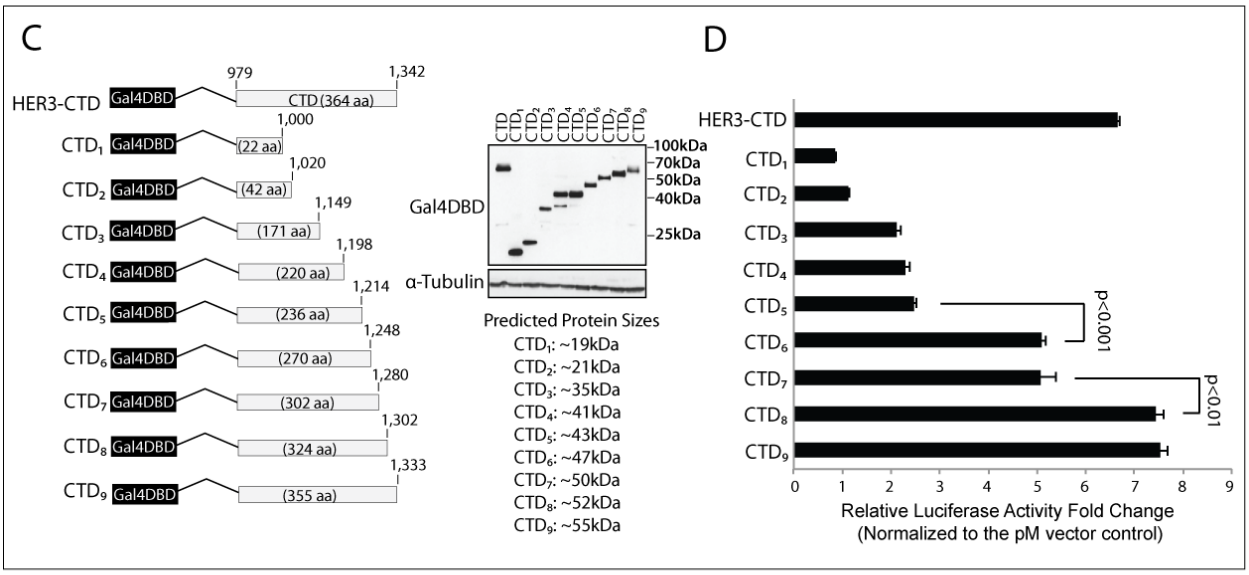

C
HER3
644 667 709
979
1,342
HER3-ICD
HER3-JKD
HER3-CTD
Vector
ICD
JKD
CTD
HER3 WT
HER3
Gal4DBD
α-Tubulin
130kDa
100kDa
70kDa
50kDa
Predicted Protein Sizes
ICD: ~90kDa
JKD: ~50kDA
CTD: ~58kDa
D
CHOK1
H226R
SKBr3
p<.001
Relative Luciferase Activity Fold Change
(Normalized to the pM vector control)

FIGURE 4: Minimal mapping of the HER3 C-terminus demonstrates the existence of a bipartite transactivation domain. (**A) HER3-CTD proline deletion mapping and validation.** The HER3-CTD deleted of proline-rich region 1 ($CTD\Delta P_1$), proline-rich region 2 ($CTD\Delta P_2$), and both regions ($CTD\Delta P_1\Delta P_2$) were fused to the Gal4DBD. CHOK1 cells were transfected with each construct for 48 hr prior to harvesting WC lysate and fractionation on SDS-PAGE followed by immunoblotting for Gal4DBD. (**B) The HER3-CTD proline-rich regions do not function as prominent TADs.** CHOK1 cells were transfected with the HER3-CTD, $CTD\Delta P_1$, $CTD\Delta P_2$, or $CTD\Delta P_1\Delta P_2$, UAS-luciferase and Tk-*Renilla* reporter plasmids for 48 hr prior to quantification by dual luciferase assay (n=6). The graph is representative of three independent experiments. (**C) HER3-CTD minimal transactivation domain mapping and validation.** Nine consecutive regions of the HER3-CTD of increasing length were fused to the Gal4DBD (CTD_1-CTD_9). CHOK1 cells were transfected with each construct for 48 hr prior to harvesting WC lysate and fractionation on SDS-PAGE followed by immunoblotting for Gal4DBD. (**D) HER3-CTD contains two regions with strong transactivation potential.** CHOK1 cells were transfected with CTD_1-CTD_9, UAS-luciferase and Tk-*Renilla* reporter plasmids for 48 hr prior to quantification by dual luciferase assay (n=3). The graph is representative of three independent experiments. (**E) HER3-CTD bipartite deletion mapping and validation.** The regions with strong transactivation potential were deleted from the HER3-CTD, denoted as $CTD\Delta B_1$ and $CTD\Delta B_2$. The double deletion mutant $CTD\Delta B_1\Delta B_2$ was also constructed. As a control, a region of similar size to B_1 and B_2 was deleted, denoted as $CTD\Delta R_1$. CHOK1 cells were transfected with each construct for 48 hr prior to harvesting WC lysate and fractionation on SDS-PAGE

followed by immunoblotting for Gal4DBD. (**F) HER3 contains a strong bipartite C-terminal TAD**. CHOK1 cells were transfected with CTDΔB_1, CTDΔB_2, CTDΔB_1ΔB_2, or CTDΔR_1, UAS-luciferase and Tk-*Renilla* reporter plasmids for 48hr prior to quantification by dual luciferase assay (n=6). The graph is representative of four independent experiments. Luciferase activity was normalized to DNA content, protein content, and the expression of *Renilla* luciferase in all assays. Luciferase activity detected for each construct was normalized to the pM Gal4DBD vector control. Data points are represented as mean +/- s.e.m. $P<0.05$.


A
HER3-CTD Gal4DBD CTD
CTDΔP1 Gal4DBD ΔP1 1,142 - 1,150
CTDΔP2 Gal4DBD ΔP2 1,208-1,214
CTDΔP1ΔP2 Gal4DBD ΔP1 ΔP2
CTD CTDΔP1 CTDΔP2 CTDΔP1ΔP2
GAL4DBD
α-Tubulin
70kDa
Predicted Protein Sizes
CTDΔP1: ~56kDa
CTDΔP2: ~56kDa
CTDΔP1ΔP2: ~55kDa
B
HER3-CTD
CTDΔP1
CTDΔP2
CTDΔP1ΔP2
0 1 2 3 4 5 6 7 8 9 10
Relative Luciferase Activity Fold Change
(Normalized to the pM vector control)


C
979 1,342
HER3-CTD Gal4DBD CTD (364 aa)
CTD1 Gal4DBD 1,000 (22 aa)
CTD2 Gal4DBD 1,020 (42 aa)
CTD3 Gal4DBD 1,149 (171 aa)
CTD4 Gal4DBD 1,198 (220 aa)
CTD5 Gal4DBD 1,214 (236 aa)
CTD6 Gal4DBD 1,248 (270 aa)
CTD7 Gal4DBD 1,280 (302 aa)
CTD8 Gal4DBD 1,302 (324 aa)
CTD9 Gal4DBD 1,333 (355 aa)
CTD CTD1 CTD2 CTD3 CTD4 CTD5 CTD6 CTD7 CTD8 CTD9
Gal4DBD
α-Tubulin
100kDa
70kDa
50kDa
40kDa
25kDa
Predicted Protein Sizes
CTD1: ~19kDa
CTD2: ~21kDa
CTD3: ~35kDa
CTD4: ~41kDa
CTD5: ~43kDa
CTD6: ~47kDa
CTD7: ~50kDa
CTD8: ~52kDa
CTD9: ~55kDa
D
HER3-CTD
CTD1
CTD2
CTD3
CTD4
CTD5
CTD6
CTD7
CTD8
CTD9
p<0.001
p<0.01
0 1 2 3 4 5 6 7 8 9
Relative Luciferase Activity Fold Change
(Normalized to the pM vector control)

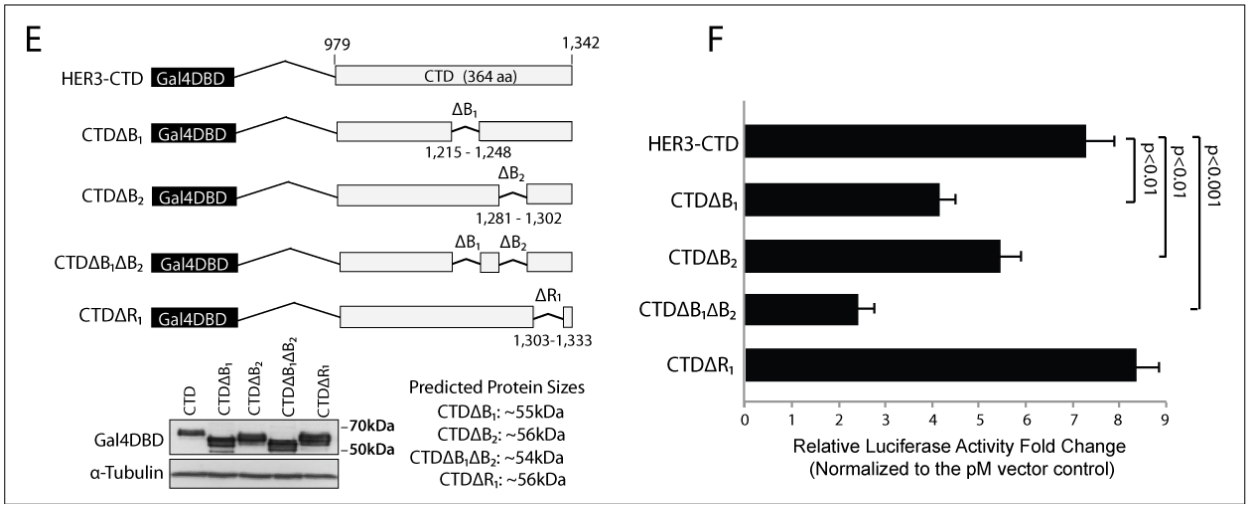

E
979 1,342
HER3-CTD Gal4DBD CTD (364 aa)
CTDΔB1 Gal4DBD ΔB1 1,215 - 1,248
CTDΔB2 Gal4DBD ΔB2 1,281 - 1,302
CTDΔB1ΔB2 Gal4DBD ΔB1 ΔB2
CTDΔR1 Gal4DBD ΔR1 1,303-1,333
CTD CTDΔB1 CTDΔB2 CTDΔB1ΔB2 CTDΔR1
Gal4DBD
α-Tubulin
70kDa
50kDa
Predicted Protein Sizes
CTDΔB1: ~55kDa
CTDΔB2: ~56kDa
CTDΔB1ΔB2: ~54kDa
CTDΔR1: ~56kDa
F
HER3-CTD
CTDΔB1
CTDΔB2
CTDΔB1ΔB2
CTDΔR1
p<0.01
p<0.01
p<0.001
0 1 2 3 4 5 6 7 8 9
Relative Luciferase Activity Fold Change
(Normalized to the pM vector control)

FIGURE 5: HER3 can associate with the cyclin D1 promoter. (**A) Nuclear EGFR and HER2 associate with the cyclin D1 promoter.** ChIP using an anti-EGFR, anti-HER2, or normal rabbit IgG antibody was performed with SKBr3 cells and isolated DNA was subsequently used for qPCR with primers flanking the 122 bp cyclin D1 promoter region (n=3). qPCR specificity for the 122 bp cyclin D1 promoter was confirmed by agarose gel electrophoresis of semi-qPCR products. DAPA analysis was performed using a biotinylated 122 bp cyclin D1 promoter probe incubated with 400 ug of nuclear lysate harvested from SKBr3 cells. Bound proteins were isolated with streptavidin agarose beads and subsequently fractionated on SDS-PAGE followed by immunoblotting for EGFR or HER2. Nuclear lysate incubated with beads only lacked association. (**B) Nuclear HER3 can associate with the cyclin D1 promoter.** ChIP using a N-TERM anti-HER3 or a human IgG antibody was performed with $H226^{R}$, SKBr3, and BT549 cells. qPCR was performed as in 5A. (**C) HER3 can associate with a cyclin D1 promoter probe.** DAPA analysis was performed using nuclear lysate harvested from $H226^{R}$, SKBr3, MCF-7, and BT549 cells as in 5A. Proteins isolated from the probe were subsequently fractionated on SDS-PAGE followed by immunoblotting for HER3. (**D) HER3 association with the cyclin D1 promoter probe is specific.** DAPA analysis was performed as in 5A using nuclear lysate harvested from $H226^{R}$, SKBr3, MCF-7, and BT549 cells transfected with either non-targeting (NT) or HER3 siRNA for 48 hr. Proteins isolated from the probe were subsequently fractionated on SDS-PAGE followed by immunoblotting for HER3. All data points for ChIP are represented as mean +/- s.e.m and normalized to the IgG control. $P<0.05$.

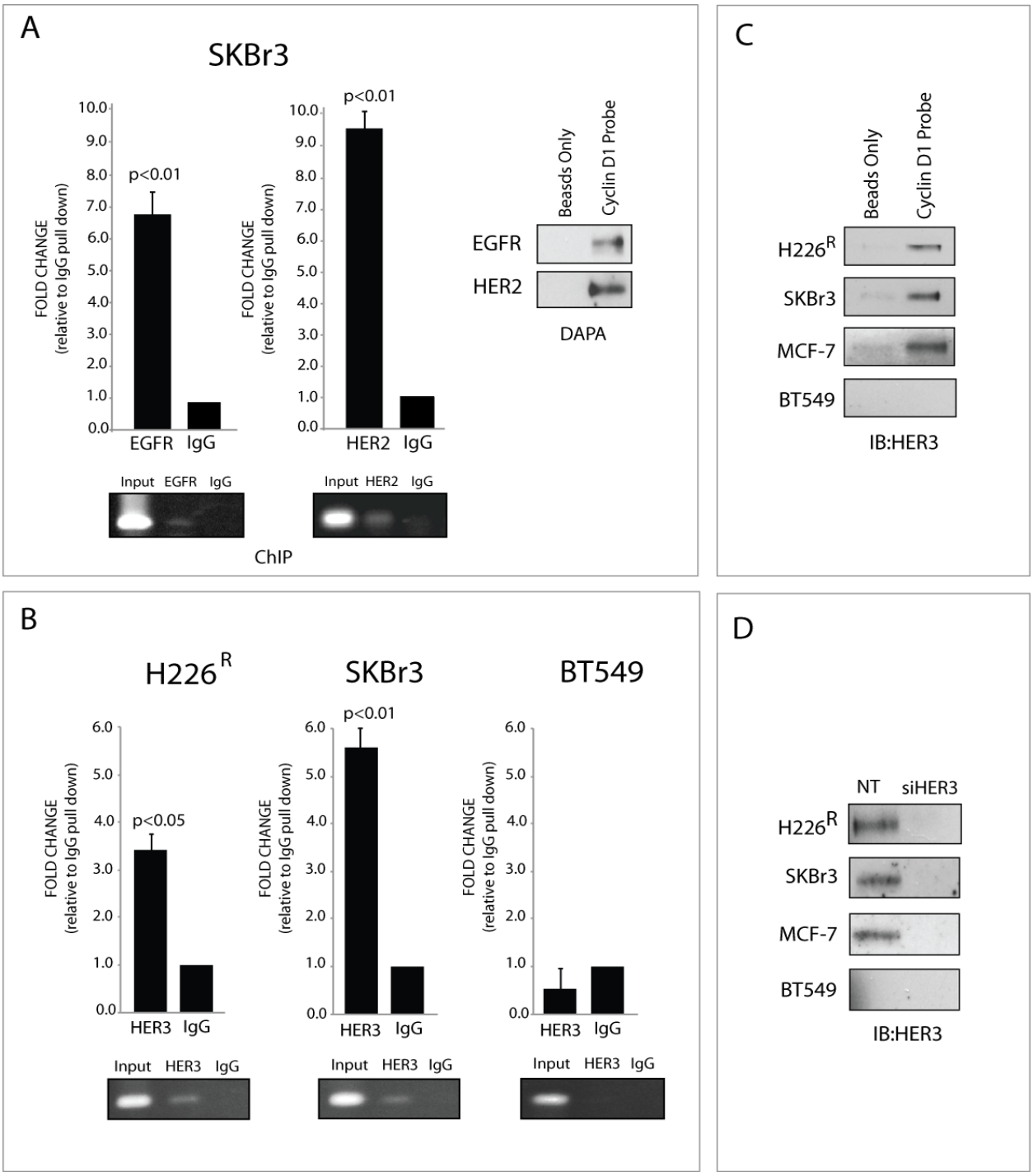

A
SKBr3
FOLD CHANGE (relative to IgG pull down)
p<0.01
EGFR IgG
p<0.01
HER2 IgG
Input EGFR IgG
Input HER2 IgG
ChIP
Beasds Only
Cyclin D1 Probe
EGFR
HER2
DAPA
C
Beads Only
Cyclin D1 Probe
H226R
SKBr3
MCF-7
BT549
IB:HER3
B
H226R
SKBr3
BT549
p<0.05
p<0.01
HER3 IgG
Input HER3 IgG
D
NT siHER3
H226R
SKBr3
MCF-7
BT549
IB:HER3

FIGURE 6: Nuclear HER3 can regulate a minimal region of the cyclin D1 promoter via its bipartite transactivation domain. (**A) HER3 knockdown decreases the activation of the cyclin D1 promoter.** $H226^R$, SKBr3, and MCF-7 cells were incubated with non-targeting (NT) or HER3 siRNA for 24 hr followed by transfection with the 122 bp cyclin D1 promoter-luciferase and Tk-*Renilla* reporter plasmids for 48 hr prior to quantification by dual luciferase assay (n=6). Percent decreases in cyclin D1 promoter-luciferase activity were normalized to NT transfected cells. The graph is representative of three independent experiments. Luciferase lysate was fractionated on SDS-PAGE followed by immunoblotting for HER3. (**B) HER3 overexpression activates the cyclin D1 promoter**. SCC6, HCC1954, SKBr3, and BT474 cells were transfected with HER3WT or control vector, 122 bp cyclin-D1-luciferase and Tk-*Renilla* reporter plasmids for 48 hr prior to quantification by dual luciferase assay (n≥3). Percent increases in cyclin D1-promoter luciferase were normalized to vector transfected cells. The graph is representative of three independent experiments. Nuclear lysate was harvested from each cell line and fractionated on SDS-PAGE followed by immunoblotting for HER3. α-tubulin and Histone H3 were used as loading and purity controls for the Nuc fraction. (**C) HER3$\Delta B_1\Delta B_2$ overexpression can prevent the activation of the cyclin D1 promoter while HER3DM remains functional.** SCC6, BT474 and HCC1954 cells were transfected with HER3WT, HER3DM, HER3$\Delta B_1\Delta B_2$ or control vector, the 122 bp cyclin-D1-luciferase and Tk-*Renilla* reporter plasmids for 48 hr prior to quantification by dual luciferase assay (n≥3). Percent increases in cyclin D1 promoter-luciferase were normalized to vector transfected cells. The graph is

representative of seven independent experiments. **Inset 1:** CHOK1 cells were transfected with HER3WT, HER3$\Delta B_1\Delta B_2$ or control vector for 48 hr prior to harvesting NN and Nuc protein, fractionation on SDS-PAGE followed by immunoblotting for HER3. α-tubulin and Histone H3 were used as loading and purity controls for Nuc fraction. **Inset 2:** CHOK1 cells were transfected with HER3WT, HER3DM, HER3$\Delta B_1\Delta B_2$ or control vector and stimulated for 40 min with 5nM neuregulin-1. Whole cell lysate was fractionated on SDS-PAGE followed by immunoblotting for indicated proteins. (**D) HER3 knockdown decreases cyclin D1 expression.** SCC6 and BT474 cells were incubated with NT or HER3 siRNA for 48 hr prior to harvesting RNA. The mRNA expression of cyclin D1 was determined by qPCR (n=3) and normalized to NT transfected cells. The graph is representative of two independent experiments**. (E) The overexpression of HER3WT enhances cyclin D1 expression while HER3$\Delta B_1\Delta B_2$ is hindered.** SCC6 and BT474 cells were transfected with HER3WT, HER3$\Delta B_1\Delta B_2$ or control vector for 72 hr prior to harvesting RNA. The mRNA expression of cyclin D1 was determined by qPCR (n=3) and normalized to vector transfected cells. The graph is representative of four independent experiments. All luciferase values were normalized to DNA content, protein content, and the expression of *Renilla* luciferase. All data points are represented as mean +/- s.e.m. $P<0.05$.

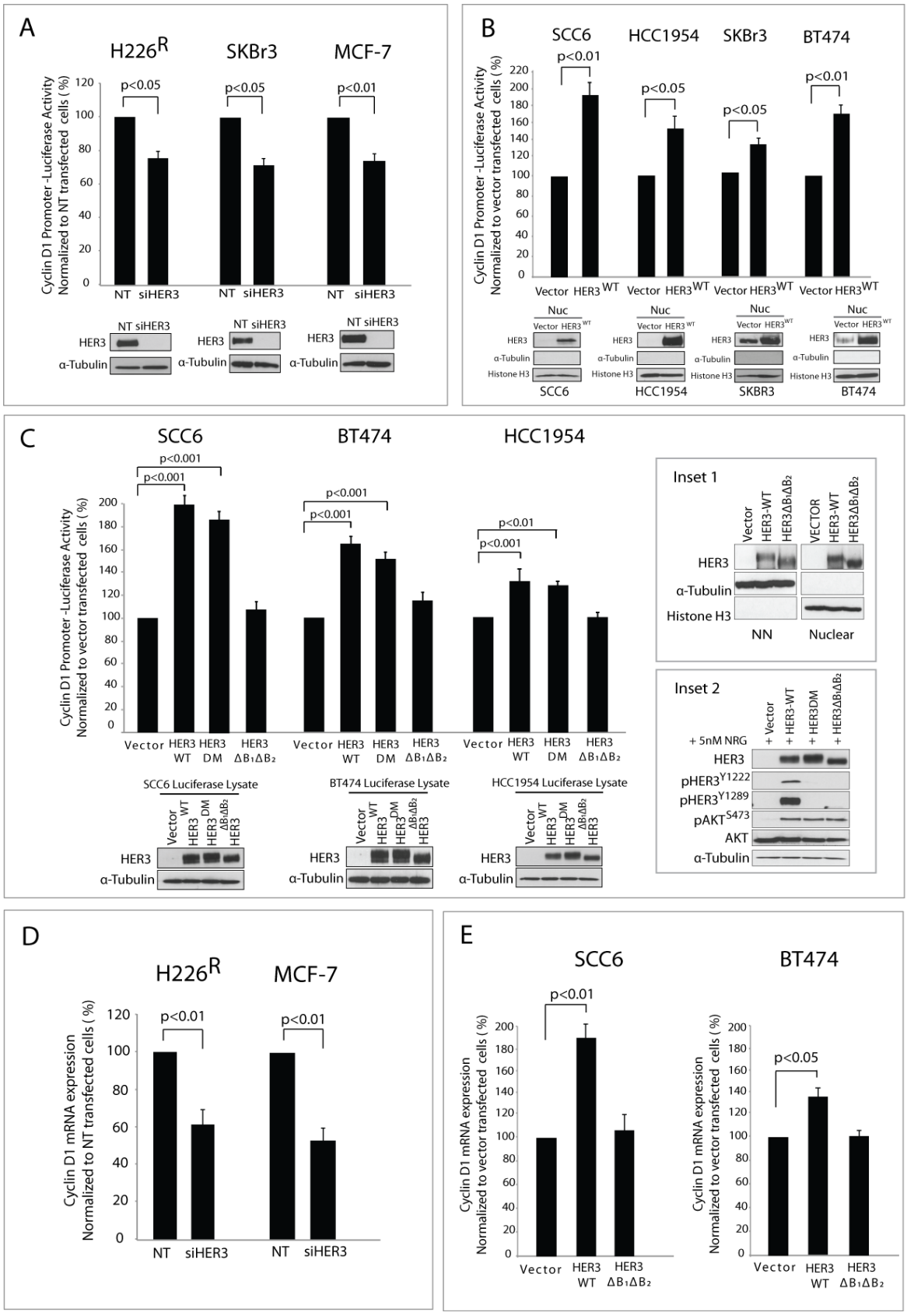
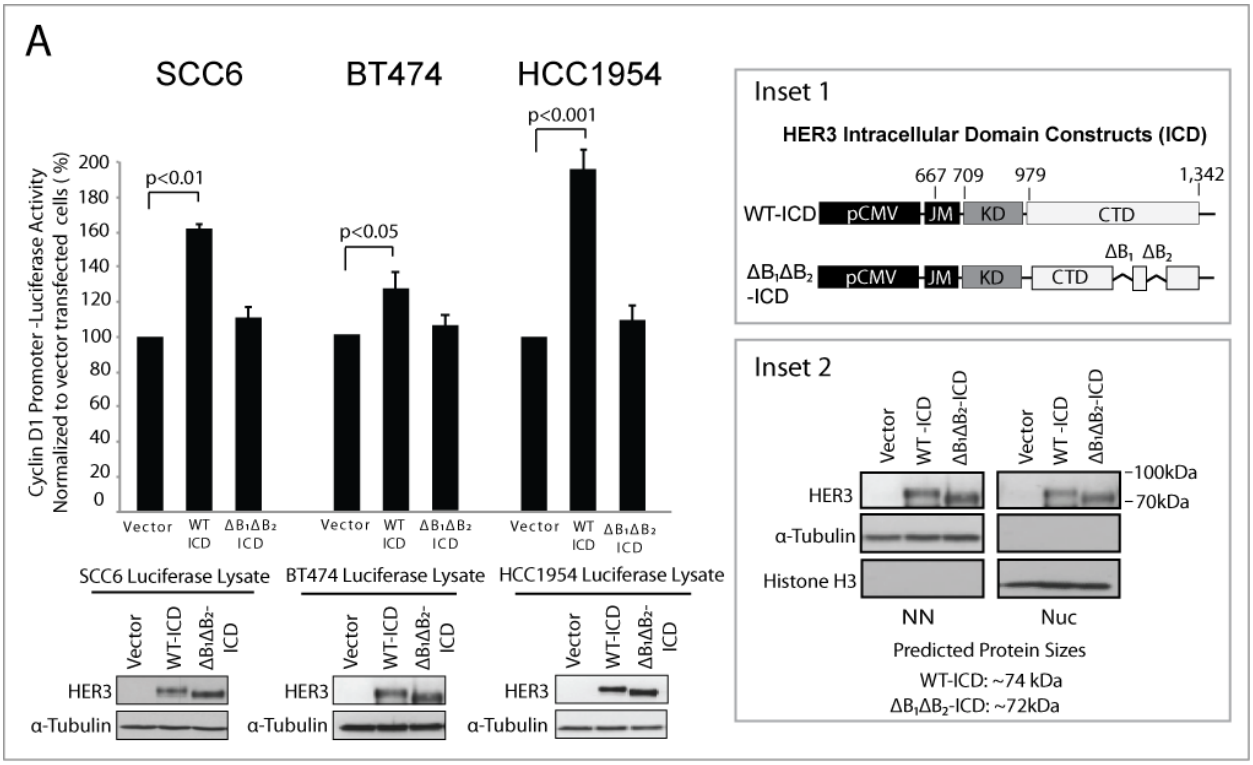
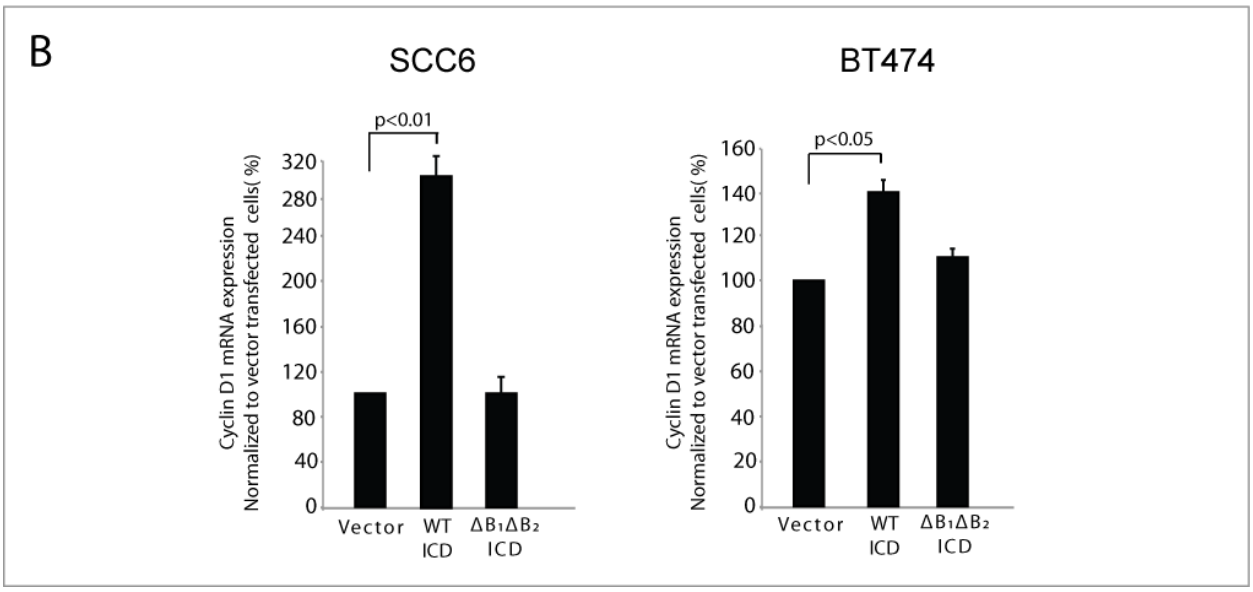

A
H226R
SKBr3
MCF-7
Cyclin D1 Promoter -Luciferase Activity
Normalized to NT transfected cells (%)
p<0.05
p<0.05
p<0.01
NT siHER3
HER3
α-Tubulin
B
SCC6
HCC1954
SKBr3
BT474
Cyclin D1 Promoter -Luciferase Activity
Normalized to vector transfected cells (%)
p<0.01
p<0.05
p<0.05
p<0.01
Vector HER3WT
Nuc
Histone H3
C
SCC6
BT474
HCC1954
Cyclin D1 Promoter -Luciferase Activity
Normalized to vector transfected cells (%)
p<0.001
p<0.001
p<0.001
p<0.001
p<0.01
p<0.001
Vector HER3 WT HER3 DM HER3 ΔB1ΔB2
SCC6 Luciferase Lysate
BT474 Luciferase Lysate
HCC1954 Luciferase Lysate
Inset 1
Vector
HER3-WT
HER3ΔB1ΔB2
VECTOR
HER3
α-Tubulin
Histone H3
NN
Nuclear
Inset 2
+ 5nM NRG
+ Vector
+ HER3-WT
+ HER3DM
+ HER3ΔB1ΔB2
HER3
pHER3Y1222
pHER3Y1289
pAKTS473
AKT
α-Tubulin
D
H226R
MCF-7
Cyclin D1 mRNA expression
Normalized to NT transfected cells (%)
p<0.01
p<0.01
NT siHER3
E
SCC6
BT474
Cyclin D1 mRNA expression
Normalized to vector transfected cells (%)
p<0.01
p<0.05
Vector HER3 WT HER3 ΔB1ΔB2

FIGURE 7: An intracellular domain (ICD) mutant of HER3 can regulate a minimal region of the cyclin D1 promoter via its bipartite transactivation domain. (A) HER3WT ICD (WT-ICD) can activate the cyclin D1 promoter while HER3$\Delta B_1\Delta B_2$-ICD ($\Delta B_1\Delta B_2$-ICD) is hindered. SCC6, BT474, and HCC1954 cells were transfected with WT-ICD, $\Delta B_1\Delta B_2$-ICD or control vector, the 122 bp cyclin D1-luciferase and Tk-*Renilla* reporter plasmids for 48 hr prior to quantification by dual luciferase assay (n≥3). Percent increases in cyclin D1 promoter-luciferase were normalized to vector transfected cells. The graph is representative of three independent experiments. **Inset 1:** Illustration of both the WT-ICD and $\Delta B_1\Delta B_2$-ICD constructs. **Inset 2:** CHOK1 cells were transfected with WT-ICD and $\Delta B_1\Delta B_2$-ICD for 48 hr prior to harvesting NN and Nuc protein, fractionation on SDS-PAGE followed by immunoblotting for HER3. α-tubulin and Histone H3 were used as loading and purity controls for Nuc fraction. (**B) The overexpression of WT-ICD can enhance cyclin D1 expression while $\Delta B_1\Delta B_2$-ICD is hindered.** SCC6 and BT474 cells were transfected with WT-ICD, $\Delta B_1\Delta B_2$-ICD or control vector for 48 hr prior to harvesting RNA. The mRNA expression of cyclin D1 was determined by qPCR (n=3) and normalized to vector transfected cells. The graph is representative of three independent experiments. All luciferase values were normalized to DNA content, protein content, and the expression of *Renilla* luciferase. All data points are represented as mean +/- s.e.m. $P<0.05$. ICD (intracellular domain).


A
SCC6
BT474
HCC1954
p<0.01
p<0.05
p<0.001
Cyclin D1 Promoter -Luciferase Activity
Normalized to vector transfected cells (%)
200
180
160
140
120
100
80
60
40
20
0
Vector
WT ICD
ΔB1ΔB2 ICD
SCC6 Luciferase Lysate
BT474 Luciferase Lysate
HCC1954 Luciferase Lysate
Vector
WT-ICD
ΔB1ΔB2- ICD
HER3
α-Tubulin
Inset 1
HER3 Intracellular Domain Constructs (ICD)
667 709
979
1,342
WT-ICD
pCMV
JM
KD
CTD
ΔB1 ΔB2
ΔB1ΔB2 -ICD
Inset 2
Vector
WT -ICD
ΔB1ΔB2-ICD
HER3
α-Tubulin
Histone H3
~100kDa
~70kDa
NN
Nuc
Predicted Protein Sizes
WT-ICD: ~74 kDa
ΔB1ΔB2-ICD: ~72kDa


B
SCC6
BT474
p<0.01
p<0.05
Cyclin D1 mRNA expression
Normalized to vector transfected cells(%)
320
280
240
200
160
120
80
40
0
160
140
120
100
80
60
40
20
0
Vector
WT ICD
ΔB1ΔB2 ICD

Chapter V

General Discussion and Future Directions

The epidermal growth factor receptor represents one of the most well studied molecules in all of biology. From its early identification and cloning, to the discovery of its role in cancer, it has been at the forefront of our understanding of receptor tyrosine kinases (RTKs) and cell signals that mediate homeostasis, but when overexpressed facilitate tumorigenesis. Since its first identification of its role as a nuclear transcription factor in 2001[1], EGFR has led the way in discovery of various other nuclear localized RTKs, including HER2[2], HER3[3], HER4[4], IGF1R[5], cMET[6] and others.

The nuclear localization of HER family receptors challenges the classical dogma centered on their role as membrane bound signaling transducers and therefore has been met with much skepticism by the RTK community[7-11]. Many reviewers are skeptical of experimental nuclear fractionation procedures which may actually capture proteins associated with the endoplasmic reticulum (ER) or are present in the cytoplasm[12]. In this thesis we have overcome much of these obstacles through multiple mechanisms, supporting the existence of nuclear HER receptors and their respective roles in tumorigenesis. First, we have optimized our nuclear fractionation procedure to ensure accurate separation of cytoplasmic, ER, and nuclear proteins, maintaining nuclear envelope integrity until the last step of the fractionation protocol. Examination of our nuclear fractionate isolated from this protocol indicated that it is quantitatively clear of contaminating cytoplasmic and ER proteins (**Chapter III: Figure 1B, and Chapter IV: Figure 1B**). Second, we have performed extensive confocal immunofluorescent microscopic analyses for the detection of nuclear EGFR and HER3 (**Chapter III: Figure 1B, and Chapter IV: Figure 2**). Sequential confocal imaging was used to excite each fluorophore rather than simultaneous image acquisition that can result in crosstalk of

fluorescence emission due to broad bandwidths exhibited by many fluorophores. Additionally, both EGFR and HER3 were stained with an Alexa Fluor 546 secondary antibody, which has less overlap with the broad emission spectrum of DAPI (used to visualize the nucleus). Additionally, Z-stack images were acquired for nuclear EGFR staining and statistical co-localization analyses were performed from these images, further indicating significant overlap between the EGFR and the nucleus (**Chapter III: Figure 1B**). Further, transmission electron microscopy (EM) was performed yielding immunogold labeled EGFR localized within the nucleus and around the nuclear envelope (**Chapter III: Figure 1C**). Immunohistochemical analyses of human TNBC tumors on a tissue microarray (TMA) indicated that approximately 20% of tumors expressed nuclear EGFR (**Chapter III: Figure 1C**), demonstrating specificity of this nuclear signal (a non-specific signal would yield a more homogenous staining pattern). Further, both siRNA and blocking peptide approaches were used to validate the specificity of the antibodies used for nuclear EGFR and nuclear HER3 detection (**Chapter III: Figure 1B, and Chapter IV: Figure 1C**). Finally, we have detected EGFR and HER3 association with specific gene promoter regions by chromatin immunoprecipitation (ChIP) analyses, indicating that these proteins can associate with DNA inside the nucleus (**Chapter II: Figure 6A, and Chapter IV: Figure 5**). Collectively, this body of work supports the nuclear localization of HER receptors.

Since the identification of nuclear EGFR, numerous research groups have embarked on understanding how nuclear HER receptors translocate and function from within the nucleus[13-16]. Initial studies indicated that both nuclear EGFR and HER2 were localized in the nucleus in their full-length forms (approximately 170 and 185 kDa)[1,16-18].

Studies performed in this thesis identify that HER3 is also localized to the nucleus in its full-length form, where both N- and C-terminal antibodies were used to isolate HER3 in the nucleus by nuclear fractionation and immunofluorescent microscopy (**Chapter IV, Figure 1D and Figure 2**). Additionally, clathrin/dynamin induced endocytosis are necessary for EGFR and HER2 nuclear transport, with more recent data indicating that EGFR and HER2 are localized with Sec61β in the INM prior to nuclear import[14-16,19]. These pieces of data suggest that HER receptors translocate to the nucleus in a similar manner. In the current thesis, we found that phosphorylation of EGFR on the SFK dependent tyrosine site 1101 is necessary for its nuclear translocation (**Chapter II, Figure 7**). Currently, the specific phosphorylations of HER2 and HER3 necessary for their nuclear transport are undefined, however both receptors have been described to associate with SFKs[20-22]. Additionally, the C-terminal domains of EGFR and HER2 have been shown to contain strong transactivation potential in Gal4 UAS-chloramphenicol transferase reporter assays[1,17]. We also found that the C-terminus of HER3 contained strong transactivation potential, similar to levels detected by the C-terminal region of EGFR (**Chapter IV, Figure 3**). EGFR, HER2, and HER3 have now all been identified to function as co-transcription factors in the nucleus, and to regulate similar gene targets[1,23,24]. Work in this thesis indicates that EGFR, HER2, and HER3 can associate with a 122 base pair region of the cyclin D1 promoter, suggesting that nuclear HER family dimers may exist (**Chapter IV, Figure 5**). Finally, both HER2 and HER3 have been detected in the nucleoli of cancer cells[25,26], and studies performed in this thesis indicated that high levels of nucleolar EGFR were expressed in TNBC cell lines and human tumor specimens (**Chapter III, Figure 1**). Nuclear HER2 has been shown to

associate with RNA polymerase I in the nucleoli of breast cancer cells where it can regulate rRNA synthesis and cellular translation[26], however, whether EGFR or HER3 function similarly has yet to be defined. Collectively, current data supports an interconnected mechanism of HER family nuclear translocation and function in cancer cells, all of which may contribute to tumor development and progression.

One question that remains elusive in the field of nuclear HER family biology is the role of HER family kinase activity and its necessity for nuclear transport and/or function. This question has many clinical implications because if kinase activity is necessary then tyrosine kinase inhibitors (TKIs) should be effective therapies for the elimination of nuclear HER family functions. In this thesis, we describe how the kinase defective HER family receptor, HER3, can function as a prominent co-transcription factor to enhance cyclin D1 expression (**Chapter IV, Figure 6**). Interestingly, the addition of neuregulin did not enhance cyclin D1 promoter-luciferase assays or cyclin D1 mRNA expression (data not shown). Additionally, a HER3 C-terminal domain mutant (lacking the extracellular dimerization domain and transmembrane domain, and thus not localized to the cell surface) was still capable of activating cyclin D1 promoter-luciferase and mRNA expression (**Chapter IV, Figure 7**). These data suggest that the co-transcriptional functions of HER3 are kinase independent. We also demonstrate that the overexpression of HER3 in HER family deficient Chinese hamster ovary (CHO) cells led to an accumulation of HER3 in the nucleus, suggesting that a kinase defective receptor can be effectively nuclear localized (**Chapter IV, Figure 6C, Inset 1**). This phenomenon is further described in gefitinib resistant cell lines, which have increased nuclear levels of EGFR. Treatment of these cells with gefitinib effectively inhibited the phosphorylation

of specific tyrosine sites on EGFR but did not impair nuclear EGFR translocation[27]. Furthermore, cetuximab has been shown to induce EGFR nuclear translocation by numerous investigators[28,29], even though cetuximab effectively prevents ligand binding to the EGFR. Finally, the use of an EGFR tyrosine kinase inhibitor (AG1478) did not inhibit nuclear EGFRVIII expression, but effectively inhibited the phosphorylation of EGFRVIII on tyrosine 1173[30]. Collectively, these studies indicate that TKIs targeting the HER family may not be an effective way to inhibit HER family trafficking to the nucleus or HER family transcriptional functions.

With the discovery of mechanisms of nuclear HER family receptor translocation and function, recent interest has prompted investigations into nuclear HER receptors and therapeutic response. Whether it be that HER receptors translocate to the nucleus under times of stress (such as hypoxic conditions or lack of ATP) or that they play functional redundant roles in the nucleus as their membrane-bound counterparts, evidence supports increased nuclear HER receptor expression in cancer cells resistant to chemotherapy and molecular targeted agents[18,27,29,31,32]. This reasoning is supported by the strong correlation between nuclear HER family expression and poor prognosis of several human cancers[33]. Two pivotal studies in the setting of acquired resistance to cetuximab set the platform for the studies performed in this thesis. First, was the identification that SFKs were hyperactivated in cetuximab resistant cell clones as compared to cetuximab sensitive parental controls[34]. These studies indicated that EGFR and SFKs activate each other in resistant cells, with the knockdown of EGFR resulting in a loss of SFK activity, and alternatively, SFK inhibition (with the pan-SFK inhibitor dasatinib) resulting in a loss of EGFR activity[34]. Second, was the identification

that cetuximab resistant clones had increased expression of nuclear EGFR[29]. In this study, Li et al. found that SFK activity was necessary for nuclear EGFR translocation, where SFK blockade resulted in an increase in plasma-membrane expression of EGFR. Ultimately, this study concluded that dasatinib could re-sensitize acquired cetuximab resistant cells to cetuximab[29]. Building off these studies, we found that TNBCs, a breast cancer subtype that commonly overexpress the EGFR but have proven to be intrinsically resistant to cetuximab in both clinical trials[35-38] and in Chapter III (**Figure 2**), also express nuclear EGFR. In these studies we demonstrated that inhibition of nuclear EGFR translocation via SFK blockade could greatly enhance the sensitivity of TNBC cells to cetuximab induced growth inhibition (**Chapter III, Figure 6**). This finding expands the field of nuclear HER family biology and therapy in three major ways: First, it indicates that nuclear EGFR enhances tumor biology in such a way that it can manifest in intrinsic resistance to cetuximab. We hypothesize that nuclear EGFR's co-transcriptional functions allow for maintained growth and survival signals in cetuximab treated cells even though classical EGFR signaling is effectively shut down with cetuximab. While this hypothesis has not been tested directly (**see future directions**), this initial study indicates that EGFR can function in two distinct nodes to promote tumorigenic outcomes- through both the classical plasma-membrane signaling node and the nuclear signaling node. Second, our data suggests that cetuximab treatment regimens are not accurately targeting all of EGFR's oncogenic functions in tumors. Thus, cetuximab may fail in cancer patients because nuclear EGFR is viably signaling and promoting tumor growth and survival. Finally, the data presented in this thesis suggests that patients should be selected for cetuximab response based on nuclear

EGFR status. In the most recent clinical trial by Baselga et al., metastatic TNBC patients treated with both cisplatin and cetuximab did not meet the primary end point of a greater than 20% increase in overall response rate (ORR) as compared to cisplatin only treated patients[35]. One of the major disadvantages of this clinical trial was the lack of predictive biomarkers to identify patients who would benefit from cetuximab. The data presented in this thesis suggests that nuclear EGFR expression may predict patients who would not respond to cetuximab alone, but whom may benefit from the addition of dasatinib to their treatment regimen. Interestingly, the secondary end point of this clinical trial was progression- free survival (PFS), which was significantly prolonged in dually treated patients- suggesting some clinical response of adding cetuximab to standard chemotherapy[35]. The fact that some people did respond to cetuximab and chemotherapy suggests that these people may be non-nuclear EGFR expressing patients, further highlighting the need to stratify patient selection to obtain better clinical results. Collectively, the work highlighted in this thesis broadens our knowledge of how to best target EGFR signaling in TNBC and indicates that nuclear EGFR can mediate cetuximab resistance.

Future Directions:

The studies performed in this thesis have several immediate and long term implications for nuclear HER family biology and therapeutic targeting. The major questions stemming from this work are focused on the role of tyrosine 1101 in initiating nuclear EGFR translocation, the clinical implications for targeting nuclear EGFR in TNBC, and finally using transcriptional null HER family mutants as tools for studying

nuclear HER receptor function in both tumor biology and resistance to therapeutic agents (see **Figure 1, Discussion**).

In Chapter II we identified that EGFR mutated on tyrosine 1101 is defective in nuclear entry (**Figure 7**). Since it is well established that nuclear EGFR originates from plasma membrane-bound EGFR[14,19], and that SFKs associate and phosphorylate EGFR while localized there[39], it suggests that phosphorylation on tyrosine 1101 is an early event that influences EGFR trafficking to the nucleus. However, how phosphorylated tyrosine 1101 initiates this event is unknown. It is quite possible that specific endosomal proteins or microtubule-associated proteins (such as dynein, a microtubule associated motor protein necessary for EGFR movement from the endosome to the Golgi[40]) interact with phosphorylated tyrosine 1101. Additionally, it is also possible that specific proteins necessary for COPI mediated retrograde translocation from the Golgi to the ER or for nuclear import interact with phosphorylated tyrosine 1101 of the EGFR. These important questions will provide mechanistic insight into the initial signals that mediate nuclear EGFR translocation and may uncover new proteins that can be therapeutically targeted to prevent nuclear EGFR trafficking.

In addition to understanding how tyrosine 1101 phosphorylation regulates nuclear EGFR trafficking, tyrosine 1101 may also prove to be an important biomarker for cetuximab resistant tumors. While IHC can be used to detect nuclear EGFR, it is also possible to use tyrosine 1101 activation as a surrogate, especially when examining protein lysates harvested from tumors (obtaining nuclear lysate from tumors is often difficult due to the processing of the sample and disruption of the plasma and nuclear membranes). Interestingly, we report that erlotinib and cetuximab do not block basal

levels of EGFR activation on tyrosine 1101 in TNBC cell lines whereas tyrosine 1173 activation was effectively inhibited (data not shown)[41]. These data highlight the importance of examining the phosphorylation status of tyrosine 1101 in addition to autophosphorylated tyrosine sites on the EGFR upon drug treatments, and further indicates that anti-EGFR agents do not effectively inhibit nuclear EGFR trafficking signals. Collectively, the activation of EGFR on tyrosine 1101 may be a crucial biomarker for cetuximab response, and should be evaluated upon clinical evaluation of EGFR overexpressing tumors prior to cetuximab treatment.

Approximately 15% of all breast cancers are considered TNBCs[42,43]. There are currently minimal targeted therapy options for the treatment of TNBC due to the loss of molecular targets such as the estrogen receptor and HER2. However, EGFR has been identified as a therapeutic target in TNBC since a high percentage of TNBCs over-express EGFR, which is further associated with poor patient prognosis[44-46]. In this thesis we demonstrate that TNBC cell lines are in fact sensitive to genetic ablation studies of the EGFR (**Chapter III, Figure 2**), indicating that EGFR is a driver of TNBC cell growth. Further studies in cetuximab resistant TNBC cell lines indicated that TNBC's cells could be sensitized to cetuximab upon disruption of nuclear EGFR trafficking with SFK inhibition (**Chapter III, Figure 6**). These studies indicate that cetuximab may fail in TNBC because EGFR is localized in the nucleus, protected from the growth inhibitory effects of cetuximab and functioning independently to promote survival and growth signals. The majority of these studies were performed using established TNBC cell lines and must be translated into the *in vivo* setting prior to combinatorial clinical trials. Thus, immediate future directions of this work would be to investigate if cetuximab resistant

TNBC xenografts (either flank or orthotopic fat pad models) and patient derived TNBC xenografts (PDXs) could be sensitized to cetuximab upon nuclear EGFR inhibition with dasatinib therapy. These *in vivo* models will identify if this combinatorial therapeutic regimen may benefit nuclear EGFR expressing TNBC patients. More broadly, this therapeutic strategy may prove effective for the treatment of other nuclear EGFR expressing tumors in which it has been correlated with poor prognosis.

In Chapter III, investigations of dasatinib vs. vehicle treated MDAMB468 TNBC tumors indicated that nuclear EGFR translocation was robustly inhibited with dasatinib resulting in increased plasma membrane localized EGFR (**Figure 5**). These data support further research on whether dasatinib can effectively inhibit nuclear EGFR translocation in human patients, a critical first initial step prior to combinatorial therapeutic clinical trials. This future direction has now been designed into a window trial at the University of Wisconsin Carbone Cancer Center (see **Figure 2, Discussion**). Upon diagnosis of TNBC, nuclear EGFR expression status will be evaluated by IHC analysis from the original diagnostic biopsy and consenting nuclear EGFR positive patients will undergo treatment with dasatinib for 10-14 days (the window period) prior to surgery. Surgically resected tumors will be analyzed for nuclear EGFR expression after this window period and compared to EGFR expression in the biopsied tumors prior to treatment. We hypothesize that dasatinib treated tumors will have less nuclear EGFR expression and increased plasma membrane EGFR expression as compared to the original tumor biopsy. Collectively, these studies pave the way for personalized treatment regimens for TNBC patients, a major clinical need in this cancer setting.

The studies performed in this thesis aimed to improve and refine current EGFR-centric therapeutic approaches for treatment of cancer. Building off these translational studies, the experiments performed in Chapter IV provide important tools for the future examination of nuclear HER family receptor function and resistance to therapeutics. In this chapter, the C-terminal region of the HER3 receptor was mapped to identify the key regions that function in transcription, resulting in a mutant that was transcriptionally null but functional as a receptor tyrosine kinase on the plasma membrane (**Chapter IV, Figure 6**). This is a very powerful tool for studying the role of nuclear HER3's transcriptional functions in tumor biology. For example, HER3 is required for the development of HER2 driven breast cancers[47] and is highly associated with drug resistance to anti-HER2 inhibitors in breast and ovarian cancers[48,49], with anti-estrogen therapies in ER- positive breast cancers[50], and with anti –EGFR inhibitors in NSCLC and HNSCC[51-53]. The development of a transcriptionally null HER3-$\Delta B_1\Delta B_2$ receptor allows us to ask the question of whether tumor development or resistance to these inhibitors is augmented upon the overexpression of HER3-WT vs. HER3-$\Delta B_1\Delta B_2$. A differential outcome would indicate that the nuclear transcriptional functions of HER3 are important for tumor cell development or resistance to specific therapeutic agents. Previous reports have indicated that the C-terminal regions of EGFR and HER2 also contain potent transactivation domains[1,17], however, the minimal mapping of these domains is under current investigation by the Wheeler Laboratory as well as identifying the impact they may have on tumor biology. If the transcriptional activity of nuclear HER receptors prove to be important for tumor biology and therapeutic resistance, future aims may be to develop allosteric inhibitors that augment the structure or block the C-

terminal transactivation domains of HER receptors. Collectively, the identification of transcriptionally null nuclear HER receptors will allow for the systematic examination of nuclear HER receptor transcriptional functions in both tumor biology and resistance to therapeutic agents.

The identification of transcriptionally null HER family receptors that are functionally active as signal transducers on the plasma membrane are critical for the examination of HER receptor transcriptional functions in cancer. While HER family receptors have been shown to regulate the transcription of numerous oncogenes, secondary to their ability to phosphorylate specific substrates in the nucleus, the impact that these two identified functions have on tumor biology are relatively ill defined. There have been two studies indicating that cells expressing EGFR mutated in its NLS have reduced colony formation *in vitro* and tumor growth in vivo[30,54]. However, careful examination of EGFR NLS mutants by our laboratory indicated that EGFR intrinsic kinase activity is reduced as compared to the WT EGFR and thus raises the possibility that lack of colony formation and/or tumor growth is due to decreased receptor activity (data not shown). Transcriptionally null HER receptors provide an alternative approach to identifying if the co-transcriptional functions of these receptors augment tumor biology. The discovery of a nuclear HER family function that augments tumor biology would suggest that nuclear HER's are bonafide oncogenes, independent from their membrane bound counterparts, and would greatly strengthen the field of nuclear RTKs and provide further support into their molecular targeting in the future.

The work presented in this thesis has established the foundation for future investigations into nuclear EGFR trafficking pathways, therapeutic approaches on how

to target nuclear HER receptors, and how nuclear HER receptor transcriptional functions may augment parameters of tumor biology and resistance to therapeutic agents (see **Figure 1 Discussion)**. Research over the next decade will hopefully pursue the task of identifying if a nuclear HER receptor can function as a true oncogene, a question that can be explored through the use of transcriptionally null HER family receptors. Results stemming from these findings will most likely implicate nuclear HER receptors and various other nuclear RTKs in the etiology of cancer, and thus provide strong rationale for clinically targeting this mode of signaling in the future.

References

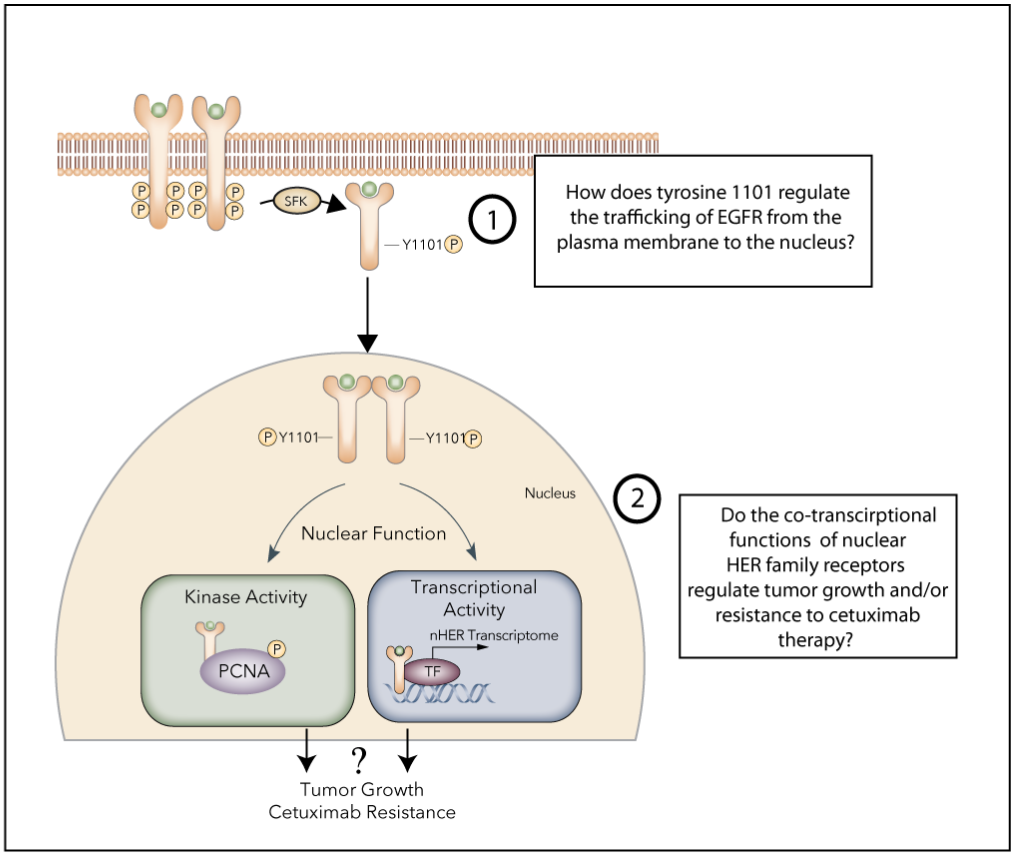
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Figure 1: Uncovering the role of nuclear HER family receptors in tumor biology and resistance to cetuximab therapy. The three main questions stemming from the work performed in this thesis are as follows: **1) How does tyrosine 1101 regulate the trafficking of EGFR from the plasma membrane to the nucleus?** These studies will include the identification of where in the trafficking process tyrosine 1101 influences nuclear EGFR trafficking events (does Y1101 influence early endosomal sorting of the EGFR or does it affect EGFR retrograde movement from the golgi, ER, or nucleus?), the specific proteins that may interact with Y1101 to influence nuclear trafficking, and will ultimately identify novel (and potentially targetable) proteins that function in this process. **2) Do the co-transcriptional functions of nuclear HER receptors regulate tumor growth and/or resistance to cetuximab therapy?** Previous research indicates that nuclear HER receptors regulate a vast array of genes[33], however, the ability for these transcriptional activities to augment tumor growth and/or resistance to cetuximab is unknown. The development of transcriptionally null HER family receptors will allow us to determine the impact that these mutants have on both tumor growth and resistance to different therapies in *in vitro* and *in vivo* model systems. **3) Can nuclear EGFR inhibition sensitize cetuximab resistant tumors to cetuximab therapy?** Our work indicates that SFK blockade can result in an accumulation of EGFR on the cell surface due to the abrogation of nuclear transport. Additionally, abrogation of nuclear EGFR transport was observed in TNBC xenografts treated with dasatinib. These data warrant further investigation of dual cetuximab and dasatinib therapies *in vivo* mouse models prior to movement into clinical trials.


SFK
Y1101
1
How does tyrosine 1101 regulate the trafficking of EGFR from the plasma membrane to the nucleus?
Y1101
Y1101
Nucleus
2
Nuclear Function
Kinase Activity
PCNA
Transcriptional Activity
nHER Transcriptome
TF
Do the co-transcirptional functions of nuclear HER family receptors regulate tumor growth and/or resistance to cetuximab therapy?
?
Tumor Growth
Cetuximab Resistance

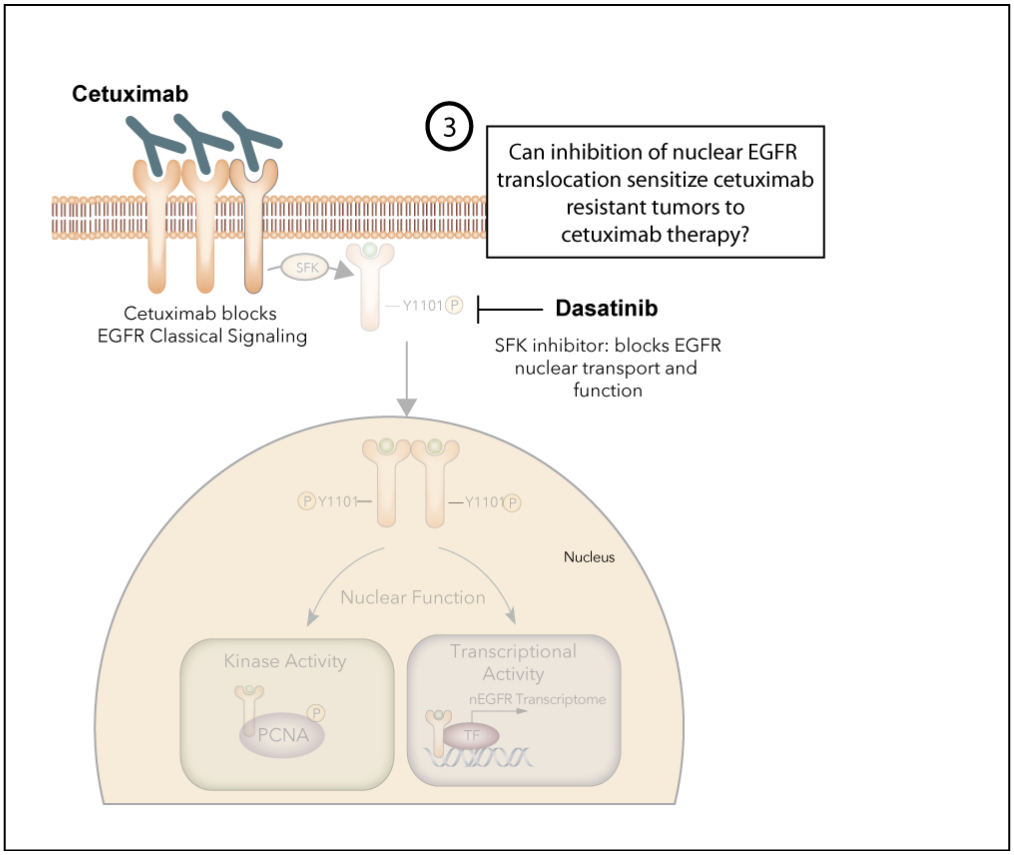
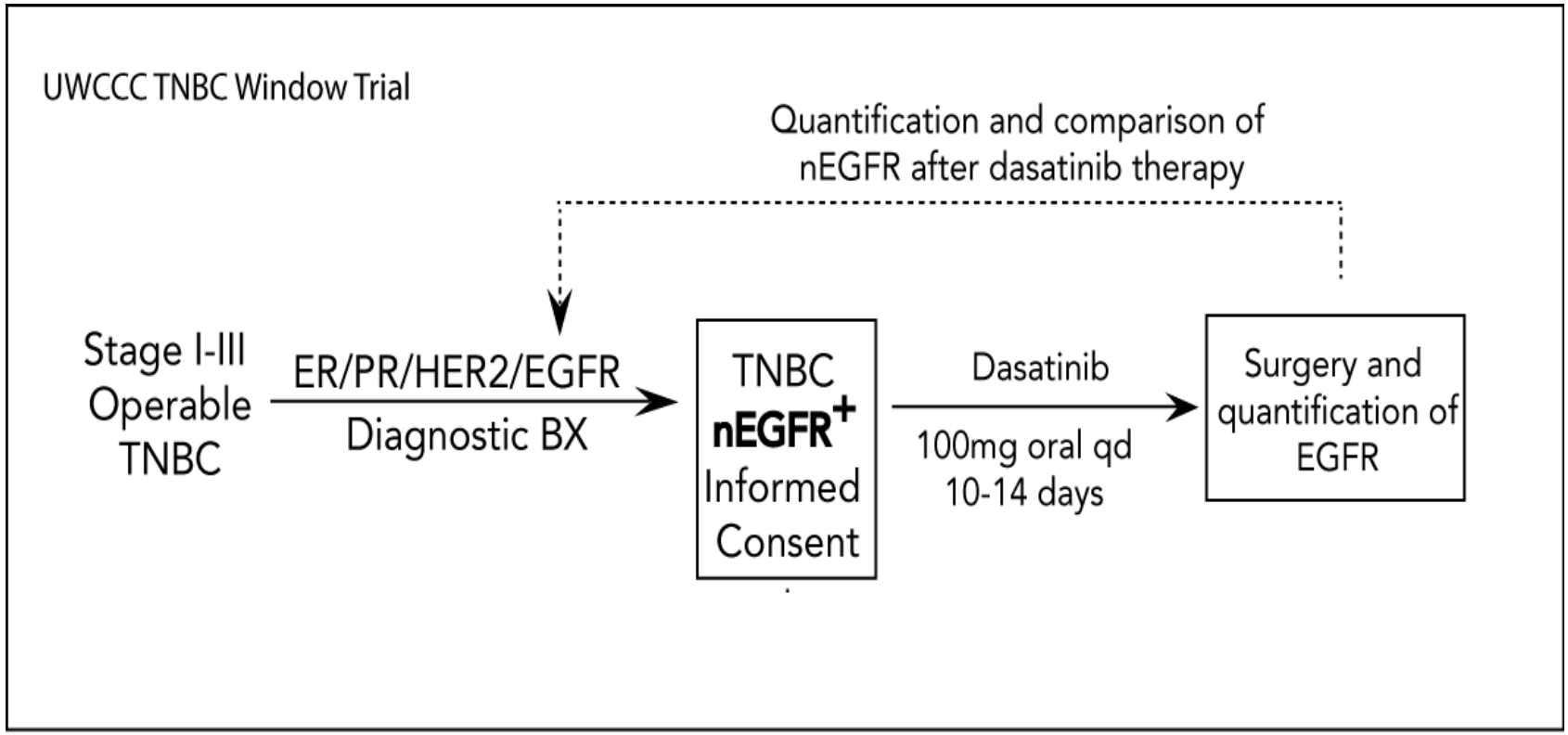

Cetuximab
3
Can inhibition of nuclear EGFR translocation sensitize cetuximab resistant tumors to cetuximab therapy?
SFK
Y1101
Cetuximab blocks EGFR Classical Signaling
Dasatinib
SFK inhibitor: blocks EGFR nuclear transport and function
Y1101
Y1101
Nucleus
Nuclear Function
Kinase Activity
PCNA
Transcriptional Activity
nEGFR Transcriptome
TF

Figure 2: University of Wisconsin Carbone Cancer Center proposed window trial for the use of dasatinib therapy in nuclear EGFR expressing operable TNBC patients. TNBC patients will be examined for nuclear EGFR expression via IHC analysis of original diagnostic biopsies. Consenting nuclear EGFR positive patients will be treated with the small molecule Src family kinase inhibitor, dasatinib, for the 10-14 day window period prior to surgery. Surgically removed TNBC tumor will be examined for EGFR expression and compared to the expression pattern of EGFR from the original diagnostic biopsy. We hypothesize that dasatinib treated tumors will have less nuclear EGFR expression and increased plasma membrane EGFR expression as compared to the original tumor biopsy.


UWCCC TNBC Window Trial
Quantification and comparison of nEGFR after dasatinib therapy
Stage I-III Operable TNBC
ER/PR/HER2/EGFR
Diagnostic BX
TNBC
nEGFR+
Informed Consent
Dasatinib
100mg oral qd
10-14 days
Surgery and quantification of EGFR