The Role of the NF-κB Pathway in Her2-overexpressing Breast Cancer

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ABSTRACT

EVAN C. MERKHOFER: The Role of the NF-κB Pathway in Her2-overexpressing Breast Cancer
(Under the direction of Dr. Al Baldwin)

Overexpression of the membrane-bound receptor tyrosine kinase Her2 (ErbB-2, EGFR2) occurs in approximately 30% of all breast cancers and typically correlates with poor prognosis. Overexpression of Her2 leads to activation of multiple downstream pathways including the MAPK, PI3K/Akt and NF-κB pathways. Her2 has been previously reported to activate the NF-κB pathway. However, the mechanism by which this occurs is poorly elucidated. In this report, we utilize an siRNA approach to investigate the role that the different Inhibitor of Kappa-B Kinase (IKK) subunits play in activation of NF-κB downstream of Her2. We show that IKKα plays a previously unreported role in NF-κB activation via the canonical pathway in Her2-overexpressing breast cancer cells. Furthermore, IKKα plays an important role in NF-κB regulated gene expression and induction of an invasive phenotype in these cells, independent of PI3K. This activation of NF-κB by Her2 also requires the NF-κB pathway kinase, TGF-β-activated kinase 1 (TAK1). Finally, we also show that inhibition of IKKα by siRNA leads to activation of SPARC, a member of the BM-40 family of genes which are often dysregulated in cancer. Activation of the NF-κB pathway can directly suppress expression of SPARC through a mechanism at the promoter of SPARC, proximal to the

transcriptional start site. In summary, we present data elucidating the role and consequences of NF-κB activation in Her2-overexpressing breast cancer.

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List of Abbreviations

AKT	v-akt murine thymoma viral oncogene homolog 1, also known as Protein Kinase B (PKB)
apoE	Apolipoprotein E
BCR	B-Cell Receptor
cAMP	Cyclic adenosine 3'5' monophosphate
ChIP	Chromatin Immunoprecipitation
cIAP	Cellular Inhibitor of Apoptosis Protein
CK2	Casein Kinase 2 (formerly)
COX-2	Cyclooxygenase-2
DCIS	Ductal Carcinoma In-Situ
DNMT	DNA methyltransferase
ErbB2	Human Epidermal Growth Factor 2, also known as Her2
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMSA	Electrophoretic Mobility Shift Assay
ER	Estrogen Receptor
ERK	Extracellular Receptor Kinase
GST	Glutathione-S-transferase
IκB	Inhibitor of KappaB
IKK	Inhibitor of KappaB Kinase
IL-1	Interleukin 1
JNK	c-Jun N-terminal Kinase

Her2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, also known asErbb2
Her3	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3
Her4	v-erb-b2 erythroblastic leukemia viral oncogene homolog 4
IRAK	IL-1R-associated Kinase
LPS	Liposaccharide
LMP-1	Latent Membrane Protein 1
MAPK	Mitogen-Activated Protein (MAP) Kinase
MEF	Mouse Embryonic Fibroblast
MEK	Mitogen Activated Protein Kinase/Extracellular Signal-regulated Kinase Kinase
MEKK	MAP Kinase Kinase Kinase
MyD88	Myeloid Differentiation Primary Response Gene (88)
NBD	NEMO Binding Domain
NEMO	NF-κB Essential Modulator
NF-κB	Nuclear Factor Kappa B
NIK	NF-κB-Inducing Kinase
NLS	Nuclear Localization Sequence
PDGF	Platelet-Derived Growth Factor
PTEN	Phosphatase and Tensin Homolog
PI3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol- 4,5-bisphosphate
PIP_3	Phosphatidylinositol- 4,5-triphosphate
RANKL	Receptor Activator for Nuclear Factor κ B Ligand

RHD	Rel-Homology Domain
RIP	Receptor-Interacting Protein
ROS	Reactive Oxygen Species
RTK	Receptor Tyrosine Kinase
SMOC	Secreted Modular Calcium-Binding Protein
SPARC	Secreted Protein Acidic and Rich in Cysteines
STAT	Signal Transducer and Activator of Transcription
TAB	TAK1-Binding Protein
TAD	Transactivational Domain
TAK1	TGF-β-Associated Kinase 1
TGF	Transforming Growth Factor
TICAM1	TIR domain-containing adaptor inducing IFN-β
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
TRADD	Tumor Necrosis Factor Receptor type 1-associated DEATH Domain
TRAF	TNF-Receptor-Associated Factor
YY1	Ying Yang 1

Chapter 1

INTRODUCTION

1.1 Summary

Her2-overexpressing breast cancer represents one of at least three subtypes of breast cancer and typically correlates with poor prognosis and/or chemoresistance. In approximately 30% of all breast cancers, the Her2 (ErbB2, EGFR2) member of the EGFR membrane bound receptor tyrosine kinase family is dramatically increased due to overexpression or amplification. The Her2 receptor tyrosine kinase has proven to be an excellent therapeutic target, with antibody-based (trastuzamab) and small molecule inhibitor-based (lapatinib) therapies showing high efficacy in treatments of this subtype of breast cancer. However, recent studies have shown that patients develop resistance to both trastuzamab and lapatinib therapies, indicating the need for other molecular targets in $Her2^{+}$ breast cancer. Here we show that Her2 can activate the NF-κB canonical pathway in a manner dependent on IKKα, which was generally not thought be involved in this pathway. Moreover, this activation leads to increased invasion of $Her2^{+}$ cells. These results suggest that IKKα may prove to be an efficacious target in this subtype of breast cancer. Another NF-κB pathway kinase, TGF-β-activated kinase 1 (TAK1), also plays an important role in NF-κB activation by Her2 overexpression upstream of IKKα, which may also be a promising therapeutic target in this disease. We also show that a the gene SPARC is downregulated upon activation of the NF-κB pathway, which has not previously been shown to be regulated by NF-κB. This downregulation occurs through modulation of transcription at the SPARC promoter proximal to the transcriptional start site. Together, these data shed light on signaling events leading to NF-κB activation downstream of the Her2 receptor tyrosine kinase.

1.2 Her-overexpressing Breast Cancer

Breast cancer is the most common malignancy among women in the United States, accounting for nearly one in four cancers diagnosed in women, and is the second leading cause of cancer mortality among this group (Herbst et al. 2006). According to the American Cancer Society (ACS), an estimated 200,000 new cases of invasive breast cancer are expected to be diagnosed in 2009, as well as approximately 63,000 cases of ductal carcinoma in-situ (DCIS). Furthermore, over 40,000 American women are expected to die from breast cancer in 2009, making this disease a critical target for further research.

Breast cancers can be divided into at least three major subtypes, as well as a normal tissue group, based upon the gene expression signature of the tumors (Perou et al. 2000). These three subtypes include the basal subtype, the luminal subtype and the $Her2^{+}/ER^{-}$ subtype. Recently, a new subgroup has been identified, termed "claudin-low" (Herschkowitz et al. 2007). The basal subtype of breast cancers, also known as "triple-negative" breast cancers, and the claudin-low subtype do not express the estrogen receptor or progesterone receptor, nor do they overexpress the Her2 receptor tyrosine kinase. The basal subtype of breast cancer typically exhibits characteristics of the basal/myoepithelial cell type (leading to the name "basal-like"), expressing high molecular weight cytokeratins 5, 14, and 17. The luminal subtype of breast cancer, the most commonly occurring subtype of breast cancer, expresses estrogen receptor and/or progesterone receptor, and while also expressing low molecular weight cytokeratins 8 and 18 (Dawson, Provenzano, and Caldas 2009; Perou et al. 2000). This luminal subtype

can be further grouped into 2 subtypes – Luminal A (good prognosis) and Luminal B (poor prognosis) (Sorlie et al. 2003). The third subtype of breast cancer, termed Her2-overexpressing (Her2^{+}) breast cancer, is defined by amplification/overexpression of the Her2 receptor tyrosine kinase (Sorlie et al. 2001).

The Her2^{+} subtype of breast cancer represents approximately 25-30% of all breast cancers and typically correlates with a poor prognosis (Slamon et al. 1989). The role of Her2 in tumorigenesis was originally discovered in the early 1980s, when it was demonstrated that the activated form of the rat Her2 homologue, *neu*, was oncogenic (Shih et al. 1981). The human homologue of this gene (Her2) was cloned a short time later (King, Kraus, and Aaronson 1985; Di Fiore et al. 1987), and the molecular mechanisms of oncogenesis by Her2 overexpression began to be elucidated. While Her2-overexpression is a common occurrence in breast cancer, overexpression or amplification of this receptor has been reported in ovarian and prostate cancers as well (Slamon et al. 1987; Reese et al. 2001), where it plays a similarly important role in oncogenesis. Thus the data presented here may have implications in other types of cancer.

The Her2 proto-oncogene encodes the class I transmembrane receptor tyrosine kinase (RTK) p185^{HER2} (Schechter et al. 1984). This gene is a member of the EGFR family of receptor tyrosine kinases, consisting of Her1 (ErbB1; EGFR), Her2 (ErbB2, *neu*), Her3 (ErbB3) and Her4 (ErbB4). These receptors are characterized by an extracellular ligand binding domain, a single hydrophobic transmembrane domain, and an intracellular region containing a highly conserved tyrosine kinase domain (Niu and Carter 2007). Activation of these receptors is highly pleiotropic; one of at least 12 specific ligands binds to these receptors. Such binding leads homodimerization or

heterodimerization of the receptors (Riese et al. 1995; Heldin 1995; King et al. 1988; Graus-Porta et al. 1997) (Fig. 1.1). This dimerization leads to receptor autophosphorylation by the receptor kinase activity, which is required for full activation (Ullrich and Schlessinger 1990). The Her3 ligand heregulin β has been shown to induce Her2/Her3 or Her2/Her4 interaction (Liao et al. 2007; Plowman et al. 1993), while the EGFR ligand epidermal growth factor (EGF) has been shown to activate EGFR/Her2 dimers (Wang, Zhang et al. 1999). Though these receptors share these common structural aspects, there are important differences between them, which play an integral role in their downstream signaling. This is evident with the Her2 and Her3 receptors, which represent functionally incomplete molecules (Moasser 2007). The Her2 receptor lacks ligand binding activity, requiring its signaling function to be engaged by ligand-bound receptor partners (Sliwkowski 2003). In contrast, the Her3 receptor lacks an ATP binding site within its catalytic domain, and therefore requires the kinase activity of its dimeric binding partner for activation of downstream pathways (Sierke et al. 1997; Kim et al. 1998). Through modulation of signal transduction, this activation plays an important role in the regulation of many cellular processes, including cell proliferation, survival, adhesion, migration and differentiation (Zhou and Hung 2003; Yarden and Sliwkowski 2001; Yu and Hung 2000).

Multiple signaling pathways are activated downstream of Her2-containing RTK dimers. Activation of Her2/Her3 dimers by heregulin/neuregulin activates extracellular-related kinase/mitogen activated kinase (ERK/MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways (Ben-Levy et al. 1994; Alimandi et al. 1995; Peles et al. 1992; Holbro et al. 2003; Zhang et al. 2009; Soltoff et al. 1994). Activation of EGFR/Her2 complexes by

EGF or TGF-α ligands has also been shown to lead to activation of MAPK/ERK, PI3K, STAT, p38 and JNK pathways (Gusterson and Hunter 2009; Yarden and Sliwkowski 2001; Hynes and MacDonald 2009). Furthermore, amplification of Her2 at very high levels, as seen in $Her2^+$ breast cancer *in vivo*, can lead to spontaneous receptor homodimerization, kinase activation and activation of the aforementioned pathways (Schaefer et al. 2007). These pathways, particularly the MAPK/ERK and PI3K pathways, have been reported to play important roles in cancer formation and progression in Her2-overexpressing breast cancer. Activation of the MAPK/ERK pathway by Her2 leads to an increase in invasion and cell cycle progression (Huang et al. 2009; Lenferink et al. 2001). Activation of the PI3K/Akt pathway downstream of Her2 leads to an increase in cell cycle progression, resistance to apoptosis, and migration (Lenferink et al. 2001; She et al. 2008; Kumar et al. 2008). The PI3K/Akt pathway is preferentially activated by Her2/Her3 dimers, due to the seven p85-binding phosphotyrosine-containing motifs on the activation domain of Her3 (Prigent and Gullick 1994; Soltoff et al. 1994; Schulze, Deng, and Mann 2005). Activation of the PI3K pathway has been reported to play a role in resistance to the EGFR/Her2 small molecule inhibitor lapatinib, which is currently used as a therapy for $Her2^+$ breast cancer (Eichhorn et al. 2008), as well as leading to Akt addiction in this cancer (She et al. 2008). Furthermore, Akt-1, a kinase activated downstream of PI3K, has been shown to have opposite effects on tumor growth and invasion to that of Akt-2, which is similarly activated downstream of PI3K (Hutchinson et al. 2004). Finally, the PI3K pathway, but not the MAPK/ERK or NF-κB pathways, has been reported to be important for radioresistance but not tumor formation; conversely the MAPK/ERK and NF-κB pathways, but not the PI3K pathway, are

important for transformation (Grana et al. 2002). Taken together, this evidence shows that activation of these different pathways downstream of Her2 has different effects of tumor promotion and progression.

Overexpression of Her2 has proven to be an excellent therapeutic target in breast cancer. The monoclonal antibody trastuzamab (Herceptin) was developed as a targeted therapy for Her2-overexpressing metastatic breast cancer, and approximately 30-35% of patients treated with trastuzamab show significant tumor regression (Vogel et al. 2002). However, a majority of patients treated with this therapy develop resistance (Slamon et al. 2001). The small molecular inhibitor lapatinib (Tykerb, GW2016; GSK), a dual EGFR/Her2 inhibitor recently approved for combination therapy with the chemotherapeutic agent capecitabine, has also shown promise as an effective therapy in Her2-overexpressing breast cancer (Lackey 2006; Geyer et al. 2006). However, there are data suggesting that resistance to lapatinib may occur as well (Chen, Xia, and Spector 2008). Therefore, additional pharmacological targets need to be elucidated. Since Her2 overexpression can activate NF-κB, which is described in greater detail later in this chapter, this pathway may harbor some of these targets.

1.3 NF-κB Activation in Cancer

Cancer is a family of diseases exhibiting dysregulated gene expression, and, as a transcription factor that regulates hundreds of genes involved in a multitude of cellular processes including cell proliferation, apoptosis and migration, we expect that aberrant activation of the NF-κB family of transcription factors plays a large role in many aspects of these diseases. Cloning of the p50/p105 subunit of NF-κB, and the recognition of its

homology to c-Rel, the cellular homologue of v-Rel (avian reticuloendotheliosis virus), suggested the first link between the NF-κB family of transcription factors and oncogenesis (Courtois and Gilmore 2006). Subsequent research has shown aberrant activation of NF-κB in multiple cancers including pancreatic cancer (Weichert et al. 2007), colon cancer (Voboril and Weberova-Voborilova 2006), lung cancer (Tang et al. 2006), prostate cancer (Sweeney et al. 2004), hematological malignancies (Baumgartner et al. 2002; Braun et al. 2006; Kirchner et al. 2003) and breast cancer (Chua et al. 2007; Biswas et al. 2004; Cogswell et al. 2000). This constitutive activation of NF-κB is often due to aberrant activation of the IKK signalsome complex, which has been reported in prostate cancer (Gasparian et al. 2002), pancreatic cancer (Liptay et al. 2003) and breast cancer (Romieu-Mourez et al. 2002).

Normal human cells need to undergo alterations to six essential cell processes to become malignant: evasion of apoptosis, immortalization, sustained angiogenesis, self-sufficiency of growth signals, insensitivity to growth inhibition and tissue invasion and metastasis (Hanahan and Weinberg 2000). NF-κB plays a role in several of these alterations (Karin et al. 2002). Through activation of target genes involved in G1 progression such as cyclin D1, NF-κB has been shown to increase cell proliferation (Guttridge et al. 1999). NF-κB also plays a key role in the inhibition of programmed cell death in cancer cells. The cytokine TNF-α is a strong inducer of NF-κB activation, and this activation leads to the transcription of genes such as cellular inhibitors of apoptosis (cIAP) and the Bcl-2 family of genes, which block apoptosis (Karin and Lin 2002). Increased transcription of NF-κB regulated genes can also lead to resistance to multiple cancer therapies, including genotoxic drugs and ionizing radiation (Wang, Mayo, and

Baldwin 1996; Wang, Cusack et al. 1999). It has also been reported by many groups, as well as in this study, that activation of NF-κB leads to an increase in the cytokine IL-8 and this can in turn lead to an increase in angiogenesis and invasion (Koch et al. 1992).

As previously noted, NF-κB has been shown to play a key role in breast cancer. In fact, NF-κB has been reported to be highly activated in breast cancers of all subtypes: basal (Yamaguchi et al. 2009), luminal (Pratt et al. 2009) and Her2-overexpressing (Biswas et al. 2004). Generally, NF-κB activity is highest in estrogen-receptor negative breast cancers, because estrogen receptor expression can block NF-κB activation through prevention of DNA binding (Galien and Garcia 1997; Ray et al. 1997), recruitment of corepressors to NF-κB containing complexes (Cvoro et al. 2006), competition for coactivators (Harnish et al. 2000; Nettles et al. 2008) or prevention of NF-κB nuclear translocation (Ghisletti et al. 2005). This activation of NF-κB in breast cancer is normally due to increased activation of one of the two NF-κB signaling pathways, the canonical or the non-canonical (or alternative) pathway.

1.4 NF-κB Signaling Pathways

NF-κB was originally discovered as a protein that binds to the immunoglobulin κ-enhancer sequence in B cells (Sen and Baltimore 1986). In the years since, five distinct members of the NF-κB family of inducible dimeric transcription factors have been characterized: RelA/p65, RelB, c-Rel, p52 (p100 precursor) and p50 (p105 precursor) (Fig. 1.2). NF-κB family members form a variety of hetero- or homodimers to differentially regulate transcription of a multitude of different genes in response to stimuli such as cytokines, bacterial products, viral expression, growth factors, cell stress and

oncogenes (Hayden and Ghosh 2004). The NF-κB family of transcription factors contains a highly conserved, 300 amino acid rel-homology domain in the N-terminus of the protein, which promotes dimerization, nuclear localization and DNA binding. RelA/p65, RelB and c-Rel also contain a transactivational domain (TAD) in the C-terminus, which facilitates transcriptional activation upon inducible post-translational modifications (Hayden and Ghosh 2004). Among these posttranslational modifications are phosphorylation events on RelA/p65 at serine 536 (Mattioli et al. 2004), serine 276 (Zhong, Voll, and Ghosh 1998) and serine 468 (Mattioli et al. 2006), all of which have been reported to regulate transcriptional activity. The mature p52 and p50 proteins derive from precursor proteins p100 and p105, respectively. These precursor proteins contain C-terminus ankyrin repeats that mask the nuclear localization sequences (NLS) of these proteins, rendering them inactive. p105 is constitutively cleaved into a mature p50 protein, while p100 processing to p52 is inducible and highly regulated (Xiao, Harhaj, and Sun 2001). Increased activation of NF-κB due to increased: 1) phosphorylation of RelA/p65, 2) nuclear translocation of NF-κB subunits, 3) DNA binding of NF-κB subunits and 4) transcriptional activation and subsequent gene expression of NF-κB regulated genes have all been shown to be crucial for cancer phenotypes discussed earlier in this section.

Activation of the NF-κB family of transcription factors can be divided into two different signaling pathways: the canonical and the non-canonical, or alternative, signaling pathways (Fig. 1.3). The canonical pathway can be activated by various stimuli such as cytokines, microbial infection, mitogens and oncogenes (Pahl 1999). This stimuli results in the activation of the IKK (Inhibitor of Kappa B Kinase) complex. The IKK

complex consists of two serine/threonine kinases, IKKα and IKKβ, and a regulatory subunit IKKγ (NEMO), which is polyubiquitinated at Lys63 leading to activation of this complex (Chen 2005). IKKβ subsequently phosphorylates IκB (Inhibitor of κB), a conserved family of proteins (IκBα, IκBβ, IκBε) containing ankyrin repeats (Delhase et al. 1999; Karin and Ben-Neriah 2000) (Fig. 1.4). Prior to stimulation, p65/RelA containing dimers are held inactive in the cytoplasm by a member of the IκB (Inhibitor of κB) family of proteins. Phosphorylation of IκB results in its ubiquitination and degradation and the release of NF-κB dimers from the inhibitory complex. These NF-κB dimers then translocate to the nucleus and bind consensus NF-κB sites in DNA, leading to transcription of NF-κB regulated genes involved in inflammation, immunity and cancer progression (Karin and Lin 2002).

The non-canonical, or alternative NF-κB pathway, is activated by stimuli such as lymphotoxin, B-cell activating factor and RANKL. This stimulus activates the NF-κB inducing kinase (NIK), which phosphorylates an IKKα homodimer complex (Xiao et al. 2006). This IKKα homodimer phosphorylates the p100 precursor protein, which, when inactive, complexes with RelB in the cytoplasm in the cell. Upon phosphorylation of IKKα, the C-terminus of p100 is cleaved, and a mature p52 protein complexed with RelB translocates to the nucleus, resulting in DNA binding and transcription of NF-κB regulated genes. The canonical pathway is generally thought to require the IKK complex, specifically IKKβ, while the non-canonical pathway is independent of the IKK complex and relies on activation by IKKα homodimers (Senftleben et al. 2001). However, we present data in this study demonstrating that IKKα plays an important role in activation of the NF-κB canonical pathway downstream of the Her2/Neu oncogene.

1.5 IKKα and IKKβ in NF-κB activation

In canonical NF-κB signaling, activation of NF-κB dimers occurs through the IKK complex described above. The IKK complex is a 900-kD complex containing the IKKα and IKKβ catalytic subunits and the IKKγ (NEMO) regulatory subunit, as well as other proteins including cdc37, hsp90 (Chen, Cao, and Goeddel 2002) and ELKS (Ducut Sigala et al. 2004). This complex is responsible for phosphorylating IκBα and IκBβ (DiDonato et al. 1997), and further studies have indicated that both IKKα and IKKβ are able to phosphorylate the NF-κB inhibitor protein IκB (Zandi et al. 1997). IKKα and IKKβ have also been shown to phosphorylate the p65/RelA NF-κB subunit as well (Karin, Yamamoto, and Wang 2004).

Although IKKα and IKKβ do have some redundant functions and share significant structural and sequence similarities, they also play largely different roles in many signaling pathways in the cell (Lee and Hung 2008). As mentioned previously, IKKβ is generally thought to be the main mediator in canonical NF-κB signaling. IKKβ has also been shown to mediate NF-κB nuclear translocation through its direct phosphorylation of the p105 precursor protein, leading to its proteolysis (Salmeron et al. 2001). Disruption of the IKKβ gene *in vivo* leads to an embryonic lethal phenotype in mice due to uncontrolled liver apoptosis (Li et al. 1999), indicating that IKKβ plays an important role in at least some developmental processes. *In vitro* studies have also shown that loss of IKKβ leads to an increase in migration and proliferation in mouse embryonic fibroblasts through an ROS-dependent mechanism (Chen et al. 2006).

IKKα is thought to play an essential role in mediating activation of the non-canonical pathway, while playing little to no role in the canonical pathway. However, recent reports, as well as data provided here, suggest that IKKα may in fact play an important role in NF-κB signaling independent of the non-canonical pathway. NF-κB activity, as measured by IκBα phosphorylation and DNA-binding assays, requires IKKα and NEMO, but not IKKβ, in MEFs stimulated with the NF-κB activator IL-1β (Solt et al. 2007). It has also been shown in our lab that TNF-α induced NF-κB transcriptional activation and IκBα phosphorylation and degradation requires IKKα in SKBr3 and HeLa cancer cell lines (Adli et al.). Furthermore, activation of IKKα or IKKβ can lead to changes in expression of different subsets of NF-κB regulated genes (Gloire et al. 2007; Massa et al. 2005), and RANKL activation of the NF-κB canonical pathway has been showed to require IKKα, but not IKKβ, in mammary epithelial cells (Cao et al. 2001).

IKKα has also recently been suggested to play important roles independent of these NF-κB pathways. IKKα can translocate to the nucleus upon cytokine activation, inducing transcription of a subset of genes (Anest et al. 2003). Nuclear IKKα can also modulate chromatin structure through interaction with cyclic AMP-responsive protein (CBP) and subsequent phosphorylation of histone H3 (Anest et al. 2003; Yamamoto et al. 2003). IKKα can mediate derepression of NF-κB regulated genes through phosphorylation of the corepressor SMRT (Hoberg, Yeung, and Mayo 2004). Furthermore, IKKα has been shown to be important in mammary gland development in regulating expression of cyclin D1 downstream of RANKL signaling (Cao et al. 2001). Finally, IKKα has also been shown to be important in osteoclast differentiation (Chaisson et al. 2004), cell cycle progression (Tu et al. 2006) and keratinocyte differentiation

(Descargues et al. 2008), as well as playing multiple different roles in cancer progression. Taken together, these data show that IKKα and IKKβ have distinct physiological roles in the cell upon diverse stimuli. We therefore investigated whether these IKK subunits are required for Her2-mediated activation of NF-κB.

1.6 Her2 in NF-κB Activation

Her2 was initially reported to activate NF-κB over a decade ago (Galang et al. 1996), when this group showed that overexpression of activated *neu*, the rat homologue of Her2, leads to an increase in NF-κB transcriptional activity. Recent research has begun to shed some light on the molecular mechanisms by which Her2 activates NF-κB using *in vitro* and *in vivo* methods. Treatment of Her2-overexpressing breast cancer cells with the Her2 monoclonal antibody trastuzamab led to an inhibition of NF-κB activation (Biswas et al. 2004), supporting previous findings that Her2 can activate NF-κB. Also, Heregulin β1, a ligand for Her3 and Her4, has been shown to activate NF-κB and this activation may be required for tumor cell growth (Tsai et al. 2003). Activation of NF-κB may also play are role in cell proliferation of $Her2^+$ tumor cells (Biswas et al. 2004). Her2 activation of NF-κB leads to an increase in vasculogenesis in mouse models of this disease (Liu et al. 2009). Furthermore, Her2-overexpression has been reported by multiple groups to lead to an increase in invasion (Xu et al. 1997; Spencer et al. 2000; Kim et al. 2009), however, it has not been previously shown whether this is due to an increase in NF-κB activation. In this study, we show that Her2-overexpression leads to an activation of the NF-κB pathway, and this activation leads to an increase in invasion in $Her2^+$ breast cancer cells.

Multiple signaling pathways have been suggested to integrate Her2 overexpression and NF-κB activation, including integrin linked kinase, protein kinase CK2 and the PI3K/Akt pathway (Makino et al. 2004; Romieu-Mourez et al. 2002; Pianetti et al. 2001). Furthermore, IKKα has also shown to be required for self-renewal of tumor-initiating cells in a transgenic mouse model of $Her2^+$ breast cancer (Cao, Luo, and Karin 2007). Taken together, these data suggests that while Her2 can clearly activate NF-κB, leading to cancer progression, the mechanisms of this activation are unclear. Therefore, we investigated the mechanisms by which Her2 activates NF-κB, including proteins upstream of the IKK complex, such as PI3K/Akt.

1.7 NF-κB and the PI3K/Akt Pathway

Activation of Akt has been suggested to play an important role in activation of NF-κB by TNF-α and platelet derived growth factor (PDGF). These studies showed that the PI3K inhibitor wortmannin, or a dominant negative Akt plasmid, can block TNF-α and PDGF-induced NF-κB activation, suggesting a requirement for Akt in NF-κB activation in 293, HeLa and ME180 cells (Ozes et al. 1999; Romashkova and Makarov 1999). Furthermore, Akt has been shown to enhance the degradation of IκBs (Kane et al. 1999), and activated Akt can associate with the IKK complex and activate IKKα through phosphorylation at threonine 23 (Ozes et al. 1999; Romashkova and Makarov 1999).

The Akt/PKB family of kinases, Akt-1, -2, and -3, plays a critical role in many different cellular processes including cell proliferation, growth, survival and metabolism (Brazil, Yang, and Hemmings 2004; Woodgett 2005; Irie et al. 2005), and is activated in a multitude of different cancers (Bellacosa et al. 2005). Akt can be activated by multiple

mechanisms, including overexpression of receptor tyrosine kinases such as Her2 (see above). Upon stimulation by EGFR family ligands such as EGF or Heregulin β1, or by constitutive activation of Her2-containing dimers, the p85 subunit of PI3K is recruited to the receptor via SH2 homology domains. The p110 catalytic subunit of PI3K then phosphorylates phosphatidylinositol- 4,5-bisphosphate (PIP_2), generating PIP_3 and leading to Akt recruitment to the plasma membrane. Akt is then phosphorylated at threonine 308 and serine 473 by phosophoinositide-dependent kinase 1 (PDK1) and mTOR, respectively, leading to its full activation (Dillon, White, and Muller 2007; El-Naggar, Liu, and Dean 2009).

It is still unclear whether activation of NF-κB by Akt is ubiquitous or specific to certain cancers or cell types. It has been reported that EGF activation of NF-κB is independent of Akt (Habib et al. 2001). Furthermore, Akt has been shown to be dispensable for NF-κB activation in endothelial, smooth muscle and fibroblast cells (Delhase, Li, and Karin 2000; Madge and Pober 2000; Rauch et al. 2000), as well as in L929sA mouse fibrosarcoma cells (Vermeulen et al. 2002). In this study, we show that, consistent with previous results, overexpression of Her2 leads to activation of Akt through PI3K. However, Her2 activation of NF-κB is independent of the PI3K/Akt pathway, as PI3K inhibitors, as well as dominant-negative Akt constructs or PTEN overexpression, fail to block NF-κB activation downstream of Her2. With this finding, we investigated other possible mediators of Her2-activation of the IKK complex.

1.8 Receptor Mediated Activation of NF-κB through IKK

There is a multitude of mediators of NF-κB activation upstream of the IKK complex, which feed into many different activation pathways. These proteins include, but are not limited to, PI3K/Akt, MEKK3, TRAF2, TRAF5, TRAF3, TRAF6, IRAK1, IRAK4, MyD88, CARMA/BCL10/MALT1, RIP1, TRADD, NIK and TAK1. The involvement of these different proteins is dependent upon the specific NF-κB stimuli. The most well-studied activator of NF-κB is tumor necrosis factor alpha (TNF-α), which can activate both the canonical and non-canonical pathway. Engagement of the TNF-α receptor (TNFR) leads to recruitment of TNFR-associated factors (TRAFs) 2 and 5, which are required for NF-κB activation (Dempsey et al. 2003). Two other molecules, TRADD and RIP1, are also recruited to the TNFR, forming a large complex sufficient for activation of the IKK signalsome (Hsu, Xiong, and Goeddel 1995; Hsu et al. 1996). MEKK3 appears to play a similar role in activation of NF-κB by TNF-α, as MEK null mouse embryonic fibroblasts (MEFs) show a defect in NF-κB activation by TNF-α and IL-1 (Ting, Pimentel-Muinos, and Seed 1996; Meylan et al. 2004; Yang et al. 2001). Similar to activation of NF-κB by the TNFR family, activation of NF-κB by members of the IL-1 receptor family, such as IL-1R, and toll-like receptor family (TLR), such as TLR4, requires multiple adaptor and signal-transduction molecules. MyD88 (myeloid differentiation primary response gene 88) and TICAM1 (TIR domain–containing adaptor inducing IFN-β) have both been shown to be required for downstream NF-κB activation (Yamamoto, Takeda, and Akira 2004; Fitzgerald et al. 2001). MyD88-mediated activation of NF-κB also requires a member of the TRAF family, TRAF6 (Cao et al. 1996; Ishida et al. 1996), which is recruited to MyD88 by IRAK1 and IRAK4 (Takatsuna et al. 2003).

NF-κB can also be activated by antigen receptors, such as T cell receptors (TCR) and B cell receptors (BCR). T cell receptors can activate NF-κB through IKK via PKCθ, while B cell receptors activate NF-κB through PKCβ (Lee et al. 2005). PKC-mediated activation requires the members of the CBM complex – CARMA1 (Card-containing MAGUK protein), BCL10, and MALT1 (mucosa-associated lymphoid tissue lymphoma translocation gene 1) (Thome and Weil 2007). It has also been shown that the kinase PDK1 plays a role by interacting with CARMA1 and facilitating activation of IKK (Lee et al. 2005). It is also possible that TRAF2 and TRAF6 play a role in TCR and BCR activation of NF-κB (Sun et al. 2004).

DNA-damaging agents, such as ionizing radiation or etoposide, which cause DNA double strand breaks, have been shown to activate NF-κB through IKKγ (NEMO) (Li and Karin 1998; Huang et al. 2002). This activation appears to occur through the important DNA damage response protein ATM (ataxia telangiectasia mutated) (Lee et al. 1998; Piret, Schoonbroodt, and Piette 1999). This wide array of molecules involved in NF-κB activation by diverse stimuli led us to investigate which proteins may be required for IKK activation, and subsequent NF-κB activation, downstream of the Her2 receptor tyrosine kinase. One protein which was not addressed above is TAK1, which we show to be an important mediator in Her2-activation of NF-κB.

TGF-β activated kinase 1 (TAK1) is a member of the mitogen-activated protein kinase (MAPK) family and was originally discovered as a kinase in the transforming growth factor β pathway (Yamaguchi et al. 1995). Though originally discovered as a member of the MAPK family, TAK1 has been shown to play an indispensable role in activation of NF-κB by multiple stimuli. The serine/threonine kinase TAK1 can be

activated by diverse NF-κB activators such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), lipopolysaccharide (LPS), and latent membrane protein-1 (LMP-1) (Irie, Muta, and Takeshige 2000; Takaesu et al. 2003; Wan et al. 2004), though TNF-α and IL-1β activation of TAK1 have been the most thoroughly studied.

Upon activation of the TNF receptor complex, the adaptor proteins TRADD, TRAF2, TRAF5 and RIP1 are recruited to the receptor complex. TRAF2 and TRAF5, RING domain-containing ubiquitin ligases, promote polyubiquitination of RIP1, which in turn activates TAK1 and the IKK complex, leading to NF-κB activation (Chen 2005; Krappmann and Scheidereit 2005; Kovalenko and Wallach 2006). In the IL-1β pathway, the binding of IL-1β to its receptor induces recruitment of multiple signaling proteins including MyD88, IRAK1, IRAK4 and TRAF6. TRAF6, another ring domain-containing ubiquitin ligase, catalyzes K63 auto-ubiquitination, leading to activation of TAK1 and subsequent phosphorylation of IKKβ on its activation loop (Deng et al. 2000; Wang et al. 2001) (Fig. 1.5). This activation of NF-κB by TAK1 requires the interaction with multiple TAK1 signaling adapter proteins (TAB1, TAB2 and TAB3) (Figure 1.6). TAB1 is constitutively bound to the activation loop of TAK1, and is responsible for mediating autophosphorylation of threonines 178 and 184, as well as serine 192 following stimulation (Kishimoto, Matsumoto, and Ninomiya-Tsuji 2000; Yu et al. 2008). TAB2 and TAB3, zinc-finger domain-containing adapter proteins, bind the C-terminal portion of TAK1 and promote recruitment of activated receptors through K63-linked ubiquitinated signaling adapters, such as TRAF6 (Besse et al. 2007; Kanayama et al. 2004; Adhikari, Xu, and Chen 2007).

As alluded to above, lysine 63 (K63)-linked ubiquitination of signaling proteins in the TAK1 activation pathway is an indispensable mechanism for activation of TAK1. Unlike lysine 48 (K48)-linked ubiquitination, which targets proteins for degradation by the proteasome, K63-linked polyubiquitination chains act as scaffolds to assemble protein kinase complexes and mediate activation of such complexes (Adhikari, Xu, and Chen 2007). Upon TNF-α activation, RIP1 is K63 polyubiquitinated, which recruits TAB2 and TAB3 containing TAK1-TAB complexes, leading to activation of the IKK signalsome (Kanayama et al. 2004). It has also been reported that, in response to TNF-α, IKKγ (NEMO) binds to K63 polyubiquitin chains, specifically to RIP1 which has been K63 polyubiquitinated, facilitating recruitment of NEMO to the TNF receptor (Ea et al. 2006; Wu et al. 2006). When cells are stimulated with IL-1β, TRAF6 catalyzes K63-linked autoubiquitination, which facilitates recruitment of TAK1-TAB1-TAB2/3 complexes (Besse et al. 2007; Lamothe et al. 2007). This K63-linked polyubiquitination of signaling proteins in the TAK1 pathway is highly regulated by deubiquitinases such as CYLD and A20 (Adhikari, Xu, and Chen 2007).

Dysregulated TAK1 activation has been shown to be associated with activation of NF-κB in multiple different cancers including head and neck squamous cell carcinoma (HNSCC), lung cancer and breast cancer (Neil and Schiemann 2008; Jackson-Bernitsas et al. 2007; Xie et al. 2009; Safina et al. 2008). Increased TAK1 signaling has been reported to lead to increased levels of interleukin-6 (IL-6) (Jackson-Bernitsas et al. 2007; Yu et al. 2008), similar to that which we have seen with Her2 overexpression. It has also recently been reported that IL-1β induced TAK1-mediated IL-6 expression is dependent upon K63-linked polyubiquitination of TAK1 at lysine 209 (Yamazaki et al. 2009). Very

little research has been done to look at possible crosstalk between the Her2 and TAK1 pathways. It has been reported that Her2 overexpression and TAK1 activation can individually upregulate the Smad signaling pathway, however, crosstalk between these pathways was not investigated (Dowdy, Mariani, and Janknecht 2003). In this study, we show that Her2 activation of NF-κB requires TAK1 and TAB1. Her2-overexpression leads to phosphorylation of TAK1 at threonine 184 and 187, and a subsequent increase in kinase activity, and siRNA inhibition of TAK1 in Her2-overexpressiong breast cancer cells blocks NF-κB activation and IL-6 transcription. Taken together, these results show that TAK1 is an important mediator of Her2-overexpression-induced NF-κB activation.

1.9 Regulation of Secreted Protein Acidic and Rich in Cysteines (SPARC) Transcription

Though NF-κB is generally thought of as a positive regulator of transcription, there is evidence that activation of the NF-κB pathway can lead to inhibition of gene expression. It has been reported that activation of the NF-κB subunit p65/RelA by cytotoxic stimuli leads to a repression of antiapoptotic gene expression (Campbell, Rocha, and Perkins 2004). The p50 subunit of NF-κB can also directly repress transcription of genes such as apolipoprotein E (apoE), TNF-α, Cox-2 and IL-8 (Yue, Christman, and Mazzone 2008; Tong et al. 2004). Repression of gene transcription by NF-κB can also occur indirectly. It has been shown that NF-κB plays a role in cell cycle progression and cell survival by inhibiting the transcription factor E2F1 and blocking transcription of the E2F-regulated gene Bnip3 (Shaw et al. 2008; Araki, Kawauchi, and Tanaka 2008). NF-κB also plays a role in skeletal myogenesis and rhabdomyosarcoma

progression through upregulation of the transcription factor Ying Yang 1 (YY1), leading to a subsequent decrease of genes negatively regulated by YY1 (Wang et al. 2007; Wang et al. 2008). Here, we show that the gene Secreted Protein Acidic and Rich in Cysteines (SPARC) is also negatively regulated by NF-κB activation.

SPARC is a member of a family of secreted extracellular matrix proteins which include other members such as thrombospondins 1 and 2, osteopontin, and tenascins C and X (Lane and Sage 1994; Sage 1997; Yan and Sage 1999) (Fig 1.6). The primary physiological role for SPARC involves modulating cell-cell and cell-matrix interactions; however, SPARC also has de-adhesive and growth inhibitory properties (Tai and Tang 2008). These properties are of great importance in multiple cancer settings, where dysregulation of SPARC has been shown to be significant in cancer progression. Regulation of SPARC can occur through modulation by microRNAs (Kapinas, Kessler, and Delany 2009), through epigenetic mechanisms such as promoter methylation (Socha et al. 2009; Cheetham et al. 2008) or by activation of signaling pathways such as TGF-β (Reed et al. 1994; Wrana, Overall, and Sodek 1991; Ford et al. 1993; Shiba et al. 1998; Shanker et al. 1999). Dysregulation of SPARC has been reported in multiple cancers, including breast cancer. Overexpression of SPARC in the basal breast cancer cell line MDA-MB-231 leads to a decrease in cell growth, as well as in metastasis (Dhanesuan et al. 2002; Koblinski et al. 2005). Furthermore, overexpression of Her2 leads to a decrease in the transcription of SPARC (Mackay et al. 2003). Taken together, these data suggest that dysregulation of the SPARC gene through Her2-overexpression induced signaling may play an important role in breast cancer pathogenesis. In this thesis, we show that

activation of NF-κB leads to a decrease in expression of SPARC, through a mechanism at the promoter region of SPARC proximal to the transcriptional start site.

1.10 Conclusions

The development of targeted therapies in cancer treatment has led to an improvement in life expectancy and quality of life for many cancer patients. This is particularly true for Her2-overexpressing breast cancer, where combined therapies of trastuzamab (Herceptin) and lapatinib (Tykerb) have been reported to extend progression-free in women with Her2-positive metastatic breast cancer (Blackwell et al.). However, due to the development of resistance to targeted therapies such as these, the discovery of other possible targets for therapy is of great importance. Due to its role in cancer initiation and progression (Basseres and Baldwin 2006), and its activation downstream of the Her2 receptor tyrosine kinase (Galang et al. 1996), the NF-κB pathway is a promising target for therapy in this disease.

This chapter has reviewed the current understanding of NF-κB activation in many different cancers, including breast cancer. NF-κB plays a diverse role in breast cancer, as it is involved in many aspects of this disease including transformation (Kim et al. 2000), inhibition of apoptosis (Biswas et al. 2004), angiogenesis (Liu et al. 2009), proliferation (Yamaguchi et al. 2009) and invasion (Cho et al. 2009). NF-κB is likely to be involved in many of these processes in Her2-overexpressing breast cancer, due to its activation in this subset of breast cancers (Cao, Luo, and Karin 2007; Biswas et al. 2004; Liu et al. 2009). TAK1 has also been shown to be important in breast cancer (Neil and Schiemann

2008), though the role it plays in the Her2-overexpressing subtype of breast cancer is currently unclear.

In the remaining chapters, we further characterize the activation and role of NF-κB in Her2-overexpressing breast cancer. The results of these studies show that IKKα plays an important role in NF-κB activation downstream of Her2, and this PI3K-independent activation leads to an induction of a subset of NF-κB regulated genes, leading to cancer cell invasion. Furthermore, we show that Her2 activates the kinase TAK1, and this is required for NF-κB activation and IL-6 transcription. Finally, we present evidence that NF-κB can negatively regulate the gene SPARC in multiple cancer cell lines through a mechanism at a proximal region of the promoter of this gene. Together, these results show that activation of NF-κB by Her2 plays a critical role in breast cancer, requiring both IKKα and TAK1, and leading to an increase in cytokine and chemokine gene expression and subsequent increase in invasion.

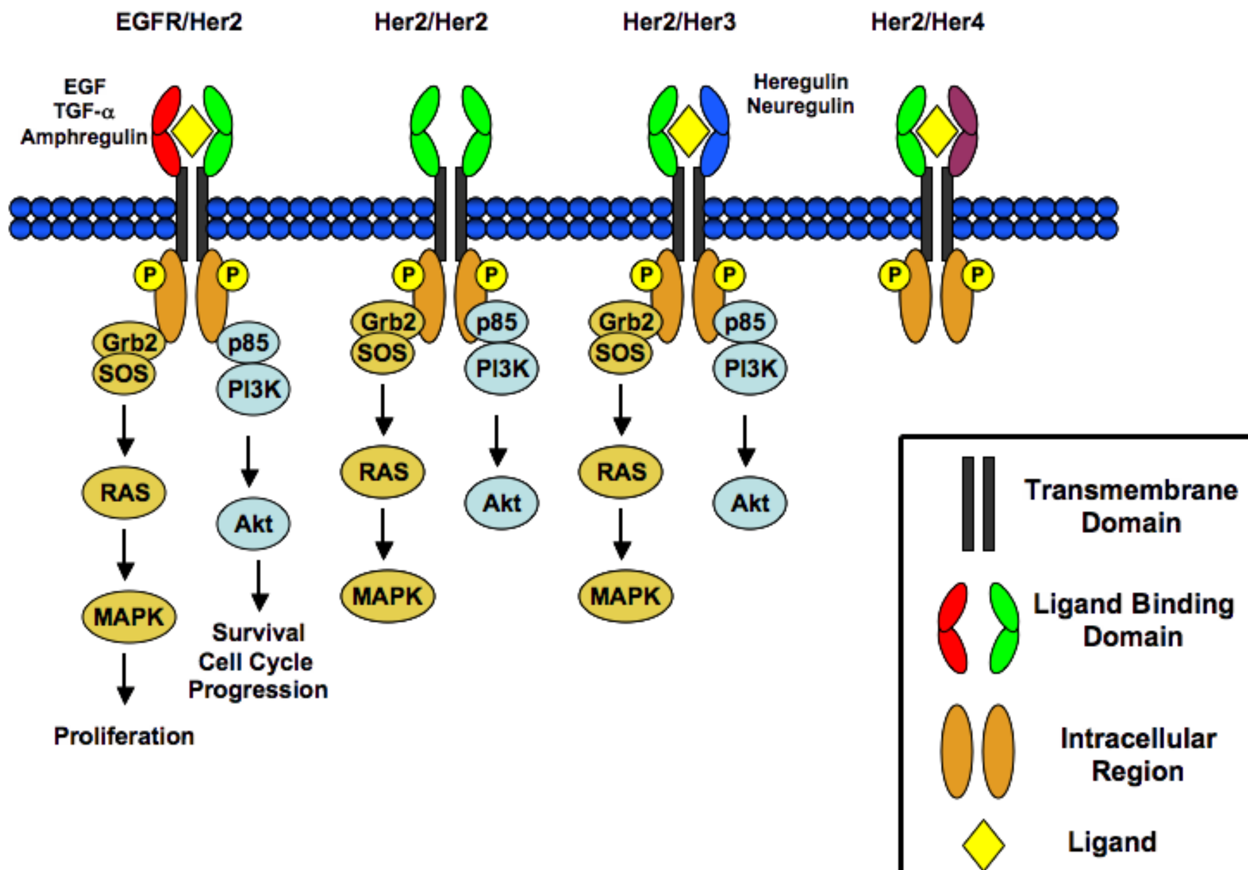

Figure 1.1 Receptor dimerization by EGFR family members. Activation of the EGFR family receptor by ligands such as epidermal growth factor (EGF) or heregulin induces receptor dimerization and subsequent receptor autophosphorylation at the intracellular kinase domain of the receptor. Autophosphorylation of the receptor activates downstream pathways such as the RAS/MAPK and PI3K/Akt pathways, leading to increases in proliferation, survival and cell cycle progression. The Her2 receptor lacks a natural ligand, though it can homodimerize upon high overexpression. The Her3 receptor contains no intrinsic kinase domain, requiring dimerization with one of the other family members. Her2-containing dimers can also activate the p38, STAT and JNK pathways (not pictured).

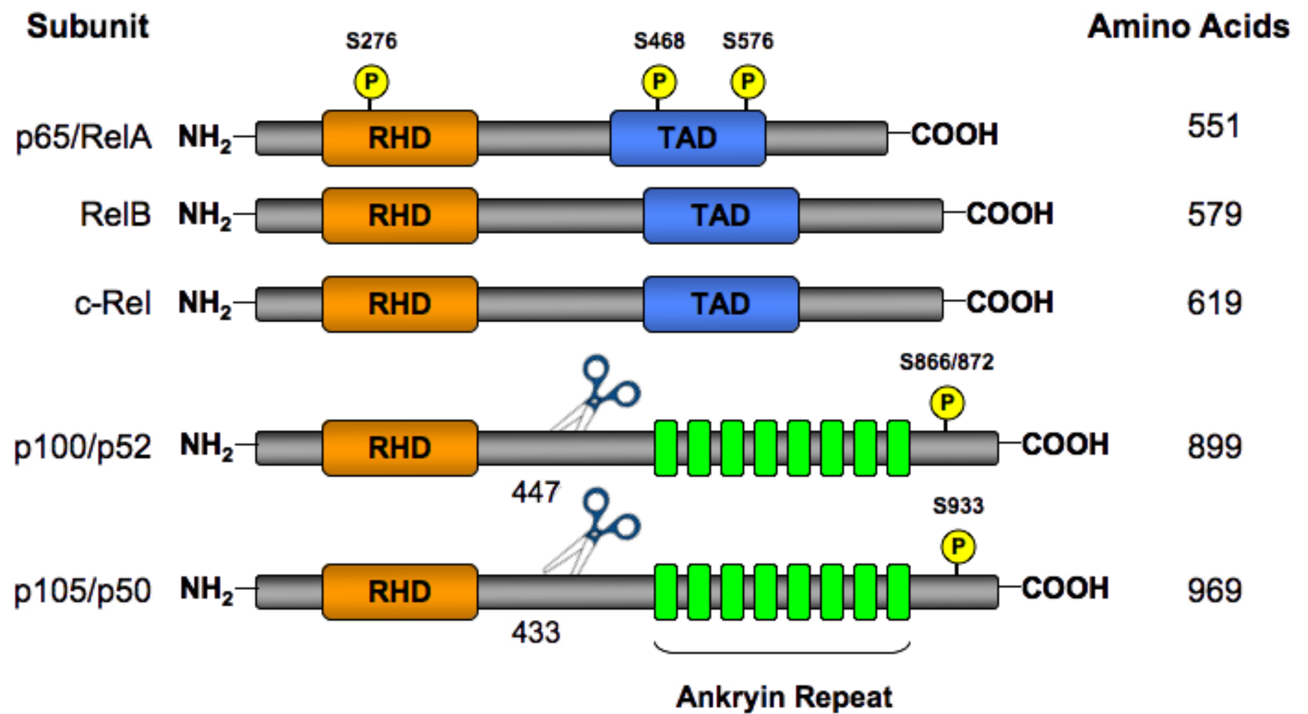

Figure 1.2 Domain organization of the NF-κB family members.
NF-κB family members form homo- or heterodimers to modulate transcriptional activation in the cell. The rel homology domain (RHD) is required for DNA binding and interactions between NF-κB subunits. The transactivational domain (TAD) is required for transcriptional activation by NF-κB family members. Phosphorylation of the p65 subunit at serine 468 and serine 536 in the TAD, as well as serine 276 in the RHD, has been shown to promote transcriptional activation. The p100 and p105 subunits of the NF-κB family exist as inactive precursors in the cell. Phosphorylation at the C-terminus of these subunits leads to proteolytic degradation to the mature p52 and p50 proteins, as well as unmasking of their nuclear localization sequences (NLS).

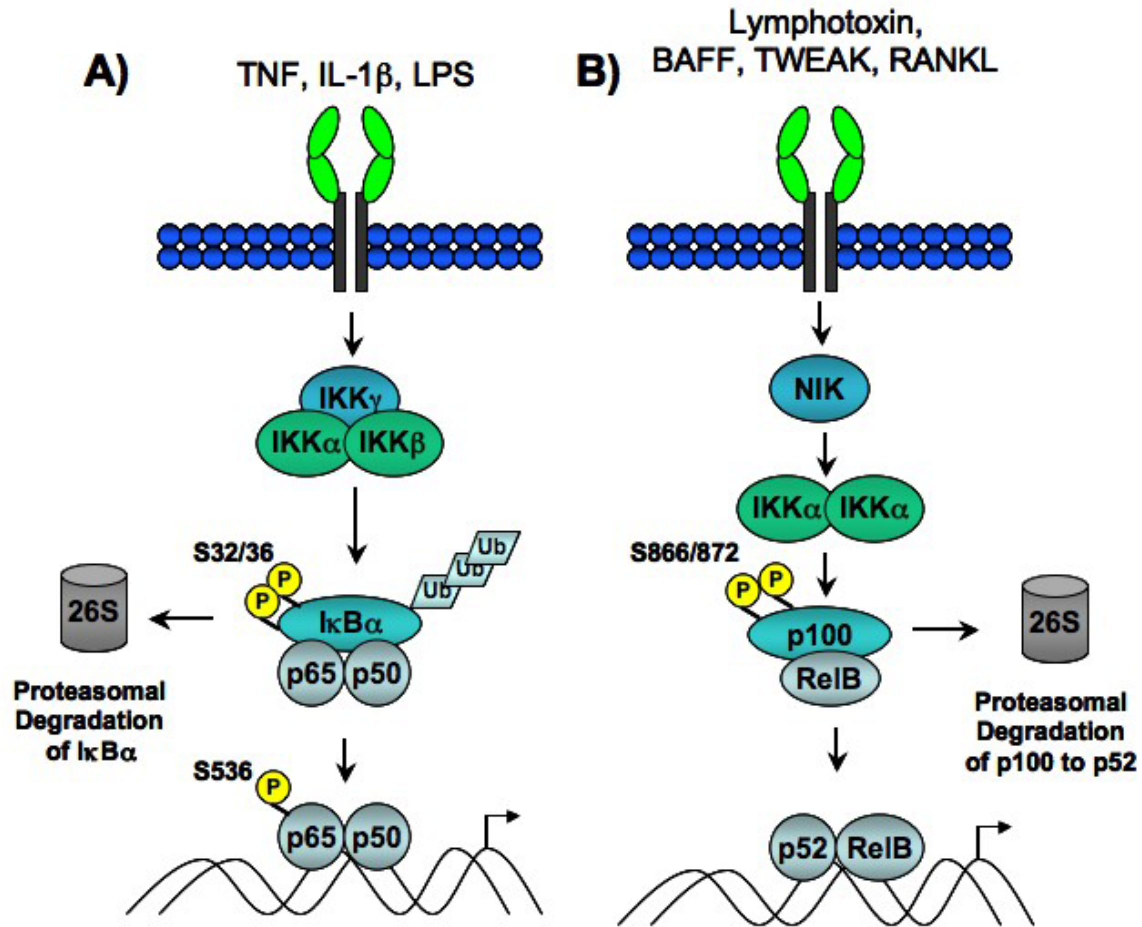

Fig. 1.3 Canonical versus non-canonical activation of NF-κB. (A) The canonical NF-κB pathway is activated by stimuli such as TNF-α or IL-1β, upon which the IKK holoenzyme (IKKα, IKKβ, IKKγ) is subsequently activated. Upon activation, the IKK complex phosphorylates IκBα at serines 32 and 36, leading to its ubiquitination and degradation by the proteasome. Unbound NF-κB complexes are then free to translocate to the nucleus and activate gene expression. (B) The non-canonical pathway is activated by stimuli such as lymphotoxin or B-cell activating factor (BAFF), upon which the NF-κB-inducing kinase (NIK) is activated. NIK activates IKKα homodimers, which subsequently phosphorylate p100. Phosphorylation of p100 induces the proteolytic cleavage to p52, which then translocates to the nucleus as an active p52/RelB homodimer.

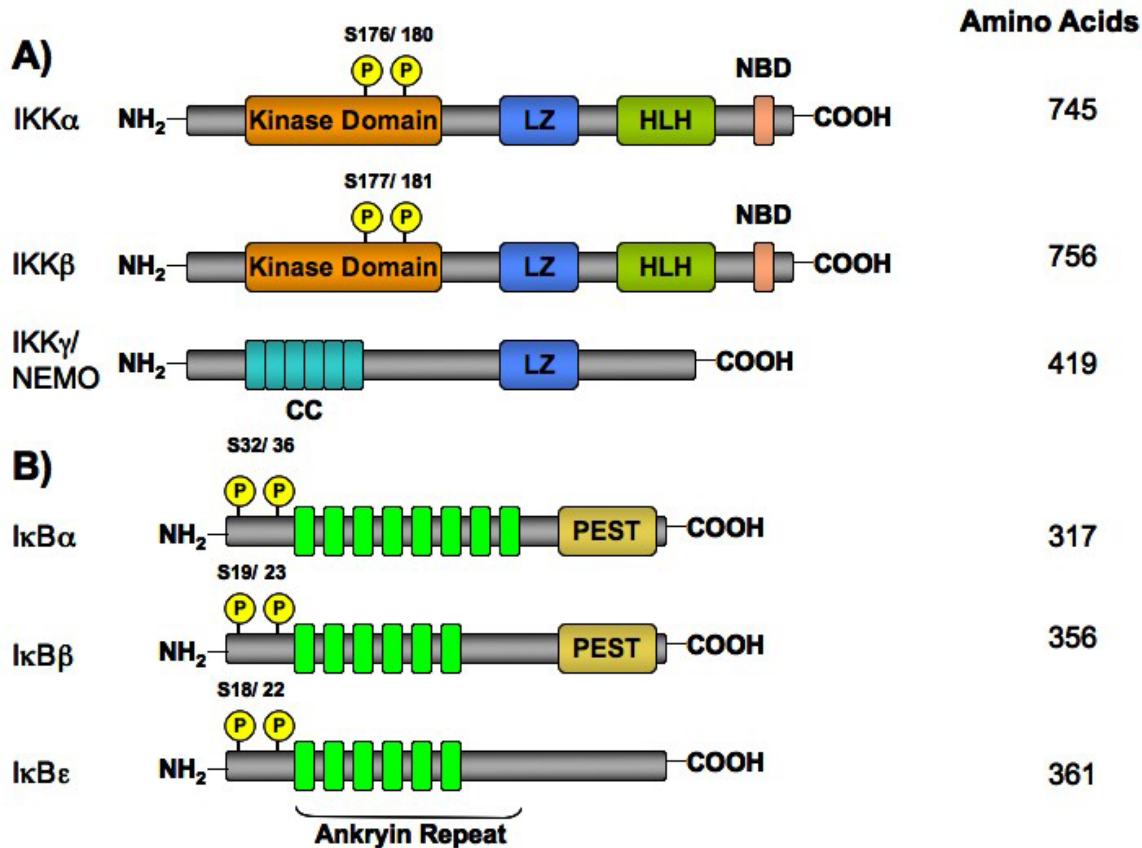

Figure 1.4 Domain organization of IKK and IκB family members. (A) The IKK complex consists of two catalytic subunits (IKKα and IKKβ) and one regulatory subunit (IKKγ/NEMO). IKKα and IKKβ contain a kinase domain, a leucine zipper (LZ) domain, a helix-loop-helix domain (HLH) and a NEMO-binding domain (NBD). The LZ and HLH domains function through facilitating IKKα/β interaction and catalytic activity (Zandi et al. 1997). Phosphorylation of IKKα and IKKβ at serines 176/180 and 177/181, respectively, in the activation loop is required for kinase activity of these proteins. The coiled-coiled (CC) domain of IKKγ facilitates binding to the NEMO binding domain (NBD) of IKKα and IKKβ. (B) The Inhibitor of κB (IκB) family consists of IκBα, IκBβ and IκBε. The ankyrin repeats in these family members mask the nuclear localization sequences (NLS) of the proteins which they interact. The PEST domain of these proteins promotes proteasomal degradation upon phosphorylation at the indicated residues.

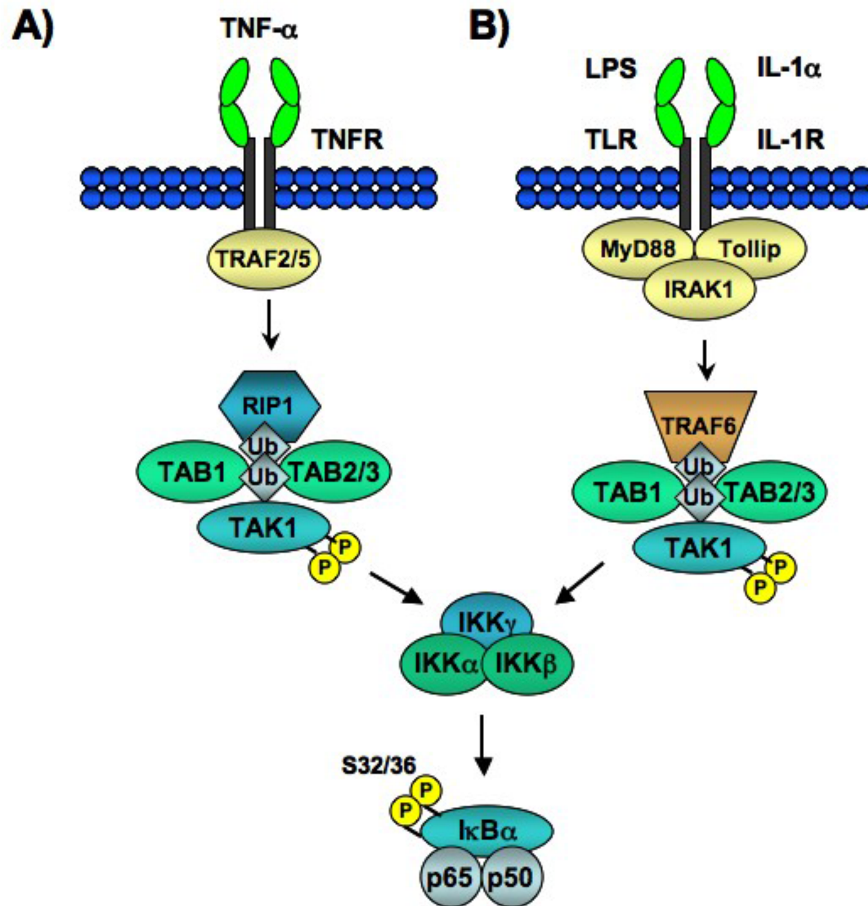

Figure 1.5. TAK-1-dependent activation of NF-κB. A) TNF-α-mediated activation occurs upon binding of TNF-α ligand to the TNFR, inducing binding of TRAF2 and TRAF5. TRAF2 and TRAF5 promote polyubiquitination of RIP1. TAK1 complexes are recruited to RIP1, leading to phosphorylation of TAK1 at serine 412 and threonines 184, 187 and 192. Phosphorylated TAK1 is able to directly phosphorylate IKKβ. B) LPS or IL-1β binding to toll-like receptors (TLRs) or IL-1 receptor, respectively, induces recruitment of the adaptor proteins MyD88, Tollip and IRAK1. TRAF6 is recruited to the receptor complex, and is concurrently autopolyubiquitinated (K63). This polyubiquitination induces autophosphorylation of TAK1, which can in turn phosphorylate IKKβ. TAK1 can also activate p38 and JNK independent of NF-κB activation (not shown).

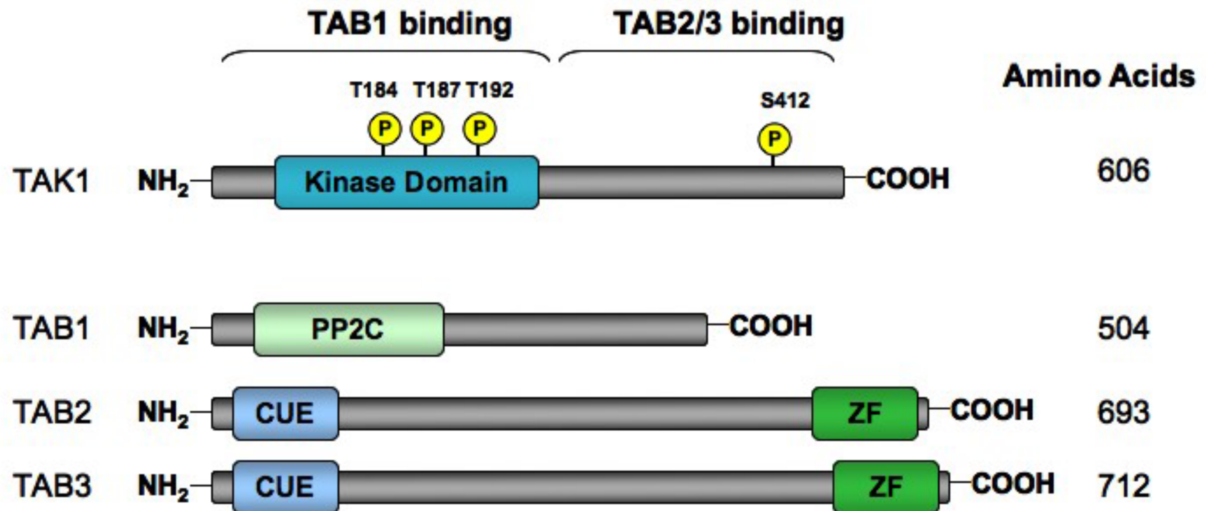

Figure 1.6. Domain organization of the TAK1 family of proteins. TAK1 is activated upon autophosphorylation at the indicated residues in the N-terminal kinase domain. TAK1 associates with TAK1 associated binding (TAB) proteins TAB1, TAB2 and TAB3. TAB1 is required for autophosphorylation of TAK1 in the kinase domain, and interacts with the N-terminus of TAK1. TAK2/3 interact with the C-terminus of TAK1. The N-terminal protein phosphotase 2C domain of TAB1 is uncharacterized, the CUE and zinc-finger domains (ZF) of TAB 2/3 facilitate interaction of polyubiquitin chains.

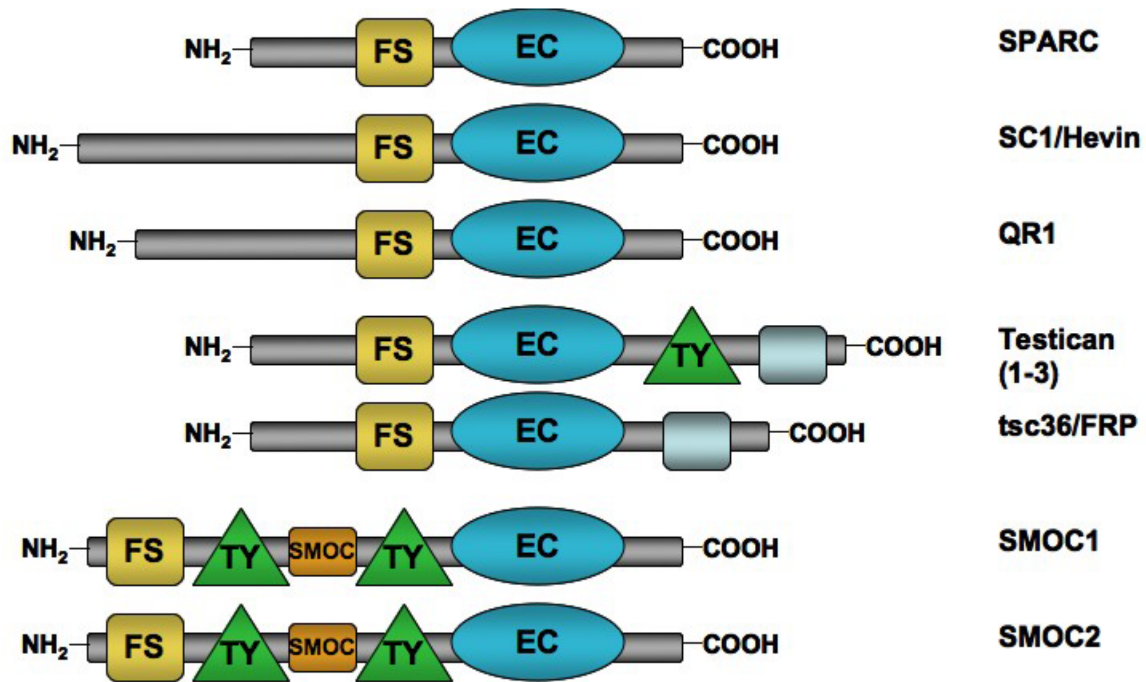

Figure 1.7. Domain organization of the BM-40 family of proteins. The SPARC family of proteins all contain an extracellular (EC) calcium-binding domain, which is necessary for the biological activity of the proteins. All BM-40 family proteins also contain a follistatin (FS) domain, which may contribute to antiangiogenic properties of these proteins (Chlenski et al. 2004). SMOC1, SMOC2 and testican also contain uncharacterized thyroglobulin-like (TY) domains. SMOC-1 and SMOC-2 contain novel SMOC domains located between the TY domains.

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CHAPTER II

HER2 ACTIVATES NF-κB AND INDUCES INVASION THROUGH THE CANONICAL PATHWAY INVOLVING IKKα

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2.1 Abstract

The membrane bound receptor tyrosine kinase Her2 is overexpressed in approximately 30% of human breast cancers which correlates with poor prognosis. Her2-induced signaling pathways include MAPK and PI3K/Akt, of which the latter has been shown to be critical for $Her2^{+}$ breast cancer cell growth and survival. Additionally, the NF-κB pathway has been shown to be activated downstream of Her2 overexpression, however the mechanisms leading to this activation are not currently clear. Using $Her2^{+}/ER^{-}$ breast cancer cells, we show that Her2 activates NF-κB through the canonical pathway which, surprisingly, involves IKKα. Knockdown of IKKα led to a significant decrease in transcription levels of multiple NF-κB-regulated cytokine and chemokine genes. siRNA-mediated knockdown of IKKα resulted in a decrease in cancer cell invasion, but had no effect on cell proliferation. Inhibition of the PI3K/Akt pathway had no effect on NF-κB activation, but significantly inhibited cell proliferation. Our study suggests different roles for the NF-κB and PI3K pathways downstream of Her2, leading to changes in invasion and proliferation of breast cancer cells. Additionally this work indicates the importance of IKKα as a mediator of Her2-induced tumor progression.

2.2 Introduction

The epidermal growth factor receptor Her2 is amplified in 20-30% of breast cancers, which typically do not express estrogen receptor, and are often correlated with poor prognosis and/or chemoresistance, making Her2 an important therapeutic target (Slamon et al. 1987; Slamon et al. 1989; Hynes and Stern 1994; Klapper et al. 2000). The Her2-specific antibody trastuzumab and the dual EGFR/Her2 inhibitor lapatinib have been shown to decrease growth of Her2-overexpressing tumors (Pegram et al. 1998; Baselga et al. 1999), however a majority of patients treated with trastuzamab develop resistance (Slamon et al. 2001), indicating the importance of elucidating alternative therapeutic targets in this disease. Her2-overexpression was first shown to activate NF-κB over a decade ago (Galang et al. 1996), however, the role NF-κB plays in development and progression of Her2-overexpressing breast cancer is still poorly understood. Additionally, the pathway leading to NF-κB activation downstream of Her2 is not well characterized.

NF-κB is an important transcription factor that has been shown to be involved in expression of genes involved in key cellular processes including innate and adaptive immunity (Bonizzi and Karin 2004), cell proliferation and survival (Papa et al. 2006), lymphoid organ development (Weih and Caamano 2003), as well as being activated in a variety different cancers, including breast cancer (Cogswell et al. 2000; Basseres and Baldwin 2006; Belguise and Sonenshein 2007). The NF-κB family of transcription factors consists of five subunits: RelA (p65), RelB, c-Rel, p105/p50 and p100/p52. These subunits are evolutionarily conserved and exist as hetero- or homodimers (Hayden and Ghosh 2004). The p65/p50 heterodimer is the most abundant NF-κB complex in the cell and is regulated by the so-called canonical pathway.

Following stimulation with activators such as TNF-α, IκB is phosphorylated by the Inhibitor of KappaB Kinase (IKK) complex. The IKK complex consists of two catalytic subunits IKKα and IKKβ, and a regulatory subunit IKKγ (NEMO), which binds both catalytic subunits at their NEMO-binding domains (NBD)(Gilmore 2006). In the canonical pathway, IKKβ phosphorylates IκBα leading to its degradation and NF-κB nuclear accumulation (Ghosh and Karin 2002). Furthermore, the p65 subunit of NF-κB can be phosphorylated on multiple residues, including serine 536, which is important for transactivation potential (Sakurai et al. 1999). NF-κB activation can also occur via the alternative, or non-canonical pathway. Activation of NF-κB in the non-canonical pathway, most common in B cells, involves Inhibitor of KappaB Kinase α (IKKα) and is IκBα-independent (Solt and May 2008). Thus most current models place IKKβ as the dominant IKK subunit in the canonical pathway with IKKα functioning in the non-canonical system. Few studies have addressed the individual roles of IKKα and IKKβ downstream of oncoprotein-dependent signaling.

Using an siRNA approach, we set out to determine how NF-κB is activated downstream of Her2, and what role the IKK complex plays in this signaling cascade, as well as how the activation of the IKK kinases may lead to a malignant state. While the classical pathway has long been thought to require IKKβ, here we show that IKKα plays a larger role than IKKβ in the activation of NF-κB in $Her2^+$ breast cancer cells, including the phosphorylation of the p65 subunit at serine 536. Using siRNA to the IKK kinases, we show that knockdown of IKKα leads to a change in the gene expression profile in $Her2^+$ cells, including a notable cytokine and chemokine gene expression signature. Furthermore, knockdown of IKKα by siRNA led to a marked decrease in invasive ability in SKBr3 cells, yet had no effect on cell proliferation. Taken

together, our data suggests that Her2 can activate NF-κB through the canonical pathway. Surprisingly, this activation occurs primarily through IKKα, a subunit typically not thought to be involved in the canonical pathway. Interestingly, we have discovered differential roles for the IKK kinases with IKKα specifically involved in an invasive oncogenic phenotype in $Her2^+$ breast cancer cells.

2.3 Materials and Methods

Cell culture and reagents

The tumor-derived SKBr3 cell lines were maintained in McCoy's 5A medium (Mediatech) supplemented with 10% fetal bovine serum (FBS) and 100 units/mL penicillin/streptomycin. The tumor-derived MCF7, MDA-MB-453 and MDA-MB-231 cell lines, as well as Mouse Embryonic Fibroblast (MEF) cell lines, were maintained in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% FBS and 100 units/mL penicillin/streptomycin. The human mammary epithelial cell lines (H16N2-pTP and H16N2-Her2) were maintained as previously described (Ethier et al. 1993). The stable 3x-κB luciferase SKBr3 cell line was established by transfection of a luciferase reporter construct containing tandem NF-κB binding sites from the MHC class I promoter region into SKBr3 cells with Fugene (Roche) and maintained under selection with G418 (Geneticin, Live Technologies). The Her2 wild-type and mutant (V654E) plasmids were constructed previously (Li et al. 2004) (Addgene plasmid 16257 and Addgene plasmid 16259). The Her2 coding sequences were subcloned into retroviral pLHCX vector (Stratagene) and virus was produced in 293T cells with cotransfection of AmphoPAK. MEFs were transduced with virus with polybrene and lysed 48 hours later. The following antibodies

were purchased from commercial sources: antibodies against phospho-p65 (Ser^{536}), phospho-Akt (Ser^{473}), Akt, phospho-IκBα ($Ser^{32/36}$) and IκBα from Cell Signaling Technology; antibodies against Her2, IKKα clone 14A231 and IKKβ clone10AG2 and p100/p52 from Millipore, antibodies against p65 and p50 (supershift), β-tubulin and IKKγ from Santa Cruz Biotechnology, antibody against total p65 from Rockland (PA, USA). LY294002 and Wortmannin were purchased from Cell Signaling Technology. Lapatinib (GW572016; Tykerb) was a gift from Dr. H. Shelton Earp (University of North Carolina at Chapel Hill).

Immunoblots

Whole cell extracts were prepared on ice with Mammalian Protein Extraction Reagent (Thermo Scientific) according to manufacturer's instructions supplemented with protease inhibitor mix (Roche, IN, USA) and phosphatase inhibitor mix (Sigma, MO, USA). Nuclear and cytoplasmic extracts were prepared as previously described (Mayo et al. 1997). Protein concentrations were determined by Bradford assay (Biorad Laboratories) and SDS-PAGE analysis was performed as previously described (Steinbrecher et al. 2005).

Small RNA interference

The following small interfering RNAs (siRNA; siGenome SMARTpool) were obtained from Dharmacon as a pool of four annealed double-stranded RNA oligonucleotides: IKKα (M-003473-02), IKKβ (M-003503-03), NEMO (M-003767-02), RelA (p65) (M-003533-02) and

nontargeting control #3 (D001201-03). Cells were grown to approximately 50% confluency and transfected with 100 nmol/L siRNA with Dharmafect 1 reagent according to manufacturer's instructions.

Quantitative Real-time PCR

Total RNA extracts were obtained from cells approximately 72 hours post-transfection by Trizol (Invitrogen) extraction. Two micrograms of RNA was reverse transcribed using random primers and MMLV-reverse transcriptase (Invitrogen). Real-time PCR was performed and analyzed as previously described (Steinbrecher et al. 2005) using Taqman Gene Expression Assay primer-probe sets IL-6 (Hs00174131_m1), IL-8 (Hs001741103_m1), CCL2 (Hs00234140_m1), TNF (Hs99999043_m1), and uPA (Hs00170182_m1).

Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assay (EMSA) and NF-κB supershift analysis were done on nuclear extracts as previously described (Steinbrecher et al. 2005) using ^{32}P-labeled oligonucleotide probe corresponding to an NF-κB site within the MHC class I promoter region.

IKK Kinase Assay

Whole cell lysates were prepared on ice for 45 minutes in lysis buffer containing 20 mmol/L Tris (pH 8.0), 500 mmol/L NaCl, 0.25% Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 1x protease inhibitor (Roche Applied Science), and 1x phosphatase inhibitor cocktail (Sigma-

Aldrich). IKK complexes were immunoprecipitated from 500-μg total protein extract using IKKγ antibody (Santa Cruz Biotechnology). An *in vitro* kinase assay was done and analyzed as previously described (Steinbrecher et al. 2005) using GST-IκBα as a substrate.

Luciferase Assay

SKBr3 cells stably expressing the 3x-κB plasmid were plated in equal number in triplicate in 24-well plates and transfected with siRNA for 72 hours or treated overnight with LY294002. Cells were lysed in MPER and luciferase activity was measured with Promega Luciferase Assay System (Promega). Luciferase levels were normalized by protein concentration using a Bradford assay. H16N2-Her2 and MDA-MB-453 cells were transfected with siRNA 72 hours before lysates were obtained, and were transfected with 3x-κB reporter plasmid and pRL-CMV (Promega) renilla plasmid 24 hours prior to lysate collection. Lysates were collected as mentioned above and luciferase levels were normalized to renilla.

Cell invasion assay

Innocyte™ Cell Invasion Assay Kit was purchased from Calbiochem (San Diego, California). Cells were transfected with siRNA for 48 hours before seeding. Invasion assay was performed as per manufacturer's protocol for 48 hours. The number of invading cells was measured fluorometrically with Calcein AM.

Cell Proliferation Assay

Cell proliferation assay was performed as previously described (Wilson and Baldwin 2008). Cells were cultured in the presence or absence of inhibitors, or transiently transfected with siRNA to IKK subunits and measured at the indicated time points post-transfection.

2.4 Results

Lapatinib inhibits Her2 activation of NF-κB and Akt

It has previously been shown that Her2-overexpression leads to activation of NF-κB family members involved in the canonical pathway, specifically the p65/p50 heterodimeric complex (Biswas et al. 2004; Galang et al. 1996). Given this result, we investigated whether the dual EGFR/Her2 inhibitor Lapatinib (Tykerb, GW572016) could block Her2-induced p65 phosphorylation at serine 536, a marker of increased NF-κB transcriptional activity (Sakurai et al. 1999). Five breast cancer cell lines were treated with 1 μM of lapatinib for 12 hours and whole cell extracts were analyzed for expression of phosphorylated p65. A marked decrease in p65 phosphorylation was observed in Her2-ovexpressing tumor cell lines (SKBr3 and MDA-MB-453) upon treatment with lapatinib, while non Her2-overexpressing tumor cell lines (MCF7 and MDA-MB-231) showed no change (Fig. 2.1A). The H16N2-Her2 cell line also showed a decrease in p65 phosphorylation upon lapatinib treatment. Overexpression of Her2 in this cell line results in NF-κB activation, as the parental cell line, H16N2-pTP, has very little basal p65 phosphorylation (Supplemental Figure 2.1). In order to further investigate how Her2 signals to NF-κB, we chose to use the tumor-derived SKBr3 cell line, as it has previously proven to be an

excellent *in vitro* model for Her2^{+}/ER^{-} breast cancer (Singh et al. 2007). SKBr3 cells were treated with 1 μM lapatinib or vehicle control over a course of 24 hours and whole cell extracts were analyzed for levels of phosphorylated IκBα. Phosphorylation of IκBα at serines 32 and 36 was inhibited within 3 hours of lapatinib treatment (Fig. 2.1B). Stabilization of IκBα was also observed, consistent with loss of phosphorylated IκBα. It has previously been shown that Her2-overexpression activates the PI3K/Akt pathway and that lapatinib can inhibit Akt phosphorylation in lapatinib-sensitive Her2-overexpressing breast cancer cell lines (Hegde et al. 2007). Similarly, we observe a decrease in phosphorylation of Akt at serine 473 in the lapatinib-sensitive SKBr3 cell line upon treatment with lapatinib (Fig. 2.1C). This indicates that Her2 can activate both the NF-κB and the PI3K/Akt pathways, and that pharmacological inhibition of Her2 leads to subsequent inhibition of these survival pathways.

Her2 activates the NF-κB canonical pathway through IKKα and IKKβ

We next examined the role of the IKK complex in the activation of NF-κB downstream of Her2. siRNA targeting the catalytic subunits of the IKK complex (IKKα and IKKβ) was transfected into Her2-overexpressing breast cancer cells and whole cell extracts were analyzed for markers of NF-κB activation. In the Her2-ovexpressing SKBr3, H16N2-Her2 and MDA-MB-453 cells, knockdown of IKKα led to a greater decrease in p65 phosphorylation than knockdown of IKKβ (Fig. 2.2A). Mouse embryonic fibroblasts (MEFs) lacking IKKα, as well as wild-type cells, were transduced with Her2 wild-type and constitutively active constructs. Transduction of these constructs resulted in increased p65 phosphorylation in wild-type MEFs, however, no increase in phosphorylation was seen in IKKα -/- cells (Supplemental Figure 2.2). In order to further

investigate the role IKKα may play in the activation of classical NF-κB complexes downstream of Her2, siRNA was again used to target IKK in SKBr3 cells stably expressing a 3x-κB luciferase reporter construct, as well as in H16N2-Her2 and MDA-MB-453 transiently transfected with the 3x-κB reporter plasmid. Knockdown of IKKα or a combination of IKKα and IKKβ led to a significant decrease in luciferase reporter activity (Student's *t*-test *<0.05 and **<0.001 respectively), while knockdown of IKKβ did not show a significant decrease in luciferase reporter activity in two of the three cell lines (Fig. 2.2B). An Electrophoretic Mobility Shift Assay (EMSA) was performed to further investigate the role of IKK in Her2 activation of NF-κB in SKBr3 cells. Knockdown of IKKα led to a greater decrease in NF-κB DNA binding activity than IKKβ knockdown (Fig. 2.2C). Supershift analysis indicated that loss of IKKα leads to a decrease in DNA binding of classical-pathway NF-κB heterodimers p65/p50.

Phosphorylation of IκBα by the catalytic subunits of the IKK complex is a hallmark of activation of the canonical NF-κB pathway, therefore we measured this kinase activity upon knockdown of IKKα or IKKβ. The IKK complex was immunoprecipitated with IKKγ, the scaffolding subunit of the IKK complex. Knockdown of IKKα led to a greater decrease of *in vitro* phosphorylation of IκBα than knockdown of IKKβ (Fig. 2.2D), further indicating IKKα plays a prominent role in the canonical pathway in Her2-overexpressing cells. Treatment with the 26S proteasomal inhibitor MG132, which effectively inhibits IκBα degradation, significantly blocked NF-κB luciferase activity in SKBr3 cells, further supporting the role of IκBα in Her2-induced NF-κB activation (Supplementary Figure 2.3). Taken together, these results demonstrate that IKKα plays a more significant role than IKKβ in activation of the NF-κB canonical pathway in Her2-overexpressing breast cancer cells.

Knockdown of IKKα and IKKβ leads to distinct gene expression profiles

We next determined if knockdown of the two IKK catalytic subunits leads to differential changes in gene expression in Her2-overexpressing cells. A chemiluminescent oligo-based array was used to measure expression of 219 genes. Upon knockdown of IKKα or IKKβ, significant decrease in expression was seen in 14 genes (Supplementary Table 2.1). Genes that showed significant changes in expression upon siRNA transfection were validated by quantitative real-time PCR. Decrease in expression of pro-inflammatory cytokines and chemokines IL-6, IL-8, CCL-2, TNF-α, and the serine-protease uPA, was greater upon siRNA knockdown of IKKα than IKKβ in both SKBr3 and H16N2-Her2 breast cancer cell lines (Fig. 2.3A). In order to demonstrate that IKK dependent changes in gene expression were occurring through modulation of NF-κB transcriptional activity, we performed RNAi against the classic subunit p65 in SKBr3 and H16N2-Her2 cells and assayed expression of mRNA by quantitative real-time PCR. Gene expression analysis showed that knockdown of p65 by siRNA led to a significant decrease in gene transcription levels of IL-8, IL-6, TNF and uPA (Fig. 2.3B). This transcriptional profile mirrors that seen upon knockdown of IKK, specifically IKKα, suggesting that induction of chemokines and cytokines in Her2 breast cancer cells occurs through IKK activation of p65. We next measured changes in expression of these genes in SKBr3 cells following treatment with lapatinib to confirm this activation of NF-κB regulated genes was induced downstream of overexpression of Her2. Treatment of SKBr3 cells with 1 μM of lapatinib led to a significant decrease in gene expression of IL-6, IL-8, CCL-2, TNF and uPA at both 8 and 16 hours post treatment (Fig. 2.3C). Taken together, this suggests that Her2 activates NF-κB through the

canonical pathway involving IKKα and leading to an increase in multiple NF-κB regulated genes involved in tumor progression.

Activation of NF-κB in Her2-overexpressing cells requires NEMO

The scaffolding subunit of the IKK complex, IKKγ (NEMO), is required for activation of NF-κB canonical pathway involving IKKβ (Gilmore 2006), and inhibition of the IKK signalsome with the NEMO Binding Domain (NBD) peptide can block NF-κB activation (Biswas et al. 2004). We used an siRNA approach to determine the importance of NEMO in NF-κB activation in Her2-overexpressing cell lines. siRNA knockdown of NEMO led to a marked decrease in p65 phosphorylation in all three Her2$^+$ cell lines (Fig. 2.4A). NF-κB luciferase reporter activity was also significantly decreased in these cell lines upon siRNA knockdown of NEMO (Fig. 2.4B). We performed quantitative real-time PCR analysis in the SKBr3 cell line upon NEMO knockdown to determine if this resulted in a similar gene expression profile as IKKα knockdown. Consequently, IL-6, IL-8, TNF and CCL2 all showed a significant decrease in expression upon NEMO knockdown, though uPA expression levels did not change (Fig. 2.4C). In order to rule out any effect loss of IKKα could have on non-classical activation of NF-κB, we analyzed processing of the p100 subunit. Cleavage of the precursor NF-κB protein p100 to p52 is a hallmark of activation of the non-canonical pathway. No significant effect was seen on p100 processing to p52 upon knockdown of either of the IKK subunits in Her2$^+$ cells (Fig. 2.4D). These results suggest that NF-κB activation in Her2$^+$ cells occurs through IKKα and this requires the NEMO subunit. Additionally, these results indicate that the non-canonical NF-κB signaling pathway is not activated in Her2$^+$ breast cancer cells.

Activation of the NF-κB canonical pathway is independent of the PI3K pathway

It has previously been reported that expression of dominant-negative PI3K and Akt plasmids can block NF-κB DNA binding (Pianetti et al. 2001). Therefore, we investigated if NF-κB activation downstream of Her2 is dependent on the PI3K/Akt pathway. Upon treatment of SKBr3 cells with lapatinib, phosphorylation of Akt at Serine 473 decreases dramatically (Fig. 1.1C). Treatment with the PI3K inhibitor LY294002 also blocked phosphorylation of Akt at serine 473, however LY294002 had no effect on the phosphorylation status of p65 at serine 536 in SKBr3, H16N2-Her2, or MDA-MB-453 cells (Fig. 2.5A-C). Furthermore, treatment of SKBr3 cells stably expressing the 3x-κB luciferase reporter with LY294002 had no effect on NF-κB transcriptional activity (Fig. 2.5D). Finally, overexpression of a constitutively active myristylated Akt construct had no effect on p65 phosphorylation of p65 at serine 536 in SKBr3 cells (Supplementary Figure 2.4). These results demonstrate that Her2 activates Akt through PI3K, and that the Her2-induced activation of NF-κB is independent of this pathway.

IKKα induces cell invasion but not cell proliferation

Having determined that overexpression of Her2 leads to IKKα-dependent activation of the NF-κB classical pathway, we next sought to determine how this signaling may promote oncogenic phenotypes. We investigated the effect IKK activation may have on proliferation of Her2-overexpressing breast cancer cells. SKBr3 cells were transfected with siRNA to the IKK

catalytic subunits and cell proliferation was measured by MTS assay. Knockdown of IKKα or IKKβ led to a slight increase in cell proliferation (Fig. 2.6A). As a control, SKBr3 cells were treated with the PI3K-inhibitor LY294002, as well as lapatinib. Inhibition of PI3K/Akt or Her2 led to a dramatic decrease in cell growth (Fig. 2.6B), consistent with what has been previously reported, suggesting that Her2 drives cell proliferation through the PI3K/Akt pathway. Our previous results have shown IKK/NF-κB dependent increases in proinflammatory cytokines downstream of Her2, and these genes have been shown to promote increased motility and invasiveness. Furthermore, overexpression of Her2 has been shown to lead to increase in invasiveness of breast cancer cells (Arora et al. 2008). We reasoned that NF-κB activity downstream of Her2 may contribute to increased invasiveness of Her2 breast cancer. To address this question, SKBr3 cells were transfected with siRNA to IKKα and IKKβ and the ability of the cells to invade through a basement membrane was measured. Knockdown of IKKα led to a significant decrease in invasiveness of SKBr3 cells while knockdown of IKKβ had no effect (Figure 2.6C). This suggests that Her2 overexpression results in activation of at least two independent oncogenic signaling pathways, one involving PI3K/Akt and another involving NF-κB, which have two different but important roles in promoting tumorigenesis (Fig. 2.6D).

2.5 Discussion

While Her2-positive breast cancer is known to activate both the NF-κB and PI3K/Akt pathways, (Biswas et al. 2004; Knuefermann et al. 2003; Pianetti et al. 2001; She et al. 2008), it has been unclear how Her2 induces NF-κB and whether PI3K is involved with this pathway. Additionally, potential roles for IKKα and IKKβ in controlling Her2-induced NF-κB have not

been addressed. The latter point is of interest since IKKα and IKKβ have previously been associated with controlling distinct NF-κB pathways, with IKKβ controlling the so-called canonical pathway and IKKα controlling the non-canonical pathway. These issues are potentially quite important in the therapeutic setting. Our data indicate the following: (i) IKKα plays an important role in controlling the ability of Her2 to activate NF-κB through the canonical pathway (including phosphorylation of IκBα, phosphorylation of RelA/p65, activation of IKK, and regulation of gene expression), (ii) IKKα controls invasion of $Her2^+$ cells, with apparent little contribution of IKKβ in this process, and (iii) PI3K-dependent pathways do not contribute to the direct activation of NF-κB in these cells.

Previous experiments from several groups have shown that IKKβ plays a major role in controlling canonical NF-κB activation downstream of inflammatory cytokines such as TNF (Verma et al. 1995). The potential contribution of IKKα to NF-κB activation downstream of Her2-dependent signaling or to that induced by other oncoproteins has not been fully elucidated. Lapatinib has been shown to be effective in its inhibition of the Akt and Erk pathways in Her2 overexpressing breast cancer cell lines and human tumor xenografts, but there are no reports of it having an effect on the NF-κB pathway (Zhou et al. 2004; Xia et al. 2002), although Herceptin has been shown to inhibit NF-κB activation in SKBr3 cells (Biswas et al. 2004). In our studies, treatment of Her2-overexpressing cell lines with 1 μM lapatinib led to a marked decrease in phosphorylation of NF-κB subunit p65 at serine 536 and of IκBα at serines 32 and 36 (Fig. 2.1A and B). Lapatinib also blocked NF-κB-induced gene transcription (Fig. 2.3C). Treatment of SKBr3 cells with lapatinib led to complete loss of phosphorylation of Akt at serine 473 (Fig. 2.1C), a marker for Akt activation.

To address potential contributions of IKKα and IKKβ to NF-κB activation in $Her2^+$ cells and to the oncogenic phenotype, we used an IKK knockdown approach in Her2-overexpressing cells. Knockdown of IKKα led to a more dramatic reduction in p65 phosphorylation at Ser536 than did knockdown of IKKβ (Fig. 2.2A). Furthermore, knockdown of IKKα strongly reduced NF-κB activation as measured through EMSA and NF-κB-dependent reporter assays while IKKβ knockdown had less of an effect (Fig. 2.2B and 2.2C). Similarly, knockdown of IKKα was more effective at blocking IKK activity than knockdown of IKKβ (Fig. 2.2D). SKBr3 cells exhibit low levels of p52/NF-κB2, which is derived from IKKα-dependent processing of the p100/NF-κB2 precursor. Knockdown of IKKα had little effect on p52 levels in these cells, indicating that non-canonical pathway does not appear to be active in SKBr3 cells at a measurable level. Consistent with this, very low to undetectable levels of p52 or RelB are detected in the nuclei of SKBr3 cells (data not shown). It is important to note that inhibition of IKKβ can lead to a compensatory response whereby IKKα controls canonical NF-κB activation in some cell types (Lam et al. 2008). Our studies clearly indicate that loss of IKKα leads to reduced NF-κB activation downstream of Her2-induced signaling. A study showing that IKKα is necessary for self-renewal of Her2-transformed mammary initiating tumor cells (Cao, Luo, and Karin 2007) is consistent with our results demonstrating the importance of IKKα in controlling NF-κB downstream of Her2. The way in which Her2 may selectively activate IKKα in breast cancer remains to be investigated. One possibility is selective activation of IKKα by the kinase NIK, as NIK has been shown to associate with ErbB2 family member EGFR (Habib et al. 2001), and has been shown to be recruited to EGF/heregulin receptor signaling complexes (Chen et al. 2003).

The knockdown studies were extended to analysis of NF-κB-dependent target gene expression (Fig. 2.3A). Knockdown of IKKα lead to a more dramatic reduction in gene expression of IL-6, IL-8, CCL2, TNF and uPA than did knockdown of IKKβ. Decreased expression of these genes upon knockdown of the p65 subunit of NF-κB indicates that this activation is occurring through the canonical pathway. (Fig. 2.3B). To demonstrate that these genes are controlled through Her2, and not through Her2-independent pathways, lapatinib was shown to block target gene expression (Fig. 2.3C). This increase in chemokine and cytokine gene expression by Her2, as well as the increase in the expression of the serine protease uPA, shows a large similarity to Her2 induced gene expression signatures which have been previously reported, and this increase has been implicated in progression of multiple different cancers, including breast cancer (Vazquez-Martin, Colomer, and Menendez 2008; Chavey et al. 2007; Arihiro et al. 2000; Wang et al. 1999). Therefore, our gene expression data suggests that IKKα plays in important role in regulating genes involved in breast cancer progression, and this requires the scaffolding subunit NEMO (Fig. 2.4).

Some studies indicate that NF-κB can be activated downstream of PI3K/Akt (Makino et al. 2004; Dan et al. 2008). However, experiments using the PI3K inhibitor LY294002 indicate that NF-κB is not activated in Her2^{+} cells downstream of PI3K (Fig. 2.5). Thus, this pathway is not a link between Her2, IKKα and NF-κB activation. We cannot rule out a PI3K-independent Akt-controlled pathway in NF-κB activation. Additionally, we cannot rule out that PI3K and/or Akt have effects on NF-κB-target gene expression that function separately from the induction of NF-κB activation as assayed through experiments described above. Future studies will address Her2-regulated pathways that lead to activation of IKK. Other studies (Dillon, White, and

Muller 2007) as well as our own (Fig. 2.6B) show that activation of the PI3K pathway plays an important role in cell proliferation/viability. Interestingly, knockdown of IKKα or IKKβ subunits (individually or together) by siRNA modestly increases cell proliferation (Fig. 2.6A). This may be due to the fact that NF-κB activation in Her2-overexpressing breast cancer cells leads to induction of a cytokine gene signature, but does not induce transcription of pro-proliferative or anti-apoptotic genes. Of note, knockdown of IKKα or IKKβ led to an increase in Bcl-2 expression, a protein known to play a role in chemoresistance in breast cancer (Fig. S2.5) (Matsuyoshi et al. 2006). Bcl-2 expression has been shown to be upregulated through the PI3K pathway (Siddiqa et al. 2008). Therefore, PI3K may be inducing cell proliferation through upregulation of genes such as Bcl-2, independent of the NF-κB pathway.

In order to determine if IKKα or IKKβ controls other oncogenic phenotypes, we used siRNA treatment and measured cell invasion of SKBr3 cells. Her2 overexpression has been shown to induce cell invasion, consistent with its ability to promote upregulation of genes such as IL-8 and uPA (Gum et al. 1995; Vazquez-Martin, Colomer, and Menendez 2008). Knockdown of IKKα, but not knockdown of IKKβ, significantly blocks the invasive phenotype of SKBr3 cells (Fig. 2.6C). This result is consistent with the regulation of target genes by IKKα that are associated with invasive phenotype. Interestingly, other factors have linked breast cancer invasion and NF-κB, including microRNAs (Ma, Teruya-Feldstein, and Weinberg 2007). MicroRNAs have been shown to negatively regulate NF-κB activity and gene expression, such as microRNA-146, which can suppress expression of IL-6 and IL-8 through a reduction in levels of IRAK1 and TRAF6 in MDA-MB-231 cells, leading to the metastatic phenotype (Bhaumik et al. 2008).

This study shows that Her2 activation of NF-κB requires IKKα, and this PI3K-independent activation leads to an increase in cytokine and chemokine expression, as well as an increase in invasive phenotype (Fig. 2.6D). This data suggests that targeting multiple pathways in $Her2^{+}$ breast cancer may be advantageous for effective therapy, and development of inhibitors of IKKα or the use of dual IKKα/IKKβ inhibitors may prove therapeutic in $Her2^{+}$ cancer cells.

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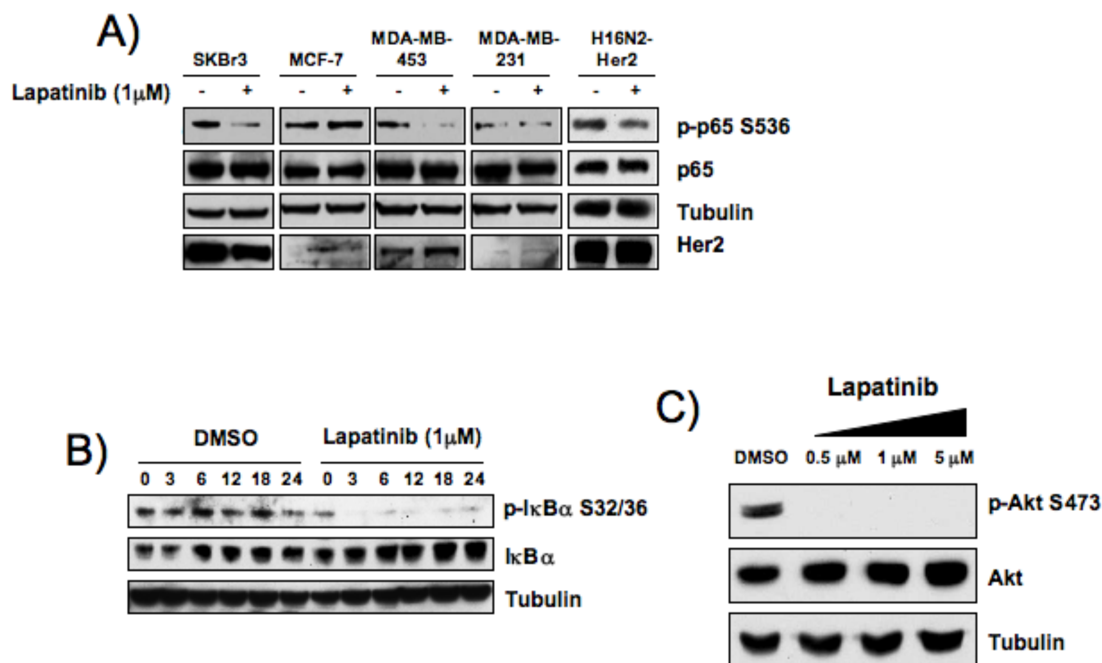


Figure 2.1. Lapatinib treatment inhibits the NF-κB and PI3K pathways in Her2-overexpressing cells. A) Western blot of phospho-p65^{S536} in multiple breast cancer cell lines treated with lapatinib. Breast cancer cell lines were treated with 1 μM dual EGFR/Her2 inhibitor lapatinib or DMSO vehicle control for 12 hours. Western blots were performed with 25 μg protein from whole cell extracts. B) Western blot of phospho-IκBα$^{S32/36}$ in SKBr3 cells treated with lapatinib. SKBr3 cells were treated with lapatinib (1 μM) or DMSO control over a course of 24 hours and levels of phospho-IκBα$^{S32/36}$ were measured by western blot of 25 μg total protein from whole cell extracts. C) Western blot of phospho-Akt^{S473} in SKBr3 cells treated with lapatinib. SKBr3 cells were treated for 12 hours with dual EGFR/Her2 inhibitor lapatinib and levels of phospho-Akt^{S473} were measured by western blot of 25 μg protein from whole cell extracts.

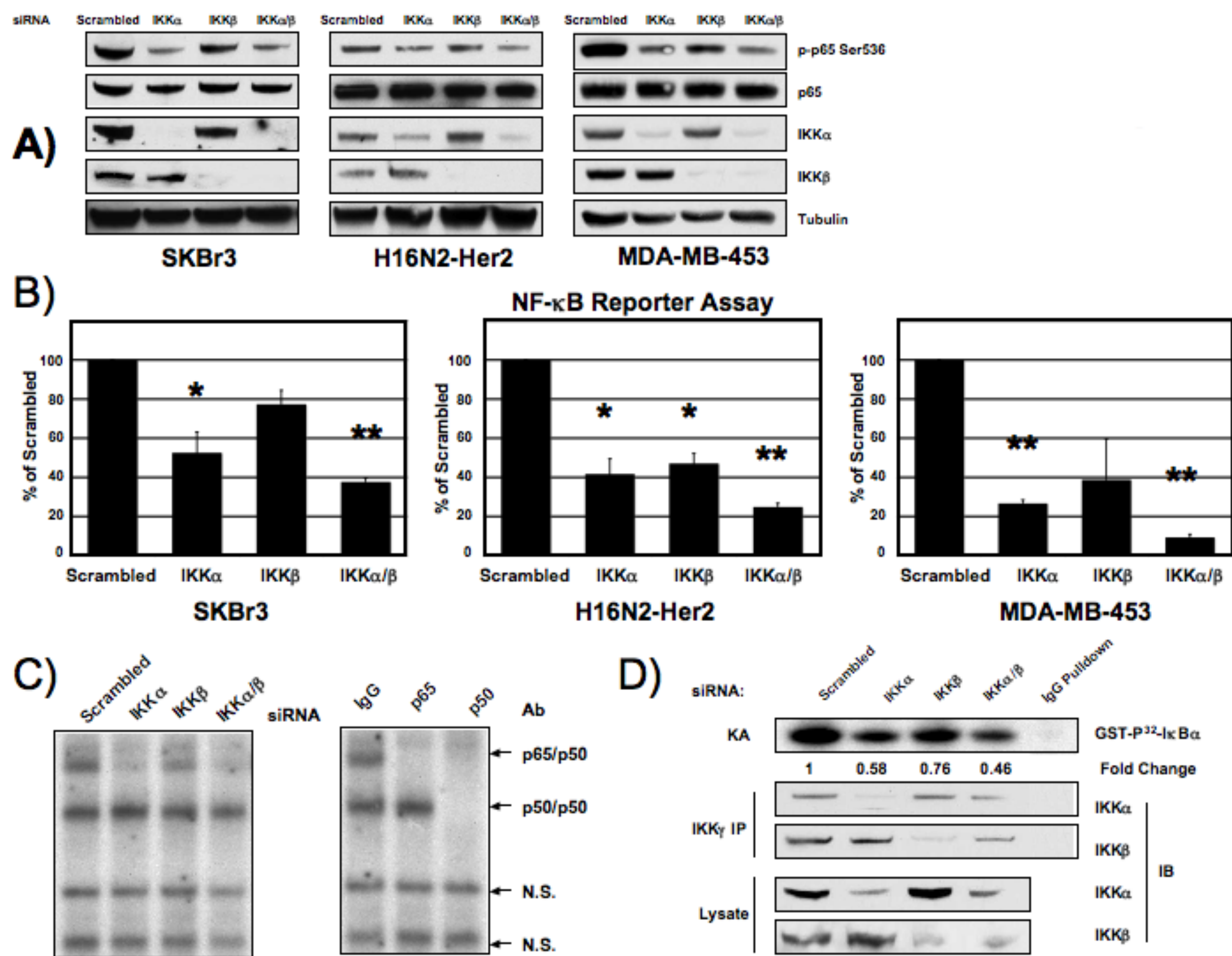


Figure 2.2. Her2 activation of NF-κB via IKKα and IKKβ involves the canonical pathway. A) Western blot of phospho-p65^{S536} in Her2-overexpressing breast cancer cells transfected with siRNA to IKK catalytic subunits. SKBr3 (left), H16N2-Her2 (center) and MDA-MB-453 (right) cells were transfected with 100 nM siRNA to IKKα and IKKβ and whole cell extracts were prepared after 72 hours and western blot analysis performed. B) NF-κB luciferase reporter assay of SKBr3, H16N2-Her2 and MDA-MB-453 cells transfected with IKK siRNA. Whole cell extracts were prepared 72 hours post-siRNA transfection and luciferase levels were measured. Statistically significant differences were determined by students t-test (*<0.05 **<0.001). Fold change of reporter activity with IKK knockdown is shown relative to scrambled siRNA treated cells. Values are the average of at least 3 experiments. Error bars are ± 1 S.E. Samples are normalized by protein concentration (SKBr3) or Renilla (H16N2-Her2 and MDA-MB-453). C) Electrophoretic mobility shift assay (EMSA) of SKBr3 cells transfected with IKK siRNA. Nuclear extracts were prepared after 72 hours. Identities of the bound complexes were determined by super-shift with antibodies to p65 and p50. Non-specific binding complexes are noted with as N.S. D) Kinase assay measuring IKK *in vitro* phosphorylation of IκBα. SKBr3

cells were transfected with IKK siRNA for 72 hours and IKKγ was immunoprecipitated from 500 μg whole cell extracts. Ability of immunoprecipitated complex to phosphorylate purified GST-IκBα was measured (KA). Amount of IKKα and IKKβ in immunoprecipitated complex (IP) and whole cell extracts (lysate) were measured. Fold change in kinase activity was calculated using pixel densitometry and compared to scrambled siRNA transfected cells.

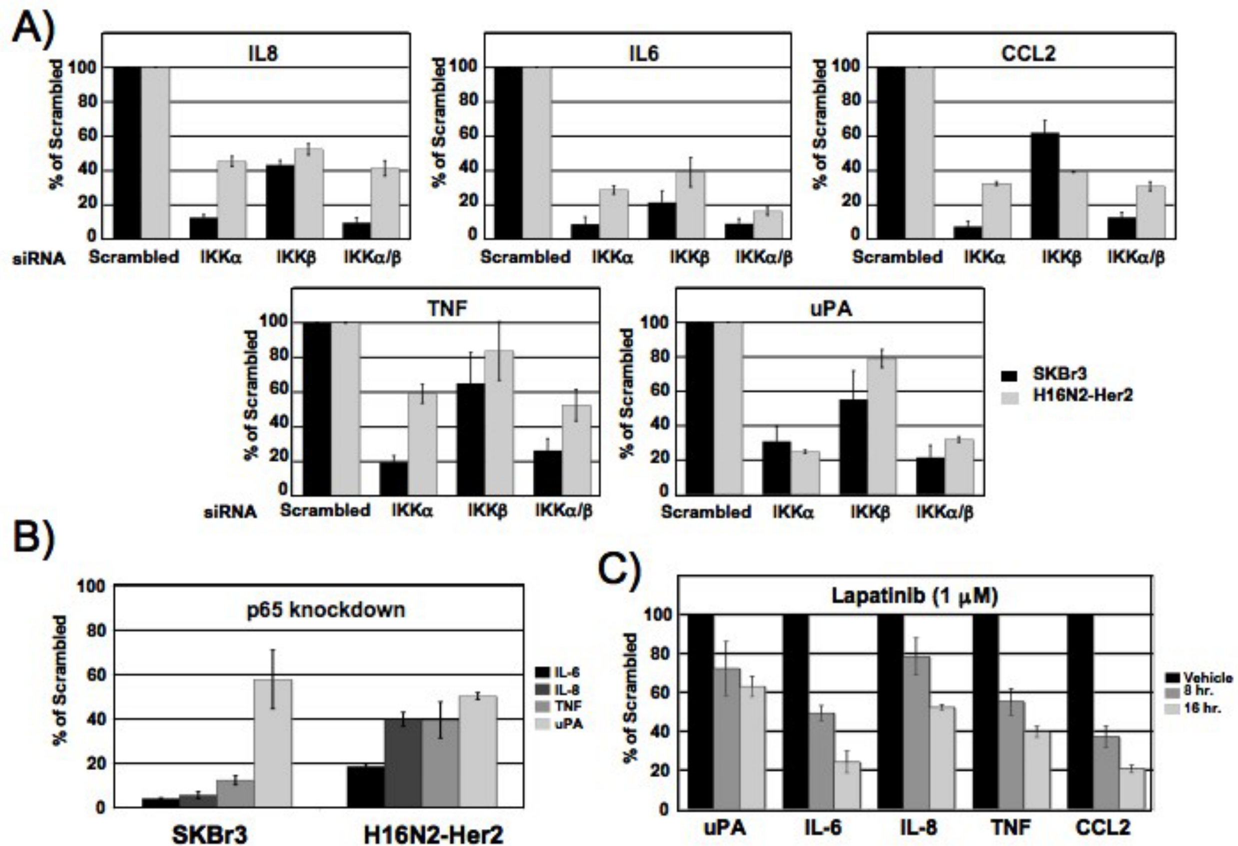


Figure 2.3. Her2 induces NF-κB-regulated gene expression through IKKα and IKKβ. (A) Quantitative real-time PCR of multiple genes shows different gene expression profiles upon IKKα or IKKβ knockdown. qRT-PCR was performed on extracts from SKBr3 (black bars) and H16N2-Her2 (gray bars) cells transfected with 100 nM IKKα or IKKβ siRNA for 72 hours. Gene expression levels were normalized to Gus or GAPDH and presented as fold change versus cells transfected with scrambled control siRNA. Values are the average of at least 3 experiments. Error bars are ± 1 S.E. (B) Quantitative real-time PCR of multiple genes upon knockdown of p65 by siRNA. SKBr3 and H16N2-Her2 cells were transfected with 100 nM siRNA for 72 hours and gene expression levels were measured. Fold change of transcript levels is shown relative to scrambled siRNA treated cells. Values are the average of at least 3 experiments. Error bars are ± 1 S.E. C) Quantitative real-time PCR shows inhibition of Her2 by lapatinib blocks NF-κB regulated gene expression. SKBr3 cells were treated with 1 μM lapatinib for 8 or 16 hours and gene expression levels of uPA, IL-6, IL-8, TNF and CCL2 were compared to DMSO treated cells. Fold change of transcript levels is shown relative to scrambled siRNA treated cells. Error bars are ± 1 S.E.

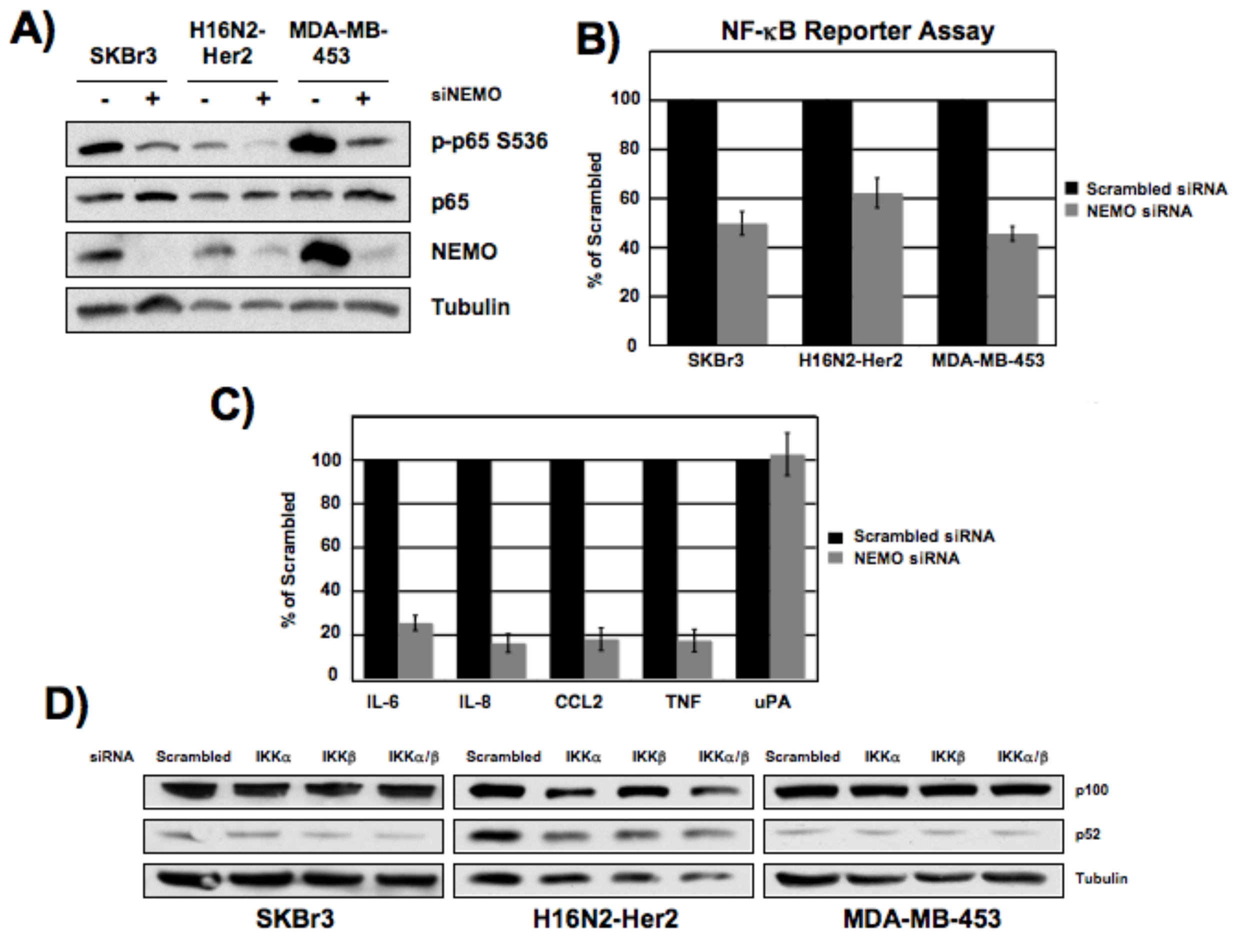


Figure 2.4. Knockdown of NEMO blocks NF-κB activation through the canonical pathway. A) Her2$^+$ breast cancer cells were transfected with 100 nM NEMO siRNA and whole cell lysates were collected 72 hours post transfection and western blot analysis of phosphorylated p65 was performed using 25 μg total protein. B) Her2$^+$ cell lines were transfected with 100 nM NEMO siRNA and whole cell extracts were prepared 72 hours post-siRNA transfection and luciferase levels were measured. Fold change of reporter activity with IKK knockdown is shown relative to scrambled siRNA treated cells. Values are the average of at least 3 experiments. Error bars are ± 1 S.E. Samples are normalized by protein concentration (SKBr3) or renilla (H16N2-Her2 and MDA-MB-453). C) SKBr3 cells were transfected with 100 nM NEMO siRNA and extracts were isolated after 72 hours and qRT-PCR was performed. Fold change of transcript levels is shown relative to scrambled siRNA treated cells. Error bars are ± 1 S.E. D) Her2-overexpressing breast cancer cells were transfected with 100 nM siRNA to IKKα or IKKβ and whole cell extracts were collected 72 hours post transfection. Levels of p100 and p52 were measured by western blot analysis using 25 μg total protein.

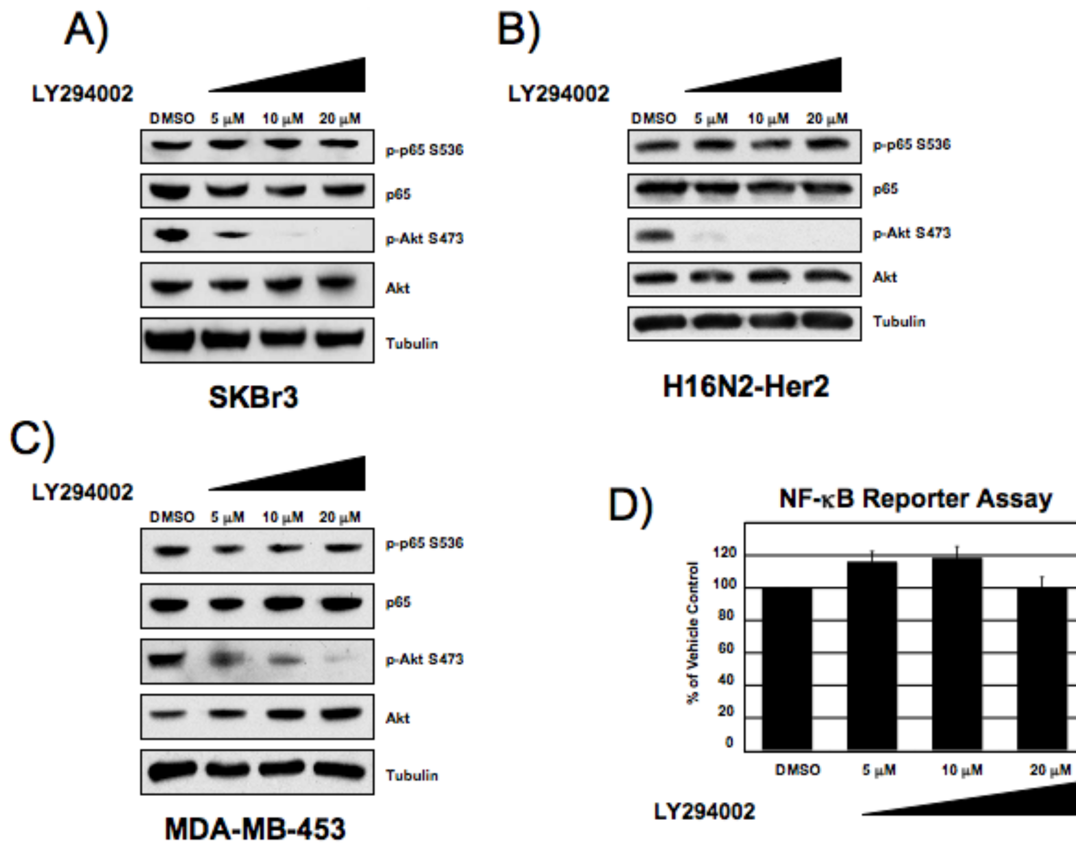


Figure 2.5. Inhibition of the PI3K-pathway does not block NF-κB activation. Western blot of phospho-p65 serine 536 from SKBr3 (A), H16N2-Her2 (B) and MDA-MB-453 (C) cells treated with PI3K-inhibitor inhibitor LY294002 for 2 hours. Western blot analysis was performed with 25 μg whole cell extracts. D) Luciferase reporter assay of SKBr3 cells treated with LY294002 overnight. Fold change of reporter activity with PI3K-inhibitor treatment is shown relative to vehicle treated cells. Values are the average of at least 3 experiments. Error bars are ± 1 S.E. Samples are normalized by protein concentration.

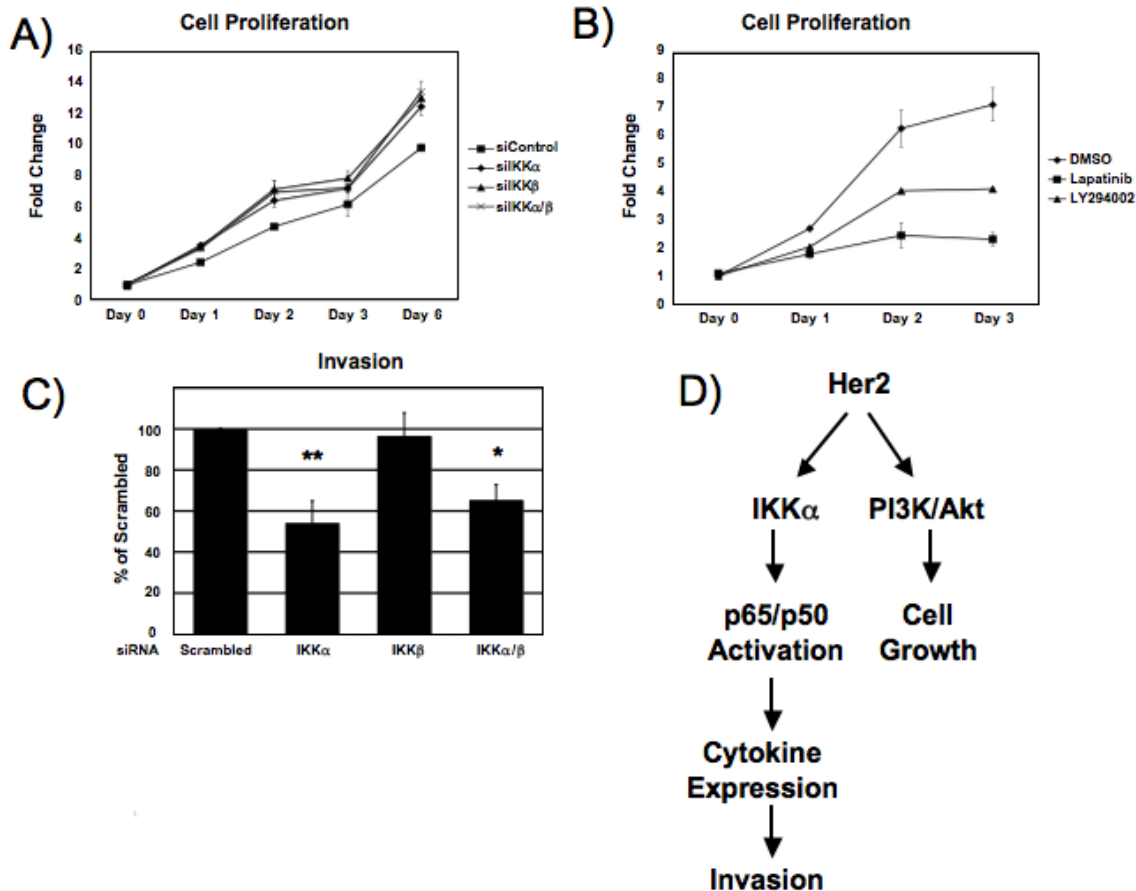
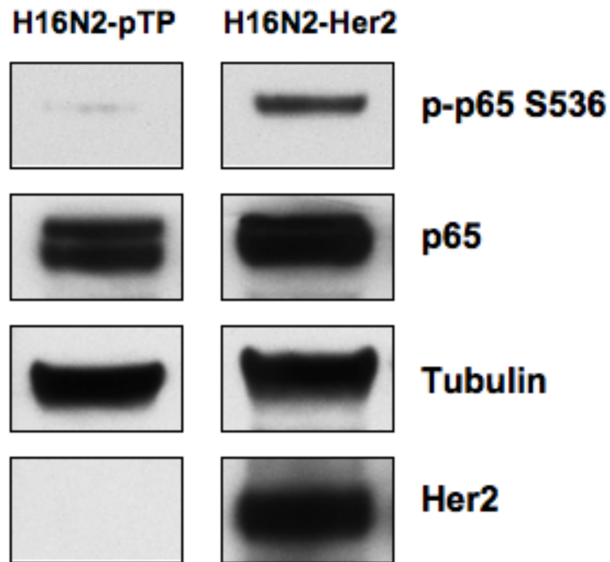
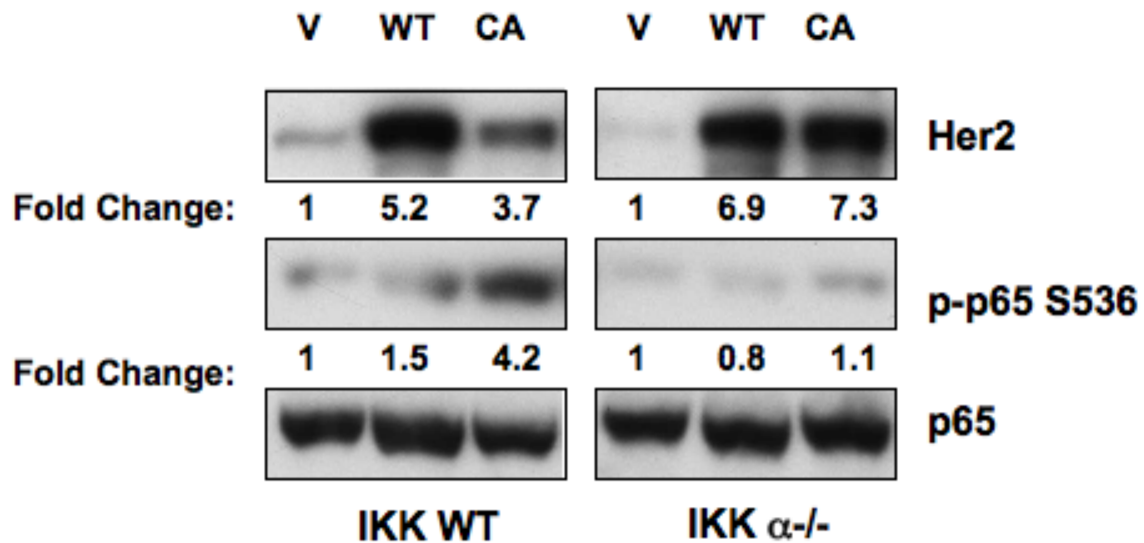


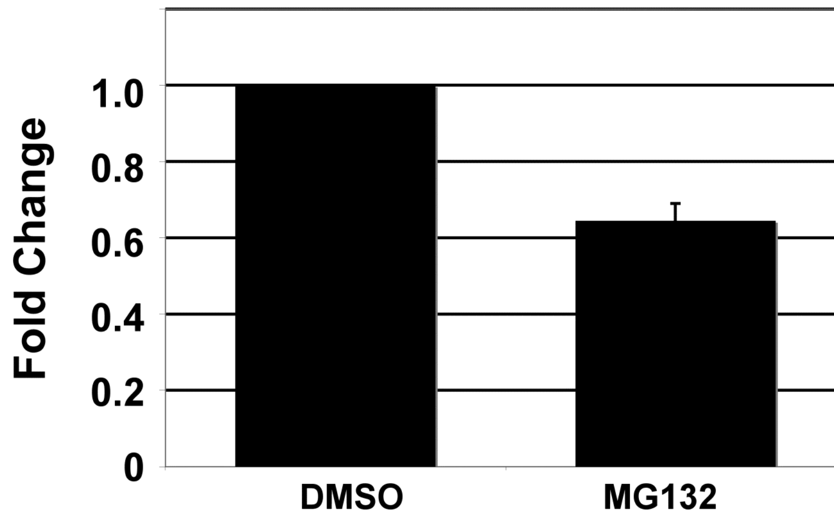
Figure 2.6. Inhibition of PI3K blocks cell proliferation, knockdown of IKKα blocks cell invasion. A) Cell proliferation of SKBr3 cells transfected with siRNA to IKKα or IKKβ was measured to for 6 days post-transfection compared to scrambled siRNA treated cells using CellTiter cell viability reagent. Knockdown of IKK by siRNA led to a slight increase in cell proliferation. Error bars represent ± 1 S.D. (B) Cell proliferation of SKBr3 cells treated with PI3K inhibitors LY294002 (10 μM) or EGFR/Her2 inhibitor lapatinib (1 μM) was measured over 3 days. Both inhibitors showed a significant decrease in cell proliferation over a course of 3 days. Error bars represent ± 1 S.D. C) SKBr3 cells were transfected with 100 nM siRNA to IKKα or IKKβ and cell invasion was measured after 48 hours fluorometrically. Statistical significance was measured by student's T-test (*<0.01, **<0.001). Error bars represent ± 1 S.D.



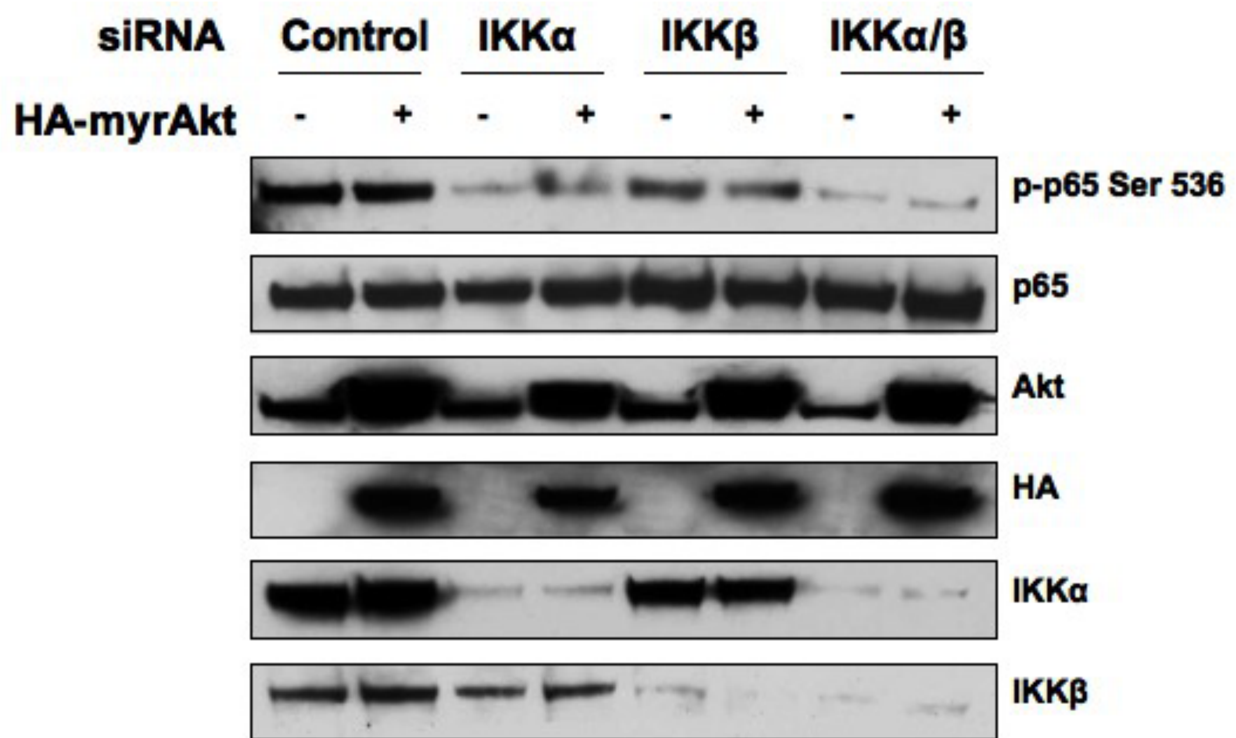
Supplementary Figure 2.1. **Stable overexpression of Her2 in breast epithelial cells activates NF-κB**. H16N2 breast epithelial cells stably expressing empty vector (pTP) or Her2 were assayed for p65 phosphorylation by western blot. Non-relevant bands were removed; exposure times are the same across cell lines for each antibody.



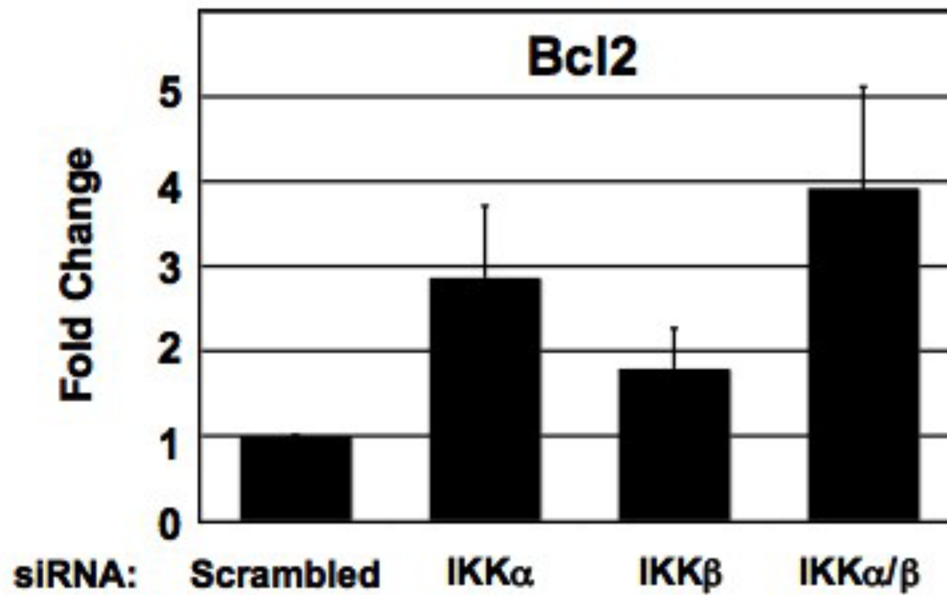
Supplementary Figure 2.2. Expression of Her2 activates NF-κB in wild-type, but not IKKα null, MEFs. Wild-type or IKKα null mouse embryonic fibroblasts were transduced with empty vector, wild-type Her2 (WT) or constitutively active Her2 (CA). Cells were lysed 48 hours post-transduction and p65 phosphorylation was measured by western blot. Fold change in Her2 and phospho-p65 levels was calculated by pixel densitometry. Band intensity was normalized to total p65 levels and fold change was calculated as change versus wild-type cells transduced with empty vector.



Supplementary Figure 2.3. Proteasomal inhibitor MG132 blocks NF-κB transcriptional activation in SKBr3 cells. SKBr3 cells stably expressing a 4x-κB reporter plasmid were treated for 12 hours with 10 μM MG132 and harvested with MPER cell lysis buffer. Luciferase assays were performed according to the manufacturer's protocol. Luciferase levels were normalized by protein levels using a Bradford assay. Luciferase levels are indicated as fold change versus vehicle (DMSO) treated cells.



Supplementary Figure 2.4. Overexpression of a constitutively active form of Akt does not affect p65 phosphorylation. SKBr3 cells were cotransfected with 1) 100 nM control, IKKα, IKKβ or IKKα and IKKβ siRNA and 2) constitutively active myristylated Akt or vector control. Whole cell lysates were prepared 72 hours post-transfection and western blot analysis was performed.



Supplementary Figure 2.5. siRNA knockdown of IKKα or IKKβ induces expression of Bcl-2. SKBr3 cells were transfected with 100 nM control, IKKα, IKKβ or IKKα and IKKβ siRNA and total RNA extracts were prepared 72 hours post transfection. Quantitative real-time PCR analysis was performed to measure Bcl-2 transcript levels. Bcl-2 levels were normalized to GUSB transcript levels. Bcl-2 transcript levels are shown as fold change versus scrambled (control) siRNA transfected cells.

Gene Symbol	Gene Name	Gene Alias	siRNA: IKKα	IKKβ	IKKα/β
CCL2	Chemokine (C-C motif) ligand 2	MCP-1	----	-	--
EFNA2	Ephrin-A2	ELF-1	----	----	----
EFNA1	Ephrin-A1	TNFAIP4	--	--	--
TNF	Tumor Necrosis Factor (TNF superfamily, member 2	TNFα	----	--	---
PLAU	Plasminogen activator, urokinase	uPA	--	+	--
CXCL9	Chemokine (C-X-C) motif ligand 9	MIG	--	+	-
BMP2	Bone morphogenic protein 2		----	---	----
IGFBP3	Insulin-like growth factor binding protein 3		----	----	----
CDKN2B	Cyclin-dependent kinase inhibitor 2B	p15	---	---	----
NFKBIA	Inhibitor of NF-kappaB alpha	IκBα	---	--	--
TNFRS10B	Tumor necrosis factor receptor superfamily, member 10b	DR5	---	--	--
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	A20	----	----	----
IRF1	Interferon regulatory factor 1	MAR	---	--	---
BIRC2	Baculoviral IAP repeat-containing 2	cIAP1	--	-	--

Supplementary Table 2.1. Superarray analysis of SKBr3 cells transfected with IKKα or IKKβ siRNA showed significant decrease in cytokines and chemokines. SKBr3 cells were transfected with siRNA to IKKα or IKKβ, and RNA was collected 3 days post-transfection. cRNA was prepared and hybridized to superarrays as per manufacturer's protocol. Arrays were developed by chemoluminescence and differences in gene expression levels were calculated by densitometry of 3 different exposures. Gene expression changes are represented as follows: ---- <0.25 fold decrease, --- 0.25 to 0.50 fold decrease, -- 0.50 to 0.75 fold decrease, - 0.75 to 1 fold decrease, + 1 to 1.5 fold increase. N.D. – No Data.
Gene expression changes are normalized to a GAPDH standard. Fold change was calculated using pixel densitometry using Adobe Photoshop.

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CHAPTER III

ACTIVATION OF NF-κB BY HER2 REQUIRES TGF-β ASSOCIATED KINASE-1 (TAK-1)

3.1 Abstract

TAK1 (transforming growth factor-β activated kinase 1) is a key mediator of signal transduction downstream of multiple cytokines including TNF-α and IL-1. Although it was initially characterized as a MAPK, it has recently been shown to play a key role in NF-κB activation upon activation of these cytokines. However, the role this kinase plays in NF-κB activation by receptor tyrosine kinases (RTKs) is currently unclear. Here, we investigate the contribution of TAK1 to NF-κB activation in Her2-overexpressing breast cancer. A luciferase assay screen using pharmacological inhibitors to multiple pathways identified TAK1 as a possible mediator of NF-κB activation in Her2-overexpressing breast cancer cells. Coexpression experiments show that Her2 overexpression can induce phosphorylation of TAK1 at threonines 184 and 187 in the activation loop, leading to an increase in autophosphorylation of TAK1. siRNA knockdown of TAK1 in the Her2-overexpressing SKBr3 cell line blocked basal, as well as EGF-and heregulin-induced, NF-κB phosphorylation and transcriptional activation. Ablation of TAK1 also blocked basal and growth factor-induced IL-6 transcription in this cell line. Finally, we present evidence that TAK1 can regulate Her2 levels in a manner dependent on its kinase activity. Taken together, we present data implicating TAK1 in Her2-mediated activation of NF-κB and subsequent regulation of IL-6 transcription.

3.2 Introduction

Transforming growth factor-β-activated kinase 1 (TAK1) is a crucial mediator of many signaling pathways in the cell. A member of the mitogen-activated protein kinase (MAPK) family, TAK1 was initially characterized as a MAPKKKK activated by TGF-β and bone morphological protein (Yamaguchi et al. 1995). However, recent studies have revealed that TAK1 plays an important role in proinflammatory activation of multiple pathways, including the JNK/p38 MAPK and NF-κB pathways, in response to signals such as IL-1 and TNF-α (Ninomiya-Tsuji et al. 1999; Takaesu et al. 2003).

IL-1β stimulation leads to the recruitment of multiple signaling proteins, including the RING domain ubiquitin ligase TRAF6, whose autoubiquitination leads to activation of TAK1 and subsequent phosphorylation of the Inhibitor of KappaB Kinase β (IKKβ) at serines 177 and 181 in its transactivation domain, and ultimately activation of NF-κB (Wang et al. 2001). Upon activation by IL-1β, TAK1 has also been shown to phosphorylate multiple members of the MKK family, which in turn activates the JNK (Jun N-terminal Kinase) and p38 kinases (Ninomiya-Tsuji et al. 1999; Wang et al. 2001). Activation by TNF-α activates multiple signaling proteins including the RING domain ubiquitin ligases TRAF2 and TRAF5, which ubiquitinate RIP1 (Receptor Interacting Protein 1). RIP1 ubiquitination activates TAK1, which in turn activates the IKK signalsome, leading to NF-κB activation (Chen 2005; Krappmann and Scheidereit 2005; Kovalenko and Wallach 2006).

NF-κB is an important transcription factor that has been shown to be involved in expression of genes involved in key cellular processes including innate and adaptive immunity (Bonizzi and Karin 2004), cell proliferation and survival (Papa et al. 2006),

lymphoid organ development (Weih and Caamano 2003), as well as being activated in a variety different cancers, including breast cancer. The NF-κB family of transcription factors consists of five subunits: RelA (p65), RelB, c-Rel, p105/p50 and p100/p52. These subunits are evolutionarily conserved and exist as hetero- or homodimers (Hayden and Ghosh 2004). NF-κB is activated via two distinct pathways known at the canonical and non-canonical, or alternative, pathways. The canonical pathway is activated upon stimulus by growth factors or cytokines, such as TNF-α, which leads to activation of the IKKβ subunit of the Inhibitor of KappaB Kinase (IKK) complex, which phosphorylates the Inhibitor of KappaB alpha (IκBα), releasing p65/RelA-containing dimers to translocate to the nucleus (Ghosh and Karin 2002). Furthermore, upon activation of the canonical pathway, the p65/RelA subunit can be phosphorylated at multiple residues, including serine 536, which is important for transactivation (Sakurai, Chiba et al. 1999). Activation of NF-κB in the non-canonical pathway, most common in B cells, involves Inhibitor of KappaB Kinase α (IKKα) and is IκBα-independent (Solt and May 2008).

We have previously shown that Her2-overexpression activates NF-κB in an IKKα dependent manner, leading to an increase in cytokine and chemokine gene expression and invasion (Merkhofer, Cogswell, and Baldwin 2009). Here we investigate TAK1 as an upstream activator of NF-κB activation in Her2-overexpressing breast cancer cells. Overexpression of Her2 induced phosphorylation of TAK1 in its activation loop, leading to an increase in autophosphorylation. Inhibition of TAK1 by pharmacological inhibitors or siRNA led to a decrease in NF-κB activation in the $Her2^{+}$ SKBr3 breast cancer cell line. In addition to basal NF-κB activation, TAK1 is also required for EGF-and heregulin-induced NF-κB phosphorylation and transcriptional activation in SKBr3 breast

cancer cells. Transcription of the cytokine IL-6, which plays a significant role in breast cancer progression (Studebaker et al. 2008; Ndlovu et al. 2009), has previously been shown to be upregulated by activation of TAK1. Notably, this leads to an increase in invasion (Xie et al. 2009). Consistent with our previously published data showing that Her2 and IKKα induce IL-6 transcription (Merkhofer, Cogswell, and Baldwin 2009), knockdown of TAK1 in Her2-overexpressing breast cancer cells blocks basal, as well as EGF-and heregulin-induced, IL-6 expression. Finally, we present evidence that TAK1 kinase activity can downregulate Her2/Neu protein levels in a proteasome-independent manner.

3.3 Materials and Methods

Cell Culture and Reagents

The tumor-derived SKBr3 cell line was maintained in McCoy's 5A medium (Mediatech) supplemented with 10% fetal bovine serum (FBS) and 100 units/mL penicillin/streptomycin. The 293T cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 100 units/mL penicillin/streptomycin. The stable 3x-κB luciferase SKBr3 cell line was established by transfection of a luciferase reporter construct containing tandem NF-κB binding sites from the MHC class I promoter region into SKBr3 cells with Fugene (Roche) and maintained under selection with G418 (Geneticin, Live Technologies). The following antibodies were purchased from commercial sources: antibodies against phospho-p65 (Ser^{536}), phospho-TAK1 (Thr^{184} and Thr^{187}) and TAK1 from Cell Signaling Technology, antibodies against β-tubulin from Santa Cruz Biotechnology, antibody against total p65

from Rockland (PA, USA), antibody against Flag from Sigma-Aldrich, antibody against Her2/Neu from NeoMarkers (CA, USA). Proteasome inhibitor MG132 was purchased from Sigma (MO, USA). Epidermal growth factor was purchased from Gibco. Recombinant heregulin β was purchased from Fitzgerald Industries. Lapatinib (GW572016; Tykerb) was a gift from Dr. H. Shelton Earp (University of North Carolina at Chapel Hill). LY294002 was purchased from Cell Signaling; SB202190, UO216 and SP600 were purchased from Sigma-Aldrich. 5Z-7 Oxozeaenol was a kind gift from Dr. Jun Ninomiya-Tsuji.

Plasmids

The NeuNT construct (Bargmann, Hung, and Weinberg 1986) was purchased from Addgene (10918). Wild-type and kinase-dead Flag-TAK1 expression constructs were previously described (Sakurai, Miyoshi et al. 1999). Flag-TAB1 expression construct was described previously (Sakurai et al. 2000). GST-IKKα and GST-IKKβ were described previously (Hutti et al. 2007).

Kinase Assay

NeuNT, Flag-TAK1 and Flag-TAB1 constructs were transfected into 293T cells using polyethylenimine (PEI). Whole cell lysates were prepared on ice for 45 minutes in lysis buffer containing 20 mmol/L Tris (pH 8.0), 500 mmol/L NaCl, 0.25% Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 1x protease inhibitor (Roche Applied Science), caliculin and 1x phosphatase inhibitor cocktail (Sigma-Aldrich). TAK1/TAB1 complexes were pulled down using Flag-conjugated agarose beads (Sigma-Aldrich). An

in vitro kinase assay was performed and analyzed as previously described (Steinbrecher et al. 2005).

Immunoblots

Whole cell extracts were prepared on ice with RIPA lysis buffer supplemented with protease inhibitor mix (Roche, IN, USA) and phosphatase inhibitor mix (Sigma, MO, USA). Protein concentrations were determined by Bradford assay (Biorad Laboratories) and SDS-PAGE analysis was performed as previously described (Steinbrecher et al. 2005).

Small RNA interference

The following small interfering RNAs (siRNA; siGenome SMARTpool) were obtained from Dharmacon as a pool of four annealed double-stranded RNA oligonucleotides: TAK1 (M-003790-06), NIK (M-003580-04) and nontargeting control #3 (D001201-03). Cells were grown to approximately 50% confluency and transfected with 100 nmol/L siRNA with Dharmafect 1 reagent according to manufacturer's instructions.

Quantitative Real-time PCR

Total RNA extracts were obtained from cells approximately 72 hours post-transfection by Trizol (Invitrogen) extraction. Two micrograms of RNA was reverse transcribed using random primers and MMLV-reverse transcriptase (Invitrogen). Real-time PCR was performed and analyzed as previously described (Steinbrecher et al. 2005) using Taqman

Gene Expression Assay primer-probe sets IL-6 (Hs00174131_m1), GUS (Hs99999908_m1) and IκBα (Hs00153283_m1).

Luciferase Assay

SKBr3 cells stably expressing the 3x-κB plasmid were plated in equal number in triplicate in 24-well plates and transfected with siRNA for 72 hours or treated overnight with inhibitors. Cells were lysed in MPER (Thermo) and luciferase activity was measured with Promega Luciferase Assay System (Promega). Luciferase levels were normalized by protein concentration using a Bradford assay.

ChIP

Chromatin immunoprecipitation (ChIP) assays were performed using chromatin immunoprecipitation reagents according to the manufacturer's protocol (Millipore) with minor modifications. SKBr3 cells were treated with vehicle or lapatinib for 16 hours and washed with phosphate buffered saline (PBS) extensively. Cells were incubated with 2mM disuccinimidyl glutarate (DSG) in PBS for 45 minutes at room temperature shaking. Cells were washed with PBS and crosslinked with 1% (v/v) formaldehyde in PBS for 15 minutes shaking. Crosslinking was quenched with 125 mM glycine for 10 minutes. Cells were washed, harvested and lysed in SDS lysis buffer on ice for 10 minutes. Cell lysate was sonicated for 15 minutes (Sonics VibraCell). Samples were precleared with Protein A/G agarose beads (Santa Cruz Technology) for one hour. Cell lysates were quantified by Bradford assay, and equal quantities of lysate were incubated with p65 antibody (Santa Cruz Biotechnology) overnight at 4 degrees. Samples were

incubated with Protein A/G agarose beads for 2 hours and bead-antibody complexes were washed per manufacturer's protocol. Crosslinks were reversed overnight and DNA was isolated using Wizard SV DNA purification kit (Promega). PCR of ChIP DNA was performed with IL-6 primers previously described (Tang et al. 2007). PCR products were run on 2% agarose gel and photographed using Kodak imaging software.

3.4 Results

TAK1 inhibitor 5Z-7 Oxozeaenol blocks NF-κB luciferase activity in Her2$^+$ Breast Cancer Cells

Her2-mediated activation of NF-κB has previously been shown by our group to require the kinases IKKα and IKKβ (Merkhofer, Cogswell, and Baldwin 2009). However, the upstream mediators of Her2-induced activation of IKK have not been elucidated. We therefore performed a screen using multiple inhibitors of possible upstream activators of IKK to measure the effect on NF-κB transcriptional activation by luciferase assay. The p38/MAPK, MEK/ERK, PI3K/Akt and JNK pathways have all been reported to be activators of NF-κB (Beyaert et al. 1996; Catley et al. 2004; Joo and Jetten 2008; Lee, Lee, and Han; Dan et al. 2008; Tsai et al. 2003). Treatment of Her2-overexpressing SKBr3 cells with p38/MAPK inhibitor SB203580, PI3K inhibitor LY294002, or MEK 1/2 inhibitor UO216 had no significant effect on NF-κB luciferase activity (Fig. 3.1 A-C). Treatment of SKBr3 cells with JNK inhibitor SP600 led to a dose-dependent significant increase in NF-κB luciferase activity (Fig. 3.1 D). This is consistent with published reports that inhibition of JNK can indirectly lead to NF-κB activation (Tan et al. 2009). We next investigated whether inhibition of TGF-β activated

kinase 1 (TAK1) modulates NF-κB transcriptional activation. SKBr3 cells were treated with increasing doses of the TAK1 inhibitor 5Z-7 Oxozeaenol (Ninomiya-Tsuji et al. 2003) and luciferase activity was measured (Fig. 3.1 E). 5Z-7 Oxozeaenol blocked NF-κB activation in dose-dependent manner, suggesting that TAK1 may be involved in NF-κB activation in Her2$^+$ breast cancer.

Overexpression of Her2 induces phosphorylation of the activation loop of TAK1

The phosphorylation of threonines 178 and 184 in the activation loop of TAK1 has been reported to be required for IL-1-mediated activation of NF-κB (Yu et al. 2008). Phosphorylation of threonine 187 has also been shown to be essential for complete TAK1 activation (Sakurai et al. 2000; Singhirunnusorn et al. 2005). We therefore investigated if Her2 overexpression can activate TAK1 and if this activation is mediated through phosphorylation of the activation loop. Increasing amounts of an activated form of the rat homologue of Her2 (NeuNT) was cotransfected into 293 cells with wild-type or kinase-dead Flag-tagged TAK1 alone or in combination with Flag-tagged TAB1 (Fig. 3.2). Cotransfection of NeuNT and Flag-TAK1 alone did not induce TAK1 phosphorylation (lanes 3-5), however, cotransfection of NeuNT, Flag-TAK1 and Flag-TAB1 led to TAK1 phosphorylation at threonines 184 and 187, which increased upon increasing levels NeuNT transfected (lanes 6-8). This phosphorylation appeared to be due to autophosphorylation by TAK1, as transfection of a kinase-dead TAK1 did not lead to phosphorylation of TAK1 (lanes 9-11 and 12-14). To confirm that this increase in phosphorylation leads to an increase in catalytic activity, we performed a kinase assay measuring autophosphorylation of TAK1/TAB1 complexes. The kinase assay using flag-

precipitated complexes shows that only complexes containing wild-type TAK1 and TAB1 have catalytic activity, and this catalytic activity is increased upon transfection of NeuNT.

siRNA Inhibition of TAK1 Blocks Basal NF-κB Activation in Her2⁺ Breast Cancer Cells

To confirm that inhibition of NF-κB activation by 5Z-7 Oxozeaenol was not due to an off target effect, we employed an siRNA strategy to investigate the role of TAK1 in NF-κB activation in SKBr3 cells. SKBr3 cells were transfected with siRNA to TAK1 and western blot analysis was performed to measure the phosphorylation levels of the RelA (p65) subunit of NF-κB. Phosphorylation of p65 at serine 536 has been shown to be a marker of NF-κB transcriptional activation (Sakurai, Chiba et al. 1999; Sakurai et al. 2003). We have previously shown that basal p65 phosphorylation in SKBr3 cells is high, and this phosphorylation is blocked by the dual EGFR/Her2 small molecule inhibitor lapatinib (Fig. 2.1A). Similarly, knockdown of TAK1 significantly blocked phosphorylation at serine 536 on the RelA subunit of NF-κB (Fig 3.3A). We next investigated whether TAK1 played a role in basal NF-κB transcriptional activation using a luciferase reporter assay. SKBr3 cells stably expressing a 4x-κB reporter construct were transfected with siRNA to TAK1 and transcriptional activation was assayed (Fig. 3.3B). Knockdown of TAK1 led to a greater than 2.5-fold reduction in luciferase activation. Taken together, these results strongly suggest that TAK1 is required for basal NF-κB activation in Her2⁺ breast cancer cells.

TAK1 is required for EGF and Heregulin activation of NF-κB in SKBr3 cells

Epidermal growth factor receptor (EGF) has been shown to activate NF-κB in multiple different normal and cancer cell types (Zhang et al. 2006; Baek et al. 2008; Huang et al. 2008; Sethi et al. 2007; Haussler et al. 2005). Heregulin, a ligand for the Her3 and Her4 receptors, has also been reported to activate NF-κB (Chen et al. 2003; Tsai et al. 2003). Importantly, heregulin is an activator of NF-κB in Her2-overexpressing SKBr3 breast cancer cells (Biswas et al. 2004). We therefore investigated whether TAK1 was required for EGF- and heregulin-induced activation of NF-κB in Her2-overexpressing breast cancer cells. SKBr3 cells were transfected with siRNA to TAK1, serum starved and treated with either EGF or heregulin and p65 phosphorylation levels were measured. Knockdown of TAK1 decreased basal NF-κB phosphorylation in serum starved cells, as well as inhibited EGF and heregulin induced phosphorylation of p65 (Figure 3.4A). We also assayed the effect of TAK1 knockdown on EGF and heregulin induction of NF-κB transcriptional activation by luciferase assay. In control siRNA transfected SKBr3 cells, serum starvation led to a slight decrease in NF-κB luciferase activity, followed by an approximate 3.5-and 3-fold induction by EGF and heregulin, respectively (Fig. 3.4B). However, TAK1 knockdown decreased NF-κB basal luciferase activation, as well as reducing EGF and heregulin induction to approximately 2-and 1.5-fold respectively. Taken together, these results show that TAK1 plays a crucial role in EGF and heregulin induction of NF-κB phosphorylation and transcriptional activation.

TAK1 is required for basal and growth-factor induced IL-6 transcription in Her2⁺ cells

It has recently been reported that TAK1 plays in important role in IL-6 transcription, requiring phosphorylation at threonines 178 and 184, as well as ubiquitination at lysine 209, of TAK1 for transcription of this cytokine (Yamazaki et al. 2009; Yu et al. 2008). To measure the effect of Her2-overexpression on IL-6 transcription levels, SKBr3 cells were treated with the dual EGFR/Her2 inhibitor lapatinib. Quantitative real-time analysis shows that inhibiting Her2 leads to a reduction of IL-6 transcript levels at 8 and 16 hours post-treatment (Fig. 3.5A). Additionally, ChIP analysis was performed in these cells upon 16 hour treatment with lapatinib. Inhibition of Her2 led to an approximately 8-fold decrease in p65 occupancy at the IL-6 promoter (Fig. 3.5B). Induction of IL-6, as well as other NF-κB regulated cytokines, occurs through Her2 activation of IKKα and IKKβ (Fig. 2.3A). To determine if TAK1 is required for EGF and heregulin induced IL-6 expression in SKBr3 cells, siRNA against TAK1 was transfected into cells and transcript levels of IL-6 were measured by quantitative real-time PCR. EGF and heregulin treatment led to an approximate 2.5- and 5-fold induction of IL-6 transcription over serum starved cells, respectively (Fig. 3.5C). Knockdown of TAK1 led to a 3-fold decrease in basal IL-6 levels, and reduced heregulin induction to 3-fold. IL-6 transcription by EGF activation still occurred, albeit at much lower total levels. Knockdown of TAK1 had no effect on transcription of IκBα, another NF-κB regulated gene (Fig. 3.4C), which this is consistent with the lack of effect on IκBα transcription with IKK knockdown in SKBr3 cells (data not shown). This data shows that TAK1 is required for basal, as well as heregulin induced, IL-6 transcription in Her2-overexpressing SKBr3 breast cancer cells.

Overexpression of TAK1 modulates Neu protein levels

Upon investigation of Her2 induction of TAK1 activation, we noticed that overexpression of TAK1 appeared to modulate levels of transfected NeuNT. To further investigate this, we transfected 293T cells with NeuNT and increasing levels of Flag-TAK1 (Fig. 3.6A). Overexpression of TAK1 clearly blocked NeuNT protein levels (compare lanes 2 and 5). This TAK1 modulation of Neu was dependent on its kinase activity, as overexpression of a kinase-dead TAK1 construct had no effect on Neu levels (lanes 9-11). This regulation also appears to be independent of the proteasome, as cells incubated with the proteasome inhibitor MG132 still showed decreased Neu protein levels (lanes 6-8). To ensure this effect was not an artifact of the Flag-TAK1 expression plasmid, 293T cells were transfected with control or TAK1 siRNA then transfected with increasing levels of NeuNT (Fig. 3.6B). Knockdown of TAK1 led to greater expression of Neu compared to control siRNA cells transfected with equal amounts of Neu, indicating that endogenous TAK1 in these cells is able to regulate exogenous Neu levels.

It has been reported that TAK1 activation can induce endosomal localization of EGFR in a p38-dependent manner (Nishimura et al. 2009). We investigated if TAK1 was inducing Her2 localization to endosomes and therefore accumulating in an insoluble fraction of the cell lysate. However, treatment of TAK1 and NeuNT cotransfected cells with a p38 inhibitor (SB202) did not rescue downregulation of Neu levels by TAK1 overexpression (Fig. 3.6C). Furthermore, western blot analysis of cells coexpressing NeuNT and TAK1 lysed in SDS protein sample buffer still showed downregulation of Neu levels by TAK1, suggesting this is not due to endosomal localization (Fig. S3.1). Cells treated with the caspase inhibitor Z-VAD also showed decreased Neu levels upon

TAK1 cotransfection, indicating this regulation is likely not due to caspase-mediated cleavage of Neu.

3.5 Discussion

TAK1 plays a critical role in multiple cellular processes, and is beginning to be considered as a viable candidate for targeted therapy in cancer (D. Melisi, Abstract 1002, ECCO15-ESMO34). Though TAK1 has been shown to be required for NF-κB activation by multiple different stimuli (Ninomiya-Tsuji et al. 1999; Takaesu et al. 2003), and NF-κB has been shown to be activated in Her2-overexpressing breast cancer (Galang et al. 1996; Biswas et al. 2004), no one has linked these pathways to date. In this study, we present compelling evidence that TAK1 is required for Her2 activation of NF-κB in breast cancer cells, leading to transcription of IL-6, a cytokine implicated in progression of breast cancer (Ndlovu et al. 2009; Studebaker et al. 2008). We also present interesting new data showing TAK1 can regulate Her2 levels through its kinase activity, independent of NF-κB and the proteasome.

Multiple signaling pathways can activate the NF-κB pathway downstream of activated cell surface receptors, including the epidermal growth factor receptor family member Her2. To determine which signaling proteins may link Her2 activation to the IKK complex, we performed a screen using an NF-κB luciferase reporter assay in Her2-overexpressing breast cancer cells. We have previously reported that inhibition of PI3K does not effect NF-κB activation in these cells (Merkhofer, Cogswell, and Baldwin 2009). Similarly, inhibition of MEKK 1/2 and p38 had no effect on NF-κB transcriptional activation, while inhibition of TAK1 significantly blocked NF-κB

activation (Fig. 3.1). We therefore further investigated the role TAK1 plays in this subtype of breast cancer. siRNA knockdown of TAK1 blocked basal NF-κB phosphorylation and transcriptional activation in SKBr3 breast cancer cells, as well as inhibiting both EGF- and heregulin-induced NF-κB activation. Taken together, these data suggest that TAK1 is required for full NF-κB activation in Her2-overexpressing breast cancer cells. TAK1 has been shown to activate both IKKα and IKKβ (Sakurai, Chiba et al. 1999). As we have shown that IKKα plays a larger role than IKKβ in Her2 activation of NF-κB, Her2 may direct preferential activation of IKKα. A possible mechanism for this activation may be through NF-κB inducing kinase (NIK). Though NIK is generally thought be active in the non-canonical NF-κB pathway, TAK1 has been reported to activate IKKα, leading to NF-κB activation downstream of IL-1, an activator of the canonical NF-κB pathway (Ninomiya-Tsuji et al. 1999). Knockdown of NIK in SKBr3 cells blocks NF-κB luciferase activity, but does not effect p65 phosphorylation (Fig. 3S2). Therefore, further investigation is required to determine if and how TAK1 may activate IKKα in Her2-overexpressing breast cancers.

The cytokine IL-6, an NF-κB regulated gene, is an important mediator of inflammation, and is dysregulated in multiple types of cancers (Naugler and Karin 2008). We have previously shown that inhibition of Her2 with lapatinib significantly blocks NF-κB regulated IL-6 expression. Here we show that blocking TAK1 by siRNA inhibits basal, as well as EGF- and heregulin-induced, IL-6 expression in SKBr3 breast cancer cells. However, inhibition of TAK1 had no effect on IκBα expression in this cell line (Fig. 3.4D). This is consistent with previous results that knockdown of IKK blocks expression of IL-6 (as well as other cytokines and chemokines), but has little effect on

expression of IκBα in SKBr3 cells (data not shown). This is likely due to upregulation of a cytokine/chemokine gene signature common in estrogen receptor-negative breast cancer (Chavey et al. 2007). Given that inflammatory genes have been shown to be overexpressed in many breast cancers, including Her2-overexpressing breast cancer (Calogero et al. 2007), leading to progression of carcinogenesis, this is of great therapeutic interest for this disease.

We present interesting new data here that TAK1 can downregulate Her2 protein levels in non-transformed cells (Fig. 3.6). Increasing levels of TAK1 transfected into 293T cells led to increasing loss of overexpressed Neu. Endogenous TAK1 was also able to regulate Her2, as knockdown of TAK1 in these cells led to greater protein levels of Neu. This regulation of Her2/Neu levels is kinase dependent, as expression a kinase-dead TAK1 was unable to affect Her2 levels. The proteasome inhibitor MG132 and the p38 inhibitor SB202 were also unable to rescue this downregulation of Her2.

The transforming growth factor-β pathway, of which TAK1 is a member, is of great interest in cancer as it can be either a tumor suppressor or induce tumor progression in breast cancer, depending on spatial and temporal expression (Barcellos-Hoff and Akhurst 2009; Bierie and Moses 2009). Activation of the TGF-β pathway has been reported to induce migration and cell survival in Her2-overexpressing breast cancer through integrin signaling (Wang et al. 2009), as well as leading to increases in invasion (Seton-Rogers et al. 2004), in Her2-ovexpressing breast cancer. However, TGF-β has also been shown to upregulate the tumor suppressor Maspin in breast cancer cells, inhibiting cell migration (Wang et al. 2007). It has also been reported that Her2-overexpression can downregulate the TGF-β pathway through loss of the TGF-β receptor

(Landis et al. 2005). We are currently investigating if this TAK1 downregulation of Her2 is through the TGF-β pathway. Interestingly, TAK1 has been shown to play opposing roles in NF-κB activation in non-transformed versus transformed breast epithelial cells (Neil and Schiemann 2008). This study showed that epithelial-mesenchymal transition (EMT) was necessary for TAK1 to induce oncogenic signaling. Therefore, it is possible that in the absence of activated TAB1, e.g. in non-transformed cells, TAK1 is able to downregulate Her2. Therefore, in transformed cells where TAB1 is active, this downregulation would not occur. This is consistent with siRNA knockdown of TAK1 in transformed SKBr3 cells, as Her2 levels are unchanged (Fig. 3.3A). We are currently investigating the role TAK1 and TAB1 may play in modulating Her2 levels in non-transformed versus transformed cells. Taken together, these data shed new light on how TAK1 may promote tumorigenesis through regulation of Her2 levels and indicates that TAK1 may be a viable target for therapy in Her2-overexpressing breast cancer.

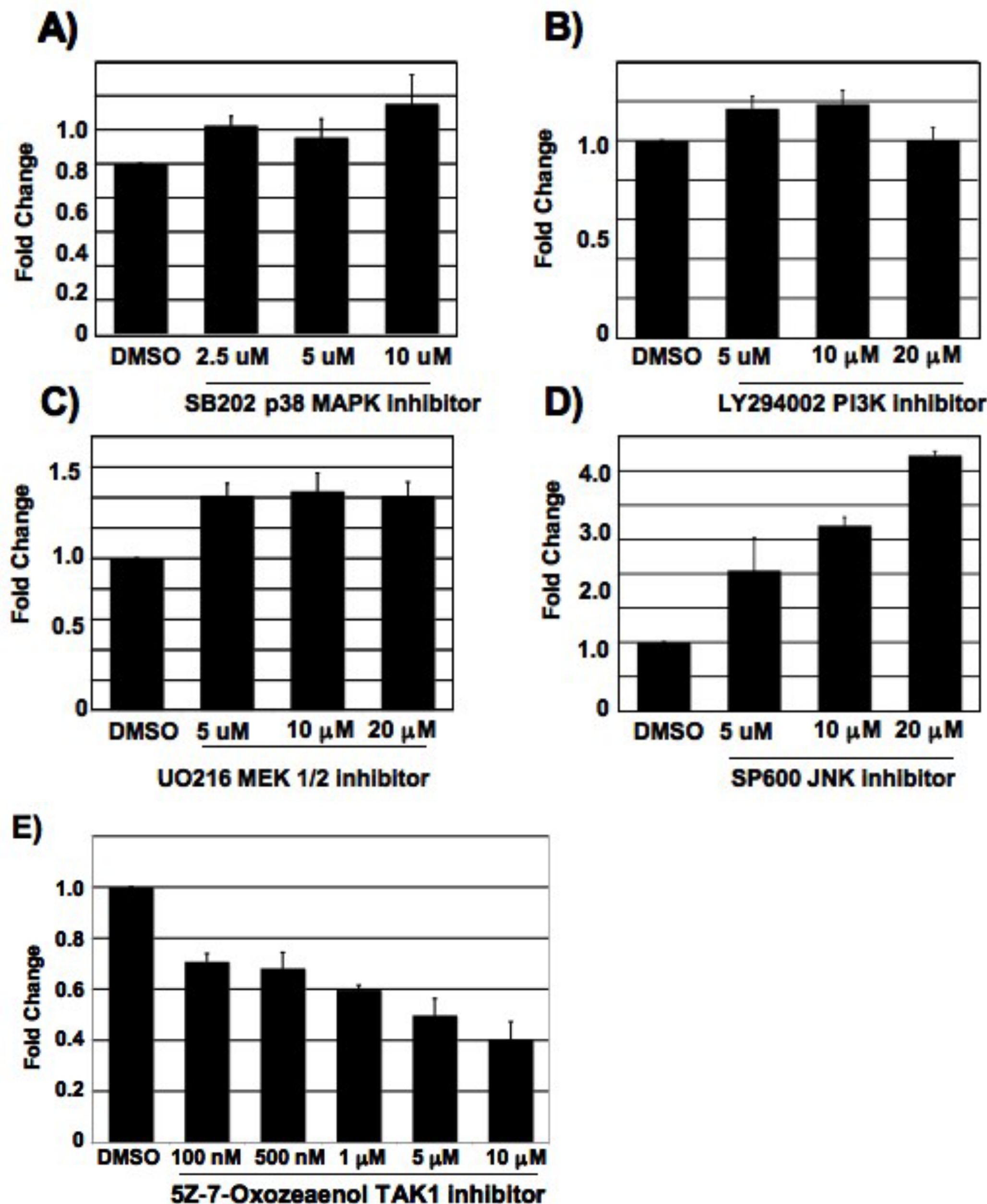


Figure 3.1. Inhibition of TAK1 with 5Z-7-Oxozeaenol blocks NF-κB transcriptional activity. $Her2^+$ SKBr3 breast cancer cells stably expressing a 4x-κB reporter plasmid were treated for 12 hours with inhibitors to (A) p38 (SB202), (B) PI3K (LY294002), (C) MEK 1/2 (UO216), (D) JNK (SP6000) or (E) TAK1 (5Z-7) at the indicated concentrations. Luciferase assays were performed to determine NF-κB transcriptional activation.

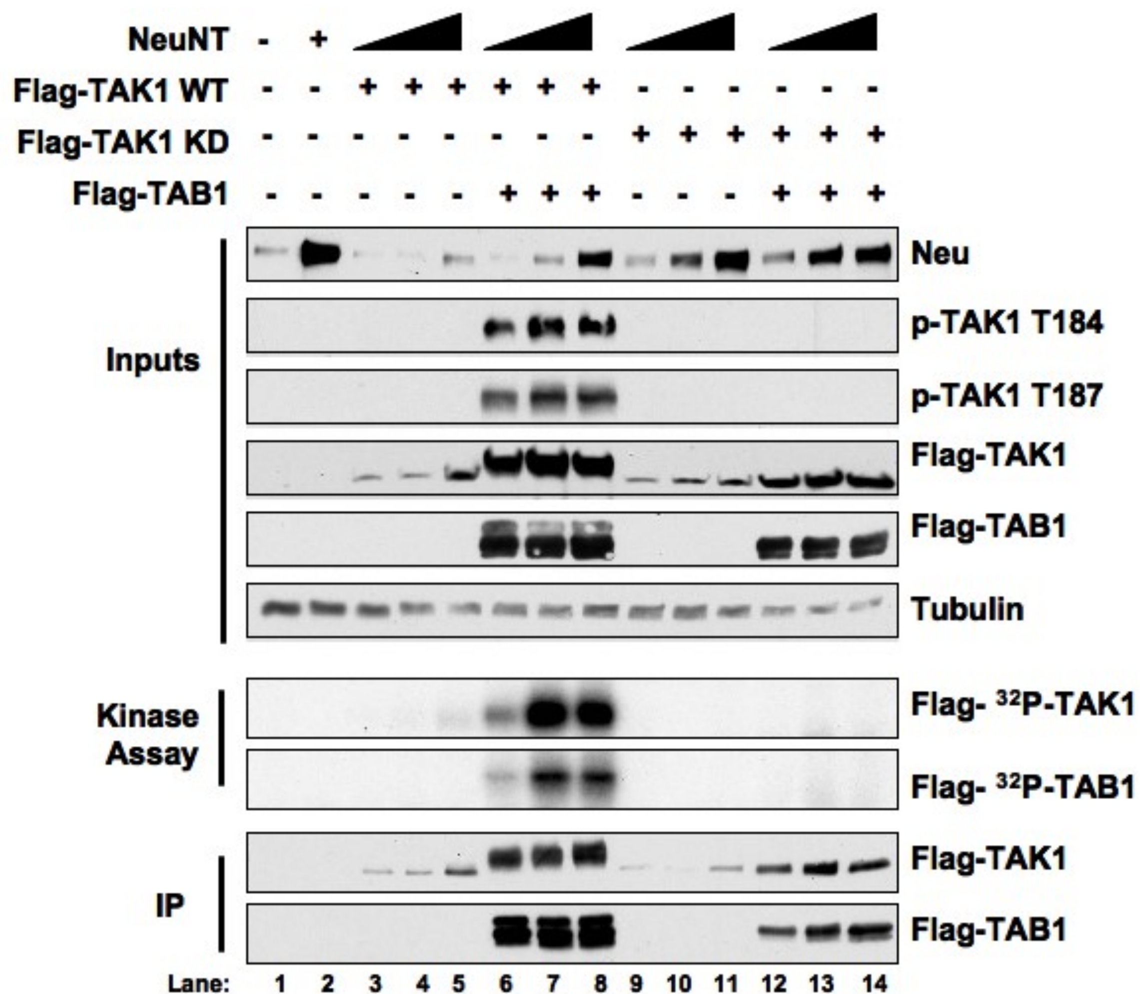


Figure 3.2. NeuNT induces TAK1 autophosphorylation in a TAB1 dependent manner. 293T cells were transfected with wild-type or kinase-dead Flag-TAK1 alone (lanes 3-5 and 9-11) or with Flag-TAB1 (lanes 6-8 and 12-14). Increasing amounts of NeuNT, the activated rat homologue of Her2, was cotransfected into cells (5, 10 or 20 μg). Cells were lysed in RIPA buffer and western blot analysis was performed to measure levels of phosphorylated TAK1. A kinase assay was performed using Flag-precipitated complexes. Levels of Flag-TAK1 and Flag-TAB1 pulled down for the kinase assay are shown indicated (IP).

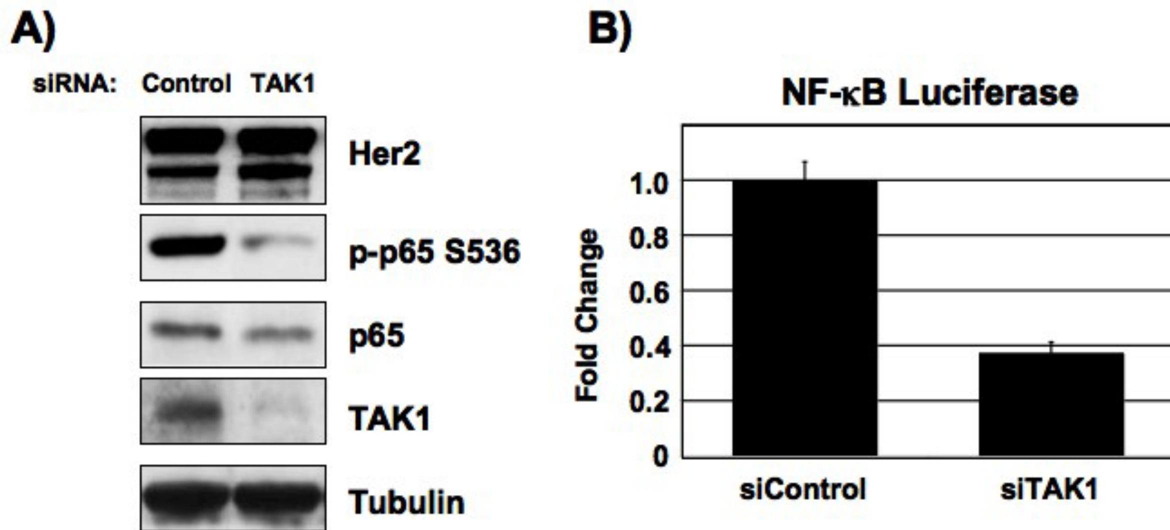


Figure 3.3. siRNA knockdown of TAK1 blocks NF-κB activation. A) Her2$^+$ SKBr3 cells were transfected with 100 nM siRNA (scrambled control or TAK1) and lysed 72 hours post-transfection. Western blot analysis was performed to measure phosphorylation of the p65 subunit of NF-κB at serine 536. B) SKBr3 cells stably expressing a 4x-κB luciferase construct were transfected with 100 nM siRNA (scrambled control or TAK1) and lysed 72 hours post-transfection with MPER. Luciferase assays were performed to measure NF-κB transcriptional activation. Luciferase activity is shown as fold change compared to control siRNA transfected cells.

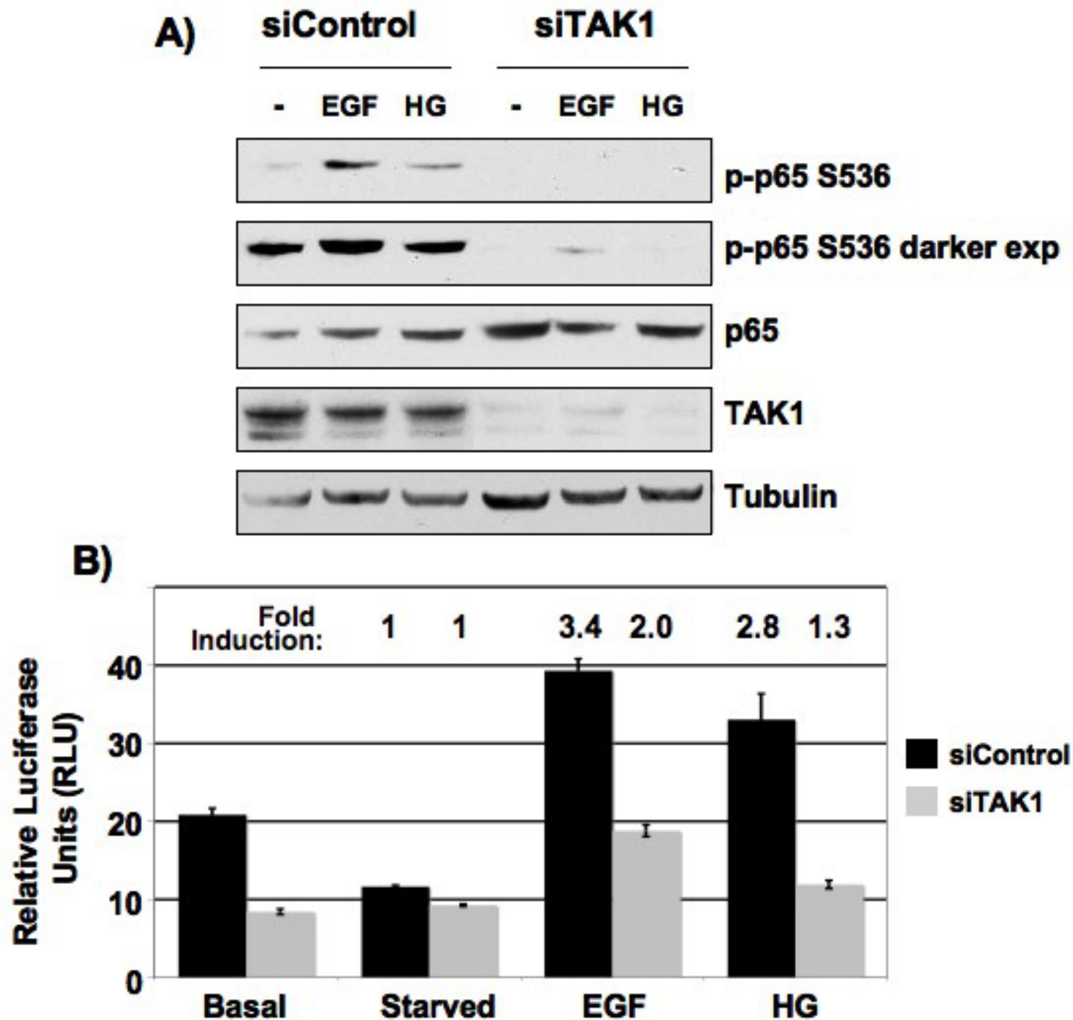


Figure 3.4. TAK1 knockdown blocks EGF and heregulin induced NF-κB activation. A) SKBr3 cells were transfected with 100 nM control or TAK1 siRNA. 72 hours post-transfection cells were treated with 100 ng/ml EGF or heregulin for 1 hour and whole cell lysates were prepared. Western blot analysis was performed to measure levels of phosphorylated p65. B) SKBr3 cells stably expressing a 4x-κB reporter plasmid were transfected with 100 nM control or TAK1 siRNA. 48 hours post-transfection cells were treated with 100 ng/ml EGF or heregulin overnight. Cells were lysed in MPER and luciferase analysis was performed. Fold induction is expressed as change in RLUs in control or TAK1 siRNA treated cells over starved levels.

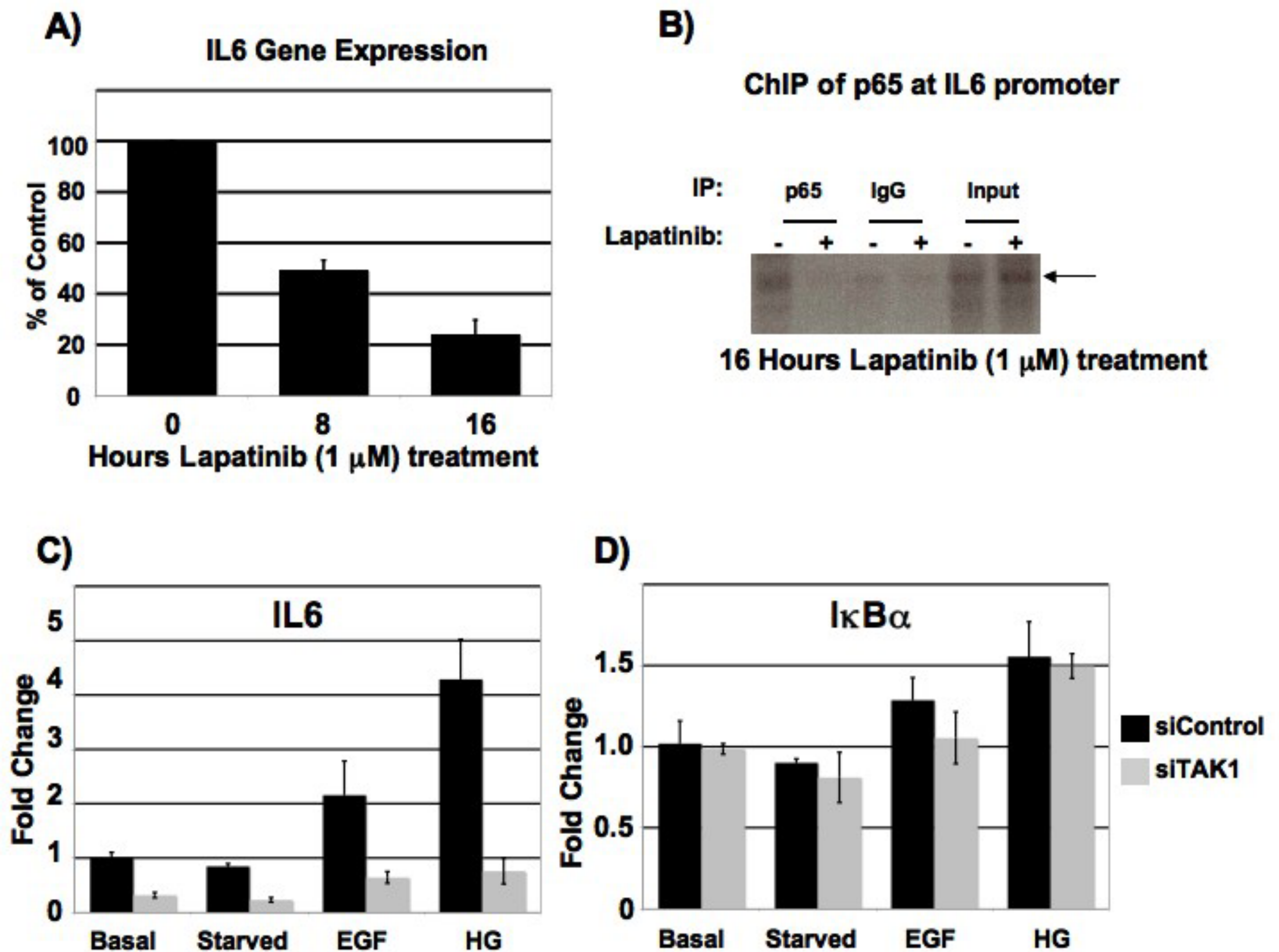


Figure 3.5. TAK1 is required for IL6 transcription in SKBr3 cells. A) SKBr3 cells were treated with 1 μM lapatinib for the indicated times and total RNA was isolated. Quantitative real-time PCR was performed to analyze IL6 transcript levels. IL6 transcript levels were normalized to GUSB transcript levels. Changes in IL6 transcript levels are indicated as percent change compared to vehicle treated cells. B) SKBr3 cells were treated with 1 μM lapatinib and chromatin immunoprecipitation was performed with an antibody to p65 (RelA). PCR was performed on immunoprecipitated chromatin with primers spanning two NF-κB binding sites in the IL-6 promoter. An arrow indicates the IL6 promoter PCR product. C and D) SKBr3 cells were transfected with 100 nM control or TAK1 siRNA and 48 hours post-transfection cells were treated with 100 ng/ml EGF or heregulin overnight. Total RNA was isolated and quantitative real-time PCR was performed to analyze IL6 transcript levels. IL6 transcript levels were normalized to GUSB transcript levels. Fold change in IL6 transcript levels is shown relative to basal control transfected cells.

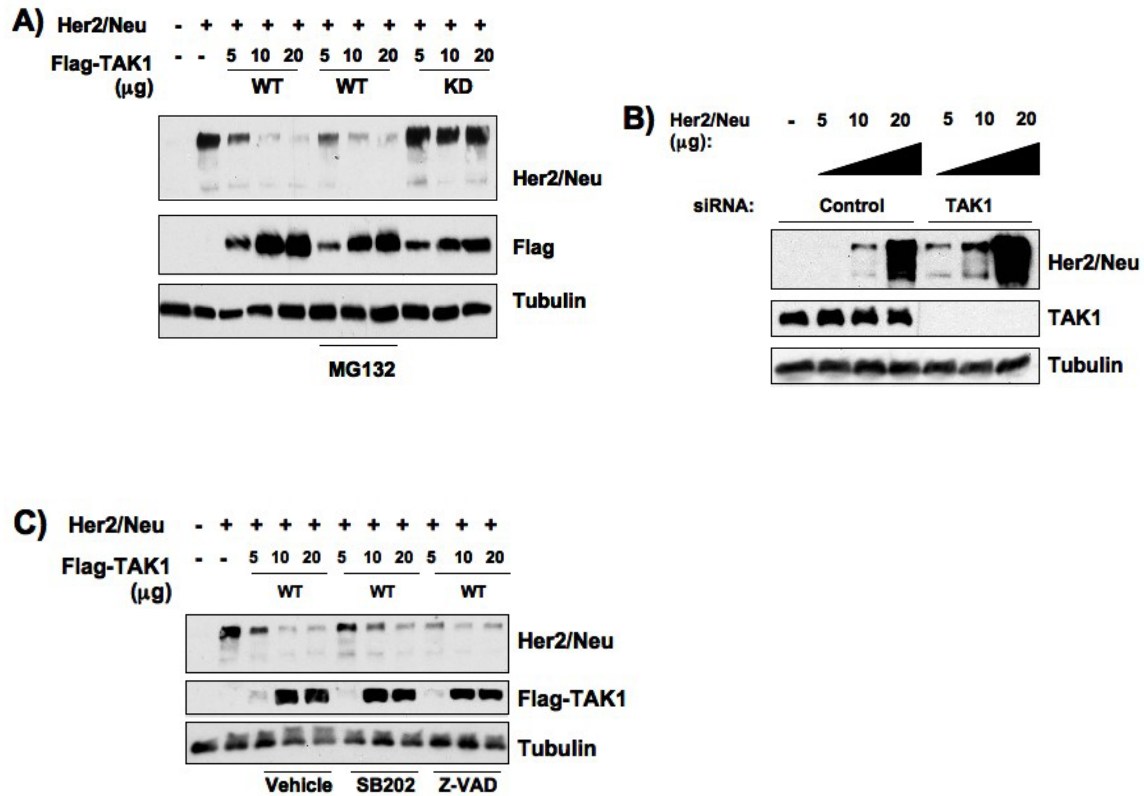
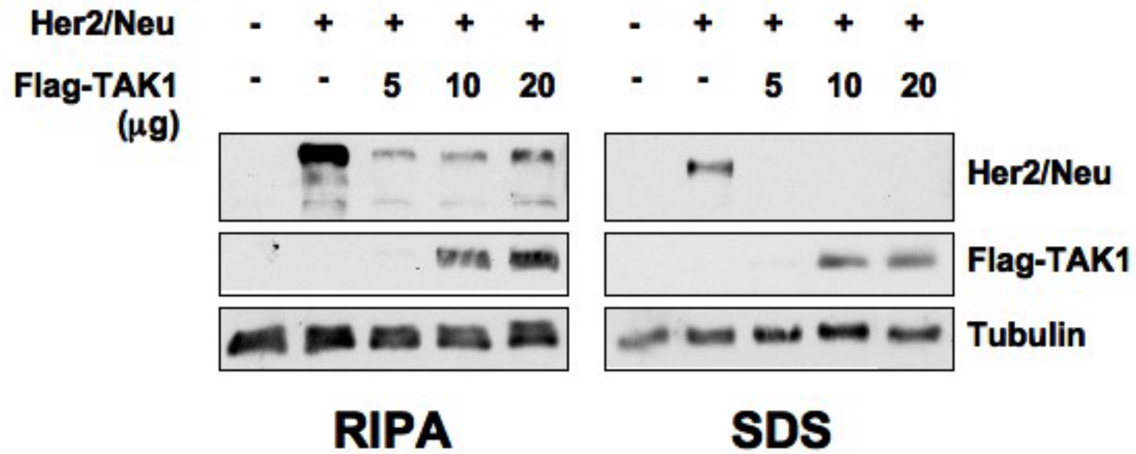
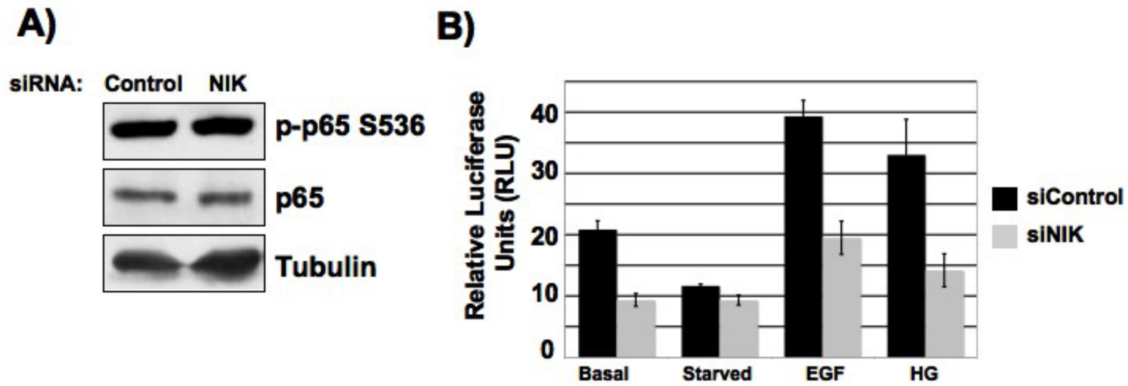


Figure 3.6. TAK1 expression downregulates Neu levels in a kinase-dependent, NF-κB and proteasomal independent manner. A) 293T cells were transfected with 5 µg of NeuNT and increasing amounts of wild-type (WT) or kinase-dead (KD) Flag-TAK1. Indicated samples were treated with 10 µM MG132 proteasome inhibitor for 12 hours. Whole cell lysates were prepared 48 hours post-transfection and western blot analysis was performed. B) 293T cells were transfected with 100 nM control or TAK1 siRNA, and transfected with increasing amounts of NeuNT 24 hours later. Whole cell lysates were prepared 48 hours after the second transfection and western blot analysis was performed. C) 293T cells were transfected with NeuNT and increasing amounts of Flag-TAK1. After 24 hours, cells were treated with 5 µM p38 inhibitor (SB202) or 10 µM caspase inhibitor (Z-VAD) for 12 hours. Whole cell lysates were prepared and western blot analysis was performed.



Supplemental Figure 3.1. Neu levels are decreased in RIPA and SDS cell lysates. 293T cells were cotransfected with 5 µg Neu and increasing levels of Flag-TAK1. Cells were lysed in RIPA (left) or SDS protein sample buffer (right) 48 hours post-transfection. Western blot analysis was performed.



Supplemental Figure 3.2. siRNA knockdown of NIK blocks transcriptional activation, but not phosphorylation, of NF-κB. A) SKBr3 cells were transfected with 100 nM control or NIK siRNA and whole cell lysates were prepared 72 hours post transfection. Western blot analysis was performed. B) SKBr3 cells stably expressing a 4x-κB luciferase reporter plasmid were transfected with 100 nM control or NIK siRNA. 48 hours post-transfection, cells were starved 6 hours and treated with EGF or heregulin for 12 hours. Cells were lysed in MPER and luciferase assays were performed according to manufacturer's instructions. Relative luciferase units were normalized to renilla.

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CHAPTER IV

NEGATIVE REGULATION OF SECRETED PROTEIN ACIDIC AND RICH IN CYSTEINES (SPARC) BY THE NF-κB PATHWAY

4.1 Abstract

Secreted Protein Acidic and Rich in Cysteines (SPARC) is a multifaceted protein involved in modulating cell-cell and cell-matrix interactions. As it is upregulated in some cancers while downregulated in others, SPARC may function as both a proto-oncogene and a tumor-suppressor. However, the molecular mechanisms involved in regulation of SPARC transcription are currently unclear. Here, we show that inhibition of the NF-κB pathway in both breast cancer and colorectal cancer cell lines leads to a significant increase in SPARC expression. Promoter analysis using a luciferase assay approach indicates that activation of the NF-κB pathway leads to significant repression of SPARC expression, and this repression occurs at a site proximal to the transcriptional start site of this gene. Similarly, activation of NF-κB blocked transcriptional activation of the promoter of another SPARC family gene, SMOC1. Effects of SPARC expression on invasion and cell proliferation were also investigated, though SPARC overexpression appeared to a have only a modest effect on these processes. This modulation of SPARC expression by NF-κB is likely due to an indirect effect, as there are no putative NF-κB binding sites in the promoter of this gene. These data suggest that NF-κB plays a role in the downregulation of SPARC through a mechanism occurring at a site proximal to the transcriptional start site of this promoter.

4.2 Introduction

Secreted protein, acidic and rich in cysteines (SPARC) (also known as osteonectin or BM-40) is a member of a family of secreted extracellular matrix proteins, which also includes thrombospondins 1 and 2, osteopontin, and tenascins C and X (Lane and Sage 1994; Sage 1997; Yan and Sage 1999). Originally identified as the bone-specific protein osteonectin (Termine et al. 1981), and later as a serum-albumin binding protein in endothelial cells (Sage, Johnson, and Bornstein 1984), this family of proteins can be divided into 3 separate modules based upon their function (Bradshaw and Sage 2001). While the primary physiological functions of SPARC involve modulating cell-cell and cell-matrix interactions, it also has de-adhesive and growth inhibitory properties (Tai and Tang 2008). SPARC has also been reported to be dysregulated in multiple types of cancer, and expression levels may play opposing roles depending upon the cancer type and the cell type in which it is underexpressed or overexpressed. SPARC has been reported to be overexpressed in gliomas and melanomas (Ledda et al. 1997; Rempel et al. 1998), while reduced SPARC expression has been seen in multiple types of cancer including ovarian (Yiu et al. 2001), colorectal (Cheetham et al. 2008; Yang et al. 2007), pancreatic (Puolakkainen et al. 2004) and acute myelogenous leukemia (DiMartino et al. 2006).

The role in which SPARC may play in breast cancer is not currently clear. SPARC has been shown to be able to inhibit breast cancer cell proliferation independent of metastasis (Dhanesuan et al. 2002), while a separate group has reported that overexpression of SPARC can inhibit colony formation and metastasis, while having no effect on cell proliferation, in the basal-subtype cell line MDA-MB-231 (Koblinski et al.

2005). However, expression of SPARC has been proposed to increase cell migration and invasion in an *in vitro* setting (Briggs et al. 2002). Furthermore, it has been reported that overexpression of the oncogene Her2 can lead to a decrease in SPARC gene expression (Mackay et al. 2003). Of note, it has also been reported that tissue specific expression of SPARC in the tumor microenvironment plays a critical factor in cancer progression (Arnold et al.; Podhajcer et al. 2008).

NF-κB is an important transcription factor that has been shown to be involved in expression of genes involved in key cellular processes including innate and adaptive immunity (Bonizzi and Karin 2004), cell proliferation and survival (Papa et al. 2006), lymphoid organ development (Weih and Caamano 2003), as well as being activated in a variety different cancers, including breast cancer (Basseres and Baldwin 2006; Belguise and Sonenshein 2007; Cogswell et al. 2000). The NF-κB family of transcription factors consists of five subunits: p65/RelA, RelB, c-Rel, p105/p50 and p100/p52. These subunits are evolutionarily conserved and exist as hetero- or homodimers (Hayden and Ghosh 2004). NF-κB is activated via two distinct pathways known at the canonical and non-canonical, or alternative, pathways. The canonical pathway is activated upon stimulus by growth factors or cytokines, such as TNF-α, which leads to activation of the IKKβ subunit of the Inhibitor of KappaB Kinase (IKK) complex, which phosphorylates the Inhibitor of KappaB alpha (IκBα), releasing p65/RelA containing dimers to translocate to the nucleus (Ghosh and Karin 2002). Furthermore, upon activation of the canonical pathway, the p65/RelA subunit can be phosphorylated at multiple residues, including serine 536, which is important for transactivation (Sakurai et al. 1999). Activation of NF-

κB in the non-canonical pathway, most common in B cells, involves Inhibitor of KappaB Kinase α (IKKα) and is IκBα-independent (Solt and May 2008).

NF-κB is a positive regulator of many genes, however, there is much interest recently in identifying genes which NF-κB may negatively regulate. It has recently been reported that NF-κB can directly downregulate antiapoptotic genes in response to cytotoxic agents through p65 (Campbell, Rocha, and Perkins 2004). The p50 subunit of NF-κB has also been shown to have a repressive effect as homodimeric complexes (Baer et al. 1998). Negative regulation of gene expression by NF-κB has also been reported to occur in an indirect manner. NF-κB-dependent transcription of the transcription factor YY1 can lead to transcriptional silencing of myofibrillar genes, and increased YY1 levels through NF-κB activation can lead to rhabdomysarcoma through dysregulation of mir-29 (Wang et al. 2007; Wang et al. 2008). Furthermore, NF-κB activation has been reported to block Bnip3 expression through antagonism of E2F-1 (Shaw et al. 2008).

In this study, we show that inhibition of NF-κB signaling in multiple cancer cell lines leads to an increase in expression of SPARC. Using a luciferase based approach, we show that this regulation of SPARC by NF-κB activation occurs through a mechanism at the promoter of SPARC, specifically in a region proximal to the transcription start site.

4.3 Materials and Methods

Cell Culture and Reagents

The tumor-derived SKBr3 and HCT-116 cell lines were maintained in McCoy's 5A medium (Mediatech) supplemented with 10% fetal bovine serum (FBS) and 100

units/mL penicillin/streptomycin. Immortalized human epithelial kidney cell line 293T was maintained in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% FBS and 100 units/mL penicillin/streptomycin. Tumor necrosis factor alpha (TNF-α) was purchased from Roche. 5-azadeoxycytidine was purchased from Sigma-Aldrich.

Plasmids

The 4x-κB luciferase reporter construct containing tandem NF-κB binding sites from the MHC class I promoter region was transfected into 293T cells with Polyethylenimine (PEI) at 4 μl per μg DNA. IKKα and IKKβ expression plasmids were constructed previously (Hutti et al. 2007). The full length SPARC promoter region spanning 1175 base pairs upstream to 72 base pairs downstream of the transcription start site was cloned from HeLa cell genomic DNA into pGL3-Basic (Promega) by restriction cloning using the following primers: forward
5' - CGG GGTACC CTAGCTAGCAGCTGGGTGTTGTGGCAT – 3', reverse 5' - CCC AAGCTT ACGCGTCGACCTCAGTGGCAGGCA – 3'. KpnI (forward) and HindIII (reverse) restriction sites were added to these previously published primers (Lopez et al. 2006). SPARC promoter deletion mutants were constructed using the following forward primers with KpnI restriction sites with the above SPARC reverse primer (+72):
Promoter 2 (-607 relative to TSS) 5'-CGGGGTACCGGTCCACCAGCTAGATGTC–3'
Promoter 3 (-345 relative to TSS) 5'-CGGGGTACCGGGCCTGGTTCTGCCCCTG-3'
Promoter 4 (-104 relative to TSS) 5'- CGGGGTACCGGCTATGGGAGAAGGAGGA GGC-3'. The full length SMOC1 promoter (-1421 to +89 relative to the TSS) was cloned using the following primers: forward 5' – CGGGGTACCGGGCCAGTGTG

GAGAGAGGGC - 3' , reverse 5' - CCCAAGCTTCATGAACCGCGCAGGAGGGG –3', containing KpnI (5') and HindIII (3') restriction sites. All promoter sequences were cloned using Platinum Taq polymerase (Invitrogen). The IκBα superrepressor described previously (Traenckner et al. 1995), under the control of the CMV promoter, was transfected with PEI as described previously. pSPORT6-SPARC expression plasmid was purchased from Origene.

Immunoblots

Whole cell extracts were prepared on ice with Mammalian Protein Extraction Reagent (Thermo Scientific) according to manufacturer's instructions supplemented with protease inhibitor mix (Roche, IN, USA) and phosphatase inhibitor mix (Sigma, MO, USA). Nuclear and cytoplasmic extracts were prepared as previously described (Mayo et al. 1997) . Protein concentrations were determined by Bradford assay (Biorad Laboratories) and SDS-PAGE analysis was performed as previously described (Steinbrecher et al. 2005).

Small RNA interference

The following small interfering RNAs (siRNA; siGenome SMARTpool) were obtained from Dharmacon as a pool of four annealed double-stranded RNA oligonucleotides: IKKα (M-003473-02), IKKβ (M-003503-03), and nontargeting control #3 (D001201-03). Cells were grown to approximately 50% confluency and transfected with 100 nmol/L siRNA with Dharmafect 1 reagent according to manufacturer's instructions.

Luciferase Assay

All 293T cells were transfected in triplicate with SPARC luciferase plasmid (full length or deletion mutant) or pGL3-Basic vector (100 ng) and pRL-CMV (Promega) (1 ng) for 48 hours in 24-well dishes. 293T cells were cotransfected with IKKα, IKKβ or vector plasmids (500 ng) described above for 48 hours, or treated with TNF-α (10 ng/ml) (Promega) overnight. Cells were cotransfected with IκBα superrepressor or vector described above (500 ng) for 48 hours. Cells were lysed in Mammalian Protein Extraction Reagent (MPER) (Thermo) and luciferase activity was measured with Promega Dual Luciferase Assay System (Promega). Luciferase activity was normalized as luciferase units divided by renilla units and normalized as fold change versus control treated or vector transfected cells.

Quantitative Real-time PCR

Total RNA extracts were obtained from cells approximately 72 hours post-transfection by Trizol (Invitrogen) extraction. Two micrograms of RNA was reverse transcribed using random primers and MMLV-reverse transcriptase (Invitrogen). Real-time PCR was performed and analyzed as previously described (Steinbrecher et al. 2005) using Taqman Gene Expression Assay primer-probe sets GUS (Hs99999908_m1) and SPARC (Hs00277762_m1).

Cell invasion assay

Innocyte™ Cell Invasion Assay Kit was purchased from Calbiochem (San Diego, California). Cells were transfected with siRNA for 48 hours before seeding. Invasion

assay was performed as per manufacturer's protocol for 48 hours. The number of invading cells was measured fluorometrically with Calcein AM.

Cell Proliferation Assay

Cell proliferation assay was performed as previously described (Wilson and Baldwin 2008). Cells were transiently transfected with siRNA to IKKα and/or transfected with pSPORT6-SPARC and measured at the indicated time points post-transfection.

4.4 Results

Inhibition of NF-κB blocks induces transcription of SPARC

As it has been previously reported that overexpression of Her2 can suppress expression of SPARC (Mackay et al. 2003), and Her2 overexpression can lead to activation of NF-κB through upstream kinases IKKα and IKKβ (Merkhofer, Cogswell, and Baldwin 2009), we sought to investigate the possible role the NF-κB pathway may play in the regulation of transcription of this gene. Her2-overexpressing breast cancer SKBr3 cells were transfected with siRNA against IKKα or IKKβ and transcript levels of SPARC were analyzed by quantitative real-time PCR. siRNA inhibition of IKKα led to a significant increase in SPARC transcript levels, while inhibition of IKKβ led to a slight decrease in SPARC transcription (Fig. 4.1 left). We next investigated whether this inhibition of SPARC transcription was limited to Her2-overexpressing breast cancer cells. The colorectal carcinoma cell line HCT-116 has been shown to have decreased SPARC levels due to hypermethylation at its promoter (Cheetham et al. 2008). HCT-116 cells were transfected with siRNA against IKKα and IKKβ, and SPARC transcript levels

were measured. Similar to the SKBr3 cell line, IKKα knockdown led to a significant increase in SPARC transcript levels, while IKKβ knockdown had no effect on SPARC transcript levels (Fig. 4.1 right).

Activation of the NF-κB Pathway Inhibits Transcriptional Activation of the SPARC Promoter

In order to determine the role NF-κB may play on transcriptional activation of the SPARC promoter, the full-length promoter sequence of SPARC was cloned into the pGL3-Basic luciferase plasmid (Lopez et al. 2006). This promoter sequence consists of a 1.2 kb genomic fragment -1175 to +72 relative to the transcription start site (Fig. 4.2A). As a control, 293T cells were transfected with an NF-κB luciferase reporter plasmid and treated with TNF-α, a strong NF-κB transcriptional activator, for 12 hours. TNF-α treatment led to an approximately 45-fold increase in transcriptional activation, and this induction was completely blocked by cotransfection of a dominant-negative inhibitor of the NF-κB pathway, IκBα superrepressor (Fig. 4.2B left). Conversely, transfection of the aforementioned SPARC promoter luciferase construct, and concurrent treatment with TNF-α, led to an approximate 2-fold decrease in transcription from the SPARC promoter as compared to vehicle treated cells (Fig. 4.2B right). Furthermore, cotransfection with the IκBα superrepressor construct blocked TNF-α induced repression of transcription from this promoter, as well as increasing transcription above basal levels in vehicle treated cells. This suggests that TNF-α treatment blocks transcription from the SPARC promoter, and inhibition of the NF-κB pathway can block TNF-α induced transcriptional

repression, as well as basal NF-κB-mediated transcriptional repression, from the SPARC promoter.

The role in which NF-κB activation may repress SPARC transcription was further investigated by measuring transcriptional repression upon overexpression of the IKKα and IKKβ kinases. Upon overexpression of IKKα or IKKβ in 293T cells, transcriptional activation of the 4x-κB luciferase construct was increased approximately 70- and 60-fold, respectively, and this induction was completely abrogated by cotransfection with IκBα-superrepressor (Fig. 4.2C left). However, overexpression of IKKα or IKKβ led to an approximate 6- and 3-fold decrease in transcriptional activation from the SPARC promoter (Fig. 4.2C right). Repression of transcriptional activation by IKK was dependent upon its catalytic activity, as kinase dead IKKα or IKKβ were unable to block SPARC promoter transcription (data not shown). This repression of transcriptional activation was suppressed by cotransfection of the IκBα-superrepressor as well, suggesting that IKK overexpression blocks transactivation at the SPARC promoter through an IκBα-dependent mechanism. Taken together, these results show that activation of the NF-κB pathway by TNF-α or overexpression of IKK negatively regulates transcriptional activation of SPARC through a mechanism at the promoter of this gene.

Activation of the NF-κB Pathway Blocks SPARC Expression Proximal to the Transcriptional Start Site of SPARC

In order to determine the minimal promoter region of SPARC responsible for the NF-κB mediated repressive effect on transcription, we constructed multiple truncations

of the SPARC promoter. Three progressive 5' truncations (SPa, SPb, and SPc) of the full promoter sequence were cloned into the pGL3-luciferase plasmid (Fig. 4.3A). Baseline promoter activity of these truncated mutants was similar to that of the full length promoter. Furthermore, overexpression of IKKα or IKKβ had a virtually identical effect on transcriptional activation of this promoter, significantly blocking transcriptional activation. Luciferase activity of shortest promoter mutant, containing a sequence -104 to +72 base pairs relative to the transcriptional start site, was significantly blocked by IKK overexpression or TNF-α treatment, and this repression was completely abrogated by cotransfection of the IκBα-SR plasmid (Fig. 4.3B). Taken together, these results suggest that NF-κB repression of SPARC transcriptional activation occurs at a highly conserved proximal region of the SPARC promoter, between 104 base pairs upstream and 72 base pairs downstream of the transcriptional start site.

Activation of NF-κB blocks transcriptional activation of SPARC family member SMOC1

A previous screen performed to identify genes upregulated or downregulated by NF-κB in Her2-overexpressing breast cancer cells identified SMOC1 as a putative NF-κB regulated gene (data not shown). Characterization of SMOC1 identified calcium-binding domains in the N-terminal portion of the protein, homologous to that of SPARC (Vannahme et al. 2002). Subsequent studies have shown SMOC1 to be important in development and differentiation (Gersdorff et al. 2006; Thomas et al. 2009), and may play a role in some cancers (Boon et al. 2004). Knockdown of IKKα in SKBr3 cells led to an increase in SMOC1 transcript levels, similar to that seen with SPARC (data not shown). A SMOC1 promoter sequence was cloned into the pGL3-luciferase plasmid,

consisting of a sequence -1421 to +89 relative to the transcriptional start site. Similar to results seen with the SPARC promoter, treatment with TNF-α or overexpression of IKK blocked transcriptional activation from the SMOC1 promoter, and this repression was rescued by cotransfection of the IκBα-superrepressor (Fig. 4.4 and data not shown).

Inhibition of DNA methylation induces SPARC expression independent of NF-κB repression

Promoter methylation is an epigenetic mechanism of regulating gene expression primarily through modification of chromatin structure, effectively silencing gene transcription (Jaenisch and Bird 2003; Weber et al. 2007). It has been reported that methylation of the SPARC promoter leads to downregulation of its expression in ovarian and colorectal cancers, including the HCT-116 cell line (Socha et al. 2009; Cheetham et al. 2008). Therefore, we investigated whether activation of the NF-κB pathway was inducing methylation of the SPARC promoter. The cancer therapeutic drug 5-azadeoxycytidine has been shown to reduce promoter methylation through inhibition of DNA methyltransferases (DNMTs) (Murgo 2005). 293T cells were pretreated with vehicle control, 2.5 μM, 5 μM or 10 μM 5-azadeoxycytidine and transfected with the SPARC promoter plasmid SPc and IKKα or IKKβ (see materials and methods). Treatment with 5-azadeoxycytidine led to a dose-dependent significant increase in promoter activation, consistent with previously published results (Fig. 4.5). However, overexpression of IKKα was still able to significantly block promoter activation, suggesting that NF-κB mediated SPARC repression is not through a DNA methylation mechanism.

Overexpression of SPARC does not inhibit invasion or cell proliferation in SKBr3 and HCT-116 cell lines

As previously noted, modulation of SPARC expression has been reported to affect different cancer phenotypes. Therefore, we next investigated if overexpression of SPARC has an effect on the invasive phenotype or on cell proliferation of SKBr3 or HCT-116 cell lines. We have previously shown that siRNA knockdown of IKKα in SKBr3 cells can inhibit invasion (Fig. 2.6C). SKBr3 cells and HCT-116 cells were cotransfected with control or IKKα siRNA and vector or SPARC plasmid. Western blot analysis was performed to confirm loss of IKKα levels and/or increase in SPARC protein levels (Fig. 4.6A). 24 hours post transfection, cells were subjected to an invasion assay for 48 hours, measuring the ability of cells to degrade a basement membrane matrix, a hallmark of invasive capability of cancerous cells. As seen previously, knockdown of IKKα blocked invasion in the SKBr3 breast cancer cell line, while overexpression of SPARC had only a slight effect on invasion (Fig. 4.6B). A combination of IKKα knockdown and SPARC overexpression revealed no additive or synergistic effect on invasion. In the HCT-116 cell line, IKKα knockdown led to a modest decrease in invasion, while overexpression of SPARC only slightly decreased invasion. Similar to the SKBr3 cell line, a combination of IKKα knockdown and SPARC overexpression showed no additive or synergistic effect on invasion. While effects of SPARC overexpression in invasion are modest, this needs to be repeated before these differences can be deemed significant. We also investigated the possible role of NF-κB and SPARC in cell proliferation in the HCT-116 cell line by MTS assay (Fig. 4.6C). Overexpression

of SPARC in HCT-116 cells had no effect on cell proliferation. However, siRNA knockdown of IKKα block proliferation in this cell line. This is contrast to the SKBr3 cell line, where IKKα knockdown slightly increased cell proliferation (Fig. 2.6B).

4.5 Discussion

The identification of genes positively and negatively regulated by the NF-κB pathway is of great importance in a cancer setting. Dysregulation of NF-κB regulated genes has been implicated in many aspects of cancer initiation and progression (Karin et al. 2002), it plays a role in resistance to chemotherapy and radiation (Wang, Mayo, and Baldwin 1996; Wang et al. 1999), and may be involved in developed resistance to targeted therapies, such as trastuzamab and lapatinib (Chen, Xia, and Spector 2008; Xia et al.; Flowers and Thompson 2009). Here, we present evidence that NF-κB can negatively regulate the expression of Secreted Protein, Acidic and Rich in Cysteines (SPARC), whose dysregulation has been linked to many cancers, including breast cancer (Dhanesuan et al. 2002; Koblinski et al. 2005).

Inhibition of IKKα, but not IKKβ, induced SPARC expression in the Her2-overexpressing breast cancer cell line SKBr3, as well as in the colorectal cancer cell line HCT-116 (Fig. 4.1). Conversely, overexpression of IKKα or IKKβ was able to block transcriptional activity from the SPARC promoter, and this repression was rescued by expression of a dominant negative form of IκBα (Fig. 4.2). This suggests that NF-κB mediated repression of SPARC likely occurs via the canonical NF-κB pathway. The canonical pathway has long been thought to require IKKβ, with IKKα playing a predominant role in the non-canonical pathway. However, recent reports have

demonstrated the requirement of IKKα in the canonical pathway downstream of specific stimuli (Solt et al. 2007). Our group has recently shown that IKKα plays an important role in activation of NF-κB downstream of Her2-overexpression in SKBr3 cells (Merkhofer, Cogswell, and Baldwin 2009). To our knowledge, the role of IKKα in the HCT-116 cell line has not been investigated. Therefore, it is plausible that preferential activation of IKKα in these cell lines may regulate expression of specific genes. Studies have shown that activation of IKKα and IKKβ can lead to changes in expression of different subsets of NF-κB regulated genes (Gloire et al. 2007; Massa et al. 2005). Of note, Her2-ovexpression has been reported to repress SPARC gene expression (Mackay et al. 2003).

Analysis of truncated mutants of the SPARC promoter indicates that NF-κB regulates expression of SPARC at a region proximal to the transcriptional start site (Fig. 4.3). Analysis of the full length SPARC promoter (Lopez et al. 2006) was performed using the University of Santa Cruz Genome Browser (v224), and the position of the full-length luciferase promoter sequence and the shortest (SPc) promoter sequence are indicated by blue and green bars, respectively, above the genomic location (Fig. S4.1). Analysis of this promoter sequence shows very high conservation across species within the SPc promoter sequence, including the 5' untranslated region, while little conservation is present upstream of the SPc promoter region, consistent with conservation of elements in the 5' end of the SPARC gene reported in *Xenopus* (Damjanovski et al. 1998). It is therefore not surprising that important transcription regulatory elements may be contained in this highly conserved region. In fact, it has been reported that the transcription factor Sp1 and/or Sp3 repress transcription of SPARC through binding in

this conserved promoter region through activation of v-Jun (Chamboredon et al. 2003). Interestingly, another group has shown that overexpression of c-Jun induces SPARC expression, possibly through Sp1/Sp3 binding in this same region (Briggs et al. 2002). Though not much is known about other transcription factors involved in SPARC expression, SOX-5 (Huang et al. 2008) and AP-1(Vial, Perez, and Castellazzi 2000) have been reported to downregulate SPARC expression. Analysis of the SPARC promoter using the TRANSFAC database revealed no putative NF-κB binding site in this region. Chromatin immunoprecipitation analysis of the promoter also did not suggest direct NF-κB binding at the SPARC promoter (data not shown). However, it is possible that NF-κB may be involved with indirect binding at the SPARC promoter, similar to that reported with c-Jun and Sp1/Sp3.

Decreased expression of SPARC due to promoter methylation has been reported in multiple cancers (Suzuki et al. 2005; Cheetham et al. 2008; Socha et al. 2009; DiMartino et al. 2006). It has been reported that the nonsteroidal anti-inflammatory drug NS398 can reactivate SPARC expression through promoter demethylation (Pan et al. 2008). This drug, a COX-2 inhibitor, has been shown by multiple groups to inhibit NF-κB (Liu et al. 2009; Chen et al. 2006). These data suggested that inhibition of SPARC expression by NF-κB may be through a mechanism involving promoter methylation. Treatment with 5-azadeoxycytidine, a DNA methyltransferase inhibitor, did in fact increase transcriptional activation of the SPARC promoter, however, activation of the NF-κB pathway by IKKα overexpression was still able to dramatically block SPARC expression with 5-azadeoxycytidine treatment (Fig. 4.5). It therefore seems unlikely that the mechanism of NF-κB repression occurs through promoter methylation.

In this study, we also investigated the role SPARC expression may play in cancer phenotypes. SKBr3 and HCT-116 cells were transfected with a combination of siRNA to IKKα and/or a SPARC expression plasmid and subjected to an invasion assay. Though overexpression of SPARC has previously been reported to block the invasive phenotype of basal breast cancer cells (Koblinski et al. 2005), overexpression of SPARC only modestly decreased invasion in SKBr3 and HCT-116 cells, while knockdown of IKKα led to a much more significant decrease in invasion (Fig. 4.6B). A combination of IKKα knockdown and SPARC overexpression did not lead to a further decrease in invasion. We have previously shown that IKKα regulates the expression of multiple genes implicated in the invasive phenotype of different cancers; therefore, it is likely that modulating expression of any one gene may not be sufficient to block tumor cell invasion. We also measured the levels of cell proliferation in HCT-116 transfected with siRNA to IKKα and/or overexpressing SPARC. Interestingly, unlike SKBr3 cells where knockdown of IKKα leads to a slight increase in cell proliferation (Fig. 2.6), knockdown of IKKα led to a significantly decreased number of cells in the HCT-116 cell line after 4 days (Fig. 4.6C). Overexpression of SPARC had no effect on cell proliferation in this cell line. The different roles IKKα plays in cell proliferation in these cell lines needs further investigation, though it may be due to differential activation of the PI3K pathway in these cell lines. Taken together, this suggests that SPARC likely does not play an important role in cell proliferation, and, though it may be involved in invasion in these cell lines, expression of SPARC alone is likely not sufficient to block this invasive phenotype.

In this study, we present data showing that activation of the NF-κB pathway blocks expression of SPARC, a gene shown to play an important role in multiple different types of cancer. Interestingly, this regulation of SPARC expression occurs at a small stretch of the promoter proximal to the transcription start site, though the mechanism of this regulation is currently unclear. Although we did not elucidate a significant biological role for SPARC in these cancer cell lines, further research will be necessary to determine its role in cancer pathogenesis.

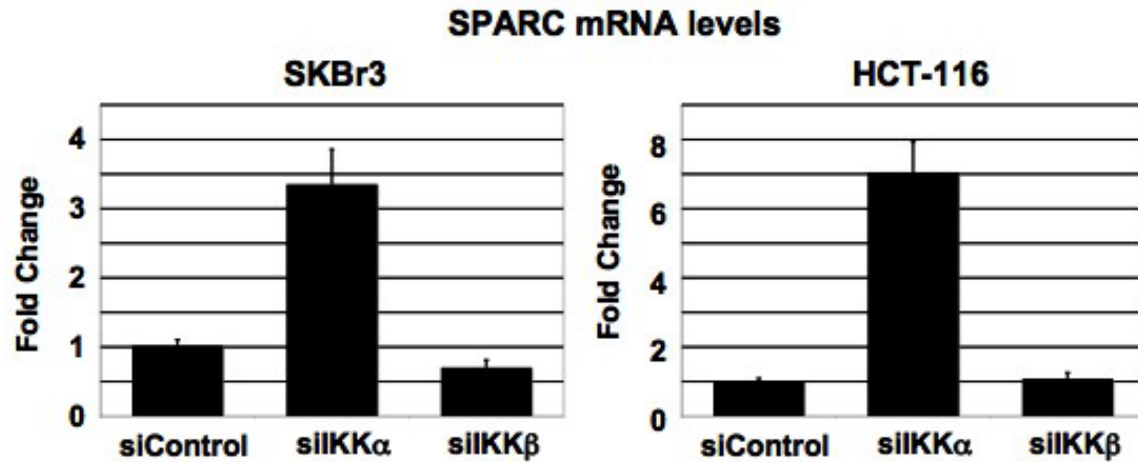


Figure 4.1. siRNA inhibition of IKKα, but not IKKβ, induces SPARC expression. SKBr3 breast cancer cells (left) and HCT-116 colorectal cancer cells (right) were transfected with 100 nM control, IKKα or IKKβ siRNA and total RNA was isolated 72 hours post-transfection. Quantitative real-time PCR analysis was performed to measure SPARC transcript levels. SPARC transcript levels were normalized to GUSB levels. SPARC transcription is shown as fold change compared to control siRNA transfected cells.

Figure 4.2. Activation of the NF-κB pathway blocks transcriptional activation of the SPARC promoter. A) The full-length SPARC promoter spanning 1175 base pairs upstream to 72 base pairs downstream of the transcriptional start site was cloned into the pGL3-luciferase plasmid. B) The 4x-κB luciferase construct (left) or the pGL3-luciferase plasmid containing the full-length SPARC promoter (pGL3-SPARC) (right) was transfected into 293T cells and cells were treated with 100 ng/ml TNF-α for 12 hours. Cells were also cotransfected with vector (black bars) or IκBα superrepressor (gray bars). Cells were lysed in MPER and luciferase assays were performed according to manufacturer's instructions. Luciferase levels are shown as fold change of relative luciferase units (luciferase/renilla) compared to vehicle treated cells. C) 4x-κB luciferase construct (left) or pGL3-SPARC (right) was cotransfected into 293T cells with GST-IKKα, GST-IKKβ or vector. Cells were also cotransfected with vector (black bars) or IκBα superrepressor (gray bars). Cells were lysed in MPER 48 hours post-transfection and luciferase assays were performed according to manufacturer's instructions. Luciferase levels are shown as fold change of relative luciferase units (luciferase/renilla) compared to vector transfected cells.

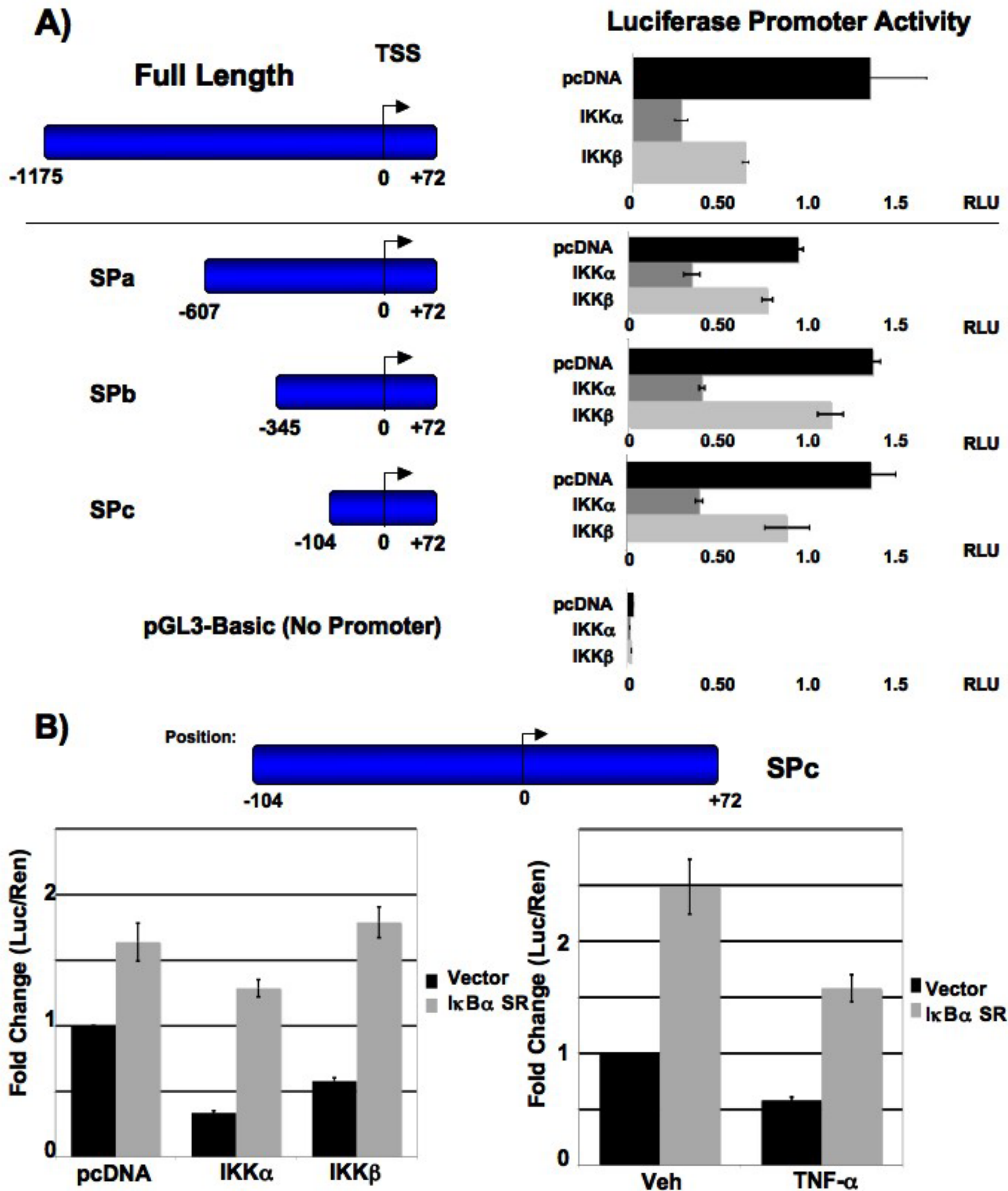


Figure 4.3. Activation of the NF-κB pathway blocks transcriptional activation of SPARC proximal to the transcriptional start site. A) N-terminal truncation mutants of the SPARC promoter were constructed and cloned into the pGL3-luciferase plasmid. Luciferase plasmids containing full-length SPARC promoter or truncated SPARC promoters (SPa, SPb, SPc) were cotransfected with IKKα, IKKβ or vector. Cells were lysed in MPER 48 hours post-transfection and luciferase assays were performed

according to manufacturer's instructions. Luciferase activity is shown as relative luciferase units (RLU). B) Luciferase reporter plasmid containing the shortest SPARC promoter mutant, SPc, was cotransfected with IKK (left) or treated with TNF-α (right). Cells were also cotransfected with vector (black bars) or IκBα superrepressor (gray bars). Cells were lysed 48 post-transfection and luciferase assays performed. Luciferase activity is shown as fold-change compared to vector transfected (left) or vehicle treated (right) cells.

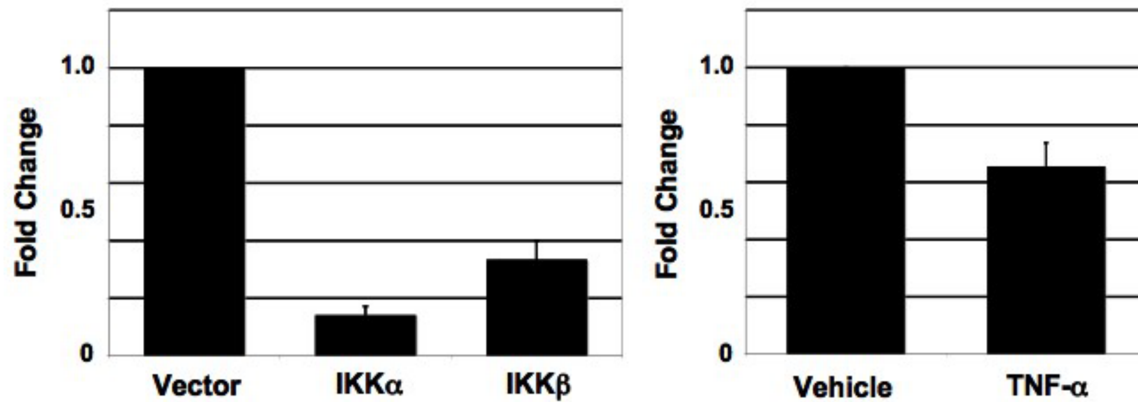


Figure 4.4. Activation of NF-κB blocks SMOC1 promoter transcriptional activation. 293T cells were transfected with pGL3-luciferase construct containing SMOC1 promoter sequence. Cells were cotransfected with IKK plasmids (left) or treated with TNF-α (right) to activate NF-κB. Cells were lysed in MPER 48 hours post-transfection and luciferase assays were performed according to manufacturer's protocol. Luciferase levels are shown as fold change versus vector transfected or vehicle treated cells.

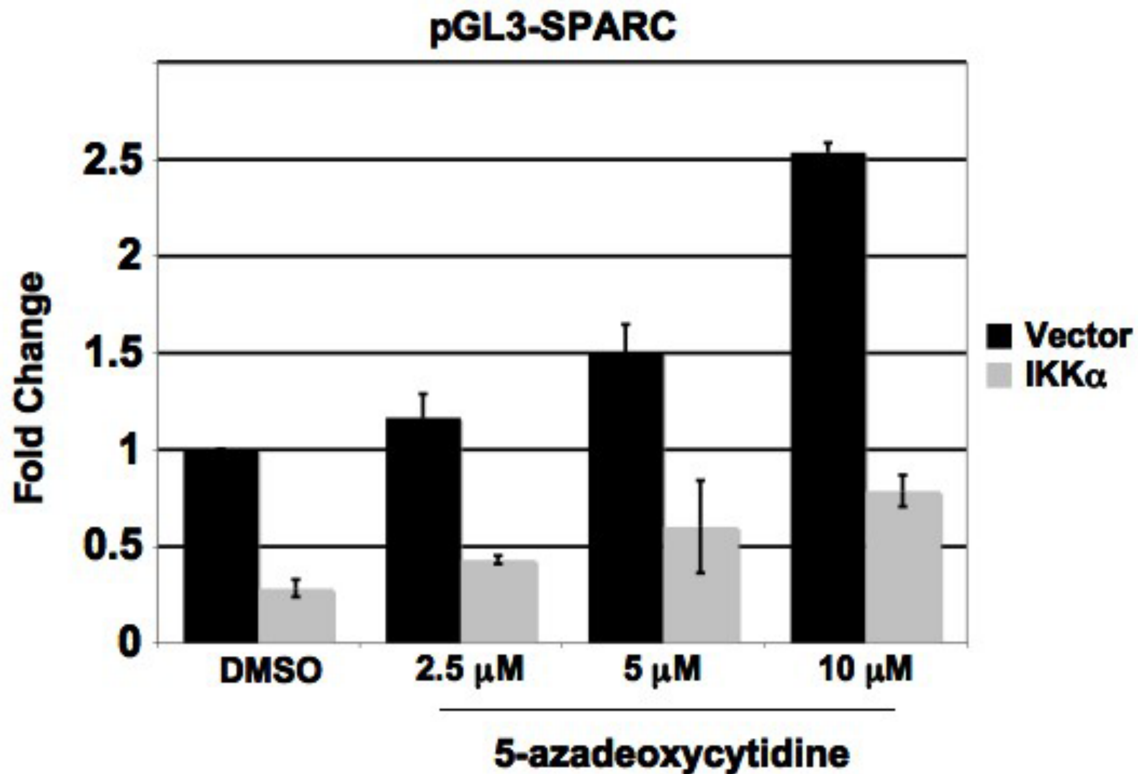


Figure 4.5. DNA-methyltransferase inhibitor 5-azadeoxycytidine induces SPARC promoter transcriptional activation. 293T cells were transfected with vector or GST-IKKα plasmid. 24 hours later, cells were treated with the indicated concentrations of 5-azadeoxycytidine for 24 hours and cells were lysed in MPER lysis buffer. Luciferase assays were performed as per manufacturer's instructions. Luciferase levels are shown as fold change versus vehicle (DMSO) treated cells.

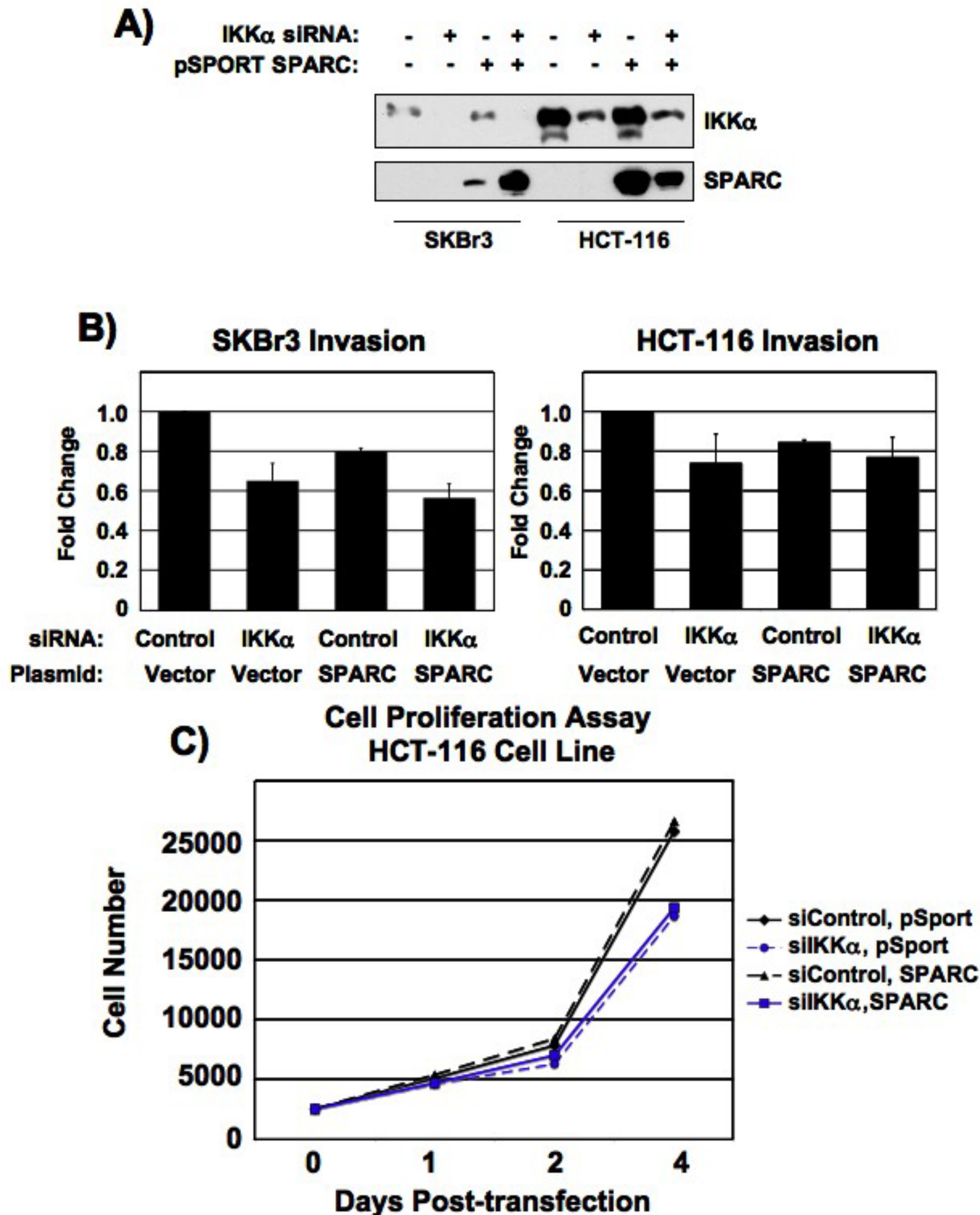
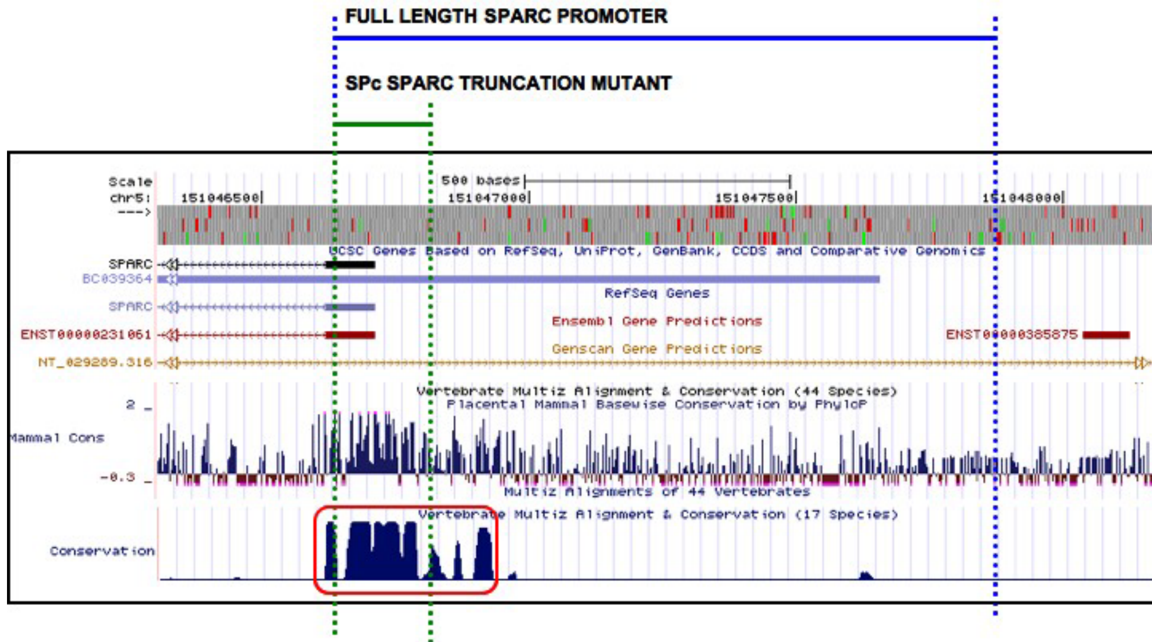


Figure 4.6. SPARC overexpression does not significantly affect invasion or cell proliferation. A) SKBr3 and HCT-116 cells were transfected with a combination of siRNA to IKKα and/or a SPARC overexpression plasmid. Western blot analysis was performed on whole cell lysates to confirm knockdown/overexpression. B) SKBr3 and HCT-116 cells were transfected and seeded in an invasion assay 24 hours post-transfection. Cell number was measured fluorometrically after 48 hours. C) Cell proliferation of HCT-116 cells transfected with siRNA and/or SPARC was measured for 4 days post-transfection using MTT assay. Cell number was calculated using a standard curve.



Supplementary Figure 1. The shortest SPARC promoter mutant, SPc, contains highly conserved promoter sequences. The full-length SPARC promoter was analyzed using the University of California Santa Cruz web browser to identify regions of conservation in the promoter. The full-length SPARC promoter is indicated by the blue line, the shortest SPARC promoter mutant, SPc, is indicated by the green line. The red rectangle indicates high levels of conservation of the promoter across 17 species.

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CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Conclusions and Future Directions

Since Her2 was identified as an oncogene almost 30 years ago, tremendous gains have been made in the elucidation of the molecular pathways downstream of Her2, as well as in the treatment of this disease (Shih et al. 1981; Olayioye 2001). The development of monoclonal antibodies such as trastuzamab (Herceptin) and small molecule inhibitors such as lapatinib (Tykerb) have dramatically increased life expectancy and quality of life of people with this disorder (Blackwell et al.). However, due to acquired resistance to these therapies (Chen, Xia, and Spector 2008; Slamon et al. 2001), it has become imperative to discover new possible targets for therapy. Since the time when NF-κB was initially discovered to be activated by Her2 (Galang et al. 1996), there have been many studies illuminating the mechanisms of activation of NF-κB downstream of Her2, as well as the critical role this transcription factor plays in the progression of this disease. However, many questions remain regarding the role of NF-κB in Her2-overexpressing breast cancer. In the previous chapters, we shed light on how members of the NF-κB signaling pathway are involved in its activation, the effect of NF-κB regulated gene expression downstream of Her2, and the biological consequences of this activation.

The Role of IKKα in Her2-mediated Activation of NF-κB

Here, we present data implicating the kinase IKKα in NF-κB activation downstream of Her2. These data represent part of a paradigm shift regarding the role of the IKK kinases in NF-κB activation. Since the discovery of the central role of IKKα in regulating the non-canonical pathway (Senftleben et al. 2001), it has been generally

accepted that this kinase is not required for activation of the canonical pathway. However, recent studies have revealed that IKKα may be required for activation by IL-1 and TNF-α (Solt et al. 2007, Adli et al. in press). Our results show that IKKα is required for activation of NF-κB downstream of Her2, consistent with a previous report implicating IKKα in self renewal of tumor initiating cells in Her2 breast cancer (Cao, Luo, and Karin 2007). Given the role of IKKα in regulation of such diverse cellular processes as osteoclast differentiation (Chaisson et al. 2004), endothelial cell motility and tumor angiogenesis (DeBusk, Massion, and Lin 2008), keratinocyte differentiation (Descargues et al. 2008), as well as mammary gland development (Cao et al. 2001), it will be of great importance to investigate the role IKKα may play in many other cancers through activation of the canonical NF-κB pathway. The development of specific inhibitors to IKKα will also be of tremendous use in the investigation of NF-κB in cancer. The specific IKKβ inhibitor Compound A had no effect on the survival or invasion of Her2-overexpressing breast cancer cells at lower concentrations (data not shown). However, this inhibitor has been reported to inhibit IKKα at higher concentrations (Ziegelbauer et al. 2005). At these higher concentrations, we have seen inhibitory effects on Her2-overexpressing breast cancer cells. These data suggest that the development of IKKα-specific inhibitors may be efficacious for therapy in Her2-overexpressing breast cancer. Interestingly, a study has shown that the proteasomal inhibitor Bortezomib (PS-341, Velcade) increases the efficacy of trastuzamab in Her2-overexpressing breast cancer cells (Cardoso et al. 2006), further supporting the idea that NF-κB inhibitors may play an important role in treatment of Her2-overexpressing breast cancer.

The Role of TAK1 in Her2-mediated Activation of NF-κB

We also present evidence in this report that the mitogen-activated protein kinase family member TAK1 is required for Her2 activation of NF-κB. Knockdown of TAK1 blocks NF-κB phosphorylation and transcriptional activation, as well as inhibiting expression of IL-6, a gene reported to play an important role in breast cancer progression (Studebaker et al. 2008; Ndlovu et al. 2009). A recent study has reported that combinatorial therapy involving gemcitabine and a specific TAK1 inhibitor reduced tumor volume and increased survival in a mouse model of pancreatic cancer (D. Melisi, Abstract 1002, ECCO15-ESMO34). The TAK1 inhibitor 5Z-7 Oxozeaenol blocked NF-κB activation in a dose-dependent manner in the SKBr3 Her2-overexpressing breast cancer cell line (Fig. 3.1), suggesting TAK1 may be a possible therapeutic target for this type of cancer. Further studies involving modulating TAK1 in Her2-overexpressing breast cancer are warranted, using both *in vitro* methods to measure effects on cell proliferation and invasion, as well as in *in vivo* mouse models of Her2$^+$ breast cancer.

Possible Mechanisms for Her2-mediated TAK1 Activation of IKKα

TAK1 can bind to both IKKα and IKKβ, leading to activation of NF-κB (Takaesu et al. 2003). Furthermore, TAK1 activation of IKKα through phosphorylation of NIK upon IL-1 stimulation was reported in 1999 (Ninomiya-Tsuji et al. 1999). Our data showing IKKα plays such an important role in NF-κB activation in Her2-overexpressing breast cancer cells indicates Her2-overexpression leads to activation of IKKα. However, the mechanism for this activation is currently unclear. NIK knockdown in Her2-overexpressing breast cancer cells blocks NF-κB transcriptional activation and IL-6

induction, yet does not affect p65 phosphorylation at serine 536 (Fig. S4.3). Therefore, it is possible that NIK is involved in IKKα activation of NF-κB downstream of Her2; this requires further investigation. It is also unclear which signaling proteins are required for TAK1 activation by Her2. The E3-ligases TRAF2 and TRAF6 are required for TNF-α and IL-1 induction of NF-κB activation, respectively (Chen 2005; Deng et al. 2000; Wang et al. 2001). Comprehensive genetic studies will be essential to determine the roles of these adaptor proteins in Her2 activation of TAK1.

TAK1 Regulation of Her2/Neu Protein Levels

An interesting new finding presented in this report shows that TAK1 can downregulate Her2 levels through its kinase activity (Fig. 3.6). Previous studies have shown that the extracellular domain of Her2 can be cleaved, resulting in a constitutively active 95 kDa C-terminal fragment, which has been implicated in therapeutic resistance (Liu et al. 2006; Chandarlapaty et al.). However, the fact that no cleavage products of Her2 were seen, and that proteasome and caspase inhibitors did not block TAK1 downregulation, suggest that this result is likely through a different mechanism. Interestingly, though expression of TAK1 downregulates exogenous activated Her2, and siRNA inhibition of TAK1 stabilizes exogenous activated Her2, in non-transformed 293T human embryonic kidney cells, siRNA knockdown of TAK1 does not affect total Her2 levels in the tumor derived SKBr3 cell line (Fig. 3.2). Therefore, the mechanism by which TAK1 modulates levels of Her2/Neu may differ depending on the malignant state of the cell. Intriguingly, a recent report has shown that in non-transformed cells, TAK1 can block NF-κB activation, but in a breast cancer cell line, TAK1 is able to activate NF-

κB through its interaction with TAB1, downstream of TGF-β (Neil and Schiemann 2008). We are currently investigating if TAK1 regulation of Her2 levels may occur through a similar mechanism.

Her2-mediated Activation of NF-κB and the PI3K/Akt Pathway

The PI3K-Akt pathway is activated by Her2 overexpression in breast cancer cell lines (Ignatoski et al. 2000; Berns et al. 2007), and is a promising target for therapy in multiple different cancers (Paz-Ares et al. 2009). A role of the PI3K-Akt pathway in the activation of NF-κB has been reported in multiple cancers (Ozes et al. 1999; Romashkova and Makarov 1999), while activation of NF-κB is independent of PI3K in other malignancies (Vermeulen et al. 2002). Here, we present data showing that NF-κB activation in Her2-overexpressing breast cancers is independent of PI3K. While inhibition of the NF-κB pathway by siRNA inhibition of IKK had no effect on cell proliferation, PI3K inhibitors LY294002 and Wortmannin were effective in blocking cell proliferation in Her2-overexpressing breast cancer cells. These results suggest that Her2 can activate the PI3K and NF-κB pathways separately, leading to different effects on tumor progression. These data indicate that targeting both of these pathways using specific inhibitors, particularly in the context of trastuzamab and lapatinib resistance, may be a promising therapy. Though these data show that inhibition of the PI3K pathway does not block NF-κB activation in Her2-overexpressing breast cancer cells, we have not investigated other mechanisms of cross talk between these two signaling pathways. There is evidence that IKKβ may downregulate PI3K signaling through phosphorylation of the p85 subunit of PI3K in some settings (W. Comb and J. Hutti,

personal communication). Therefore, further analysis of the role of these two pathways in breast cancer is required.

NF-κB Regulation of Gene Expression in Her2$^+$ Breast Cancer

In the previous chapters, we present data showing that NF-κB regulates a specific subset of genes enriched with cytokines and chemokines. NF-κB regulates the expression of genes involved in many different cellular processes, including innate and adaptive immunity (Bonizzi and Karin 2004), as well as cell proliferation and survival (Papa et al. 2006). NF-κB is activated in a variety different cancers, however, the genes which are dysregulated varies dramatically from cancer to cancer (Cogswell et al. 2000; Basseres and Baldwin 2006; Belguise and Sonenshein 2007). Here, we show that in Her2-overexpressing breast cancer, NF-κB activation results in increased transcription of the chemokines IL-6 and IL-8, while a gene strongly regulated by NF-κB, IκBα, is not significantly affected by Her2 overexpression. We also show activation of NF-κB blocks expression of the gene SPARC in multiple cancer cell lines. Further investigation of NF-κB regulation of these and other cytokines is essential due to the role of these proteins in inflammation in breast cancer. Inflammatory genes have been shown to be overexpressed in many breast cancers, including Her2-overexpressing breast cancer (Calogero et al. 2007). Tumor associated macrophages have been shown to play in important role in the progression of breast cancer (DeNardo et al. 2009). Additionally, tumor associated macrophages have been implicated in the induction of invasion and metastasis in breast cancer through upregulation of inflammatory genes by NF-κB

(Hagemann et al. 2005). The role that NF-κB plays in crosstalk with these macrophages in Her2-overexpressing breast cancer warrants further investigation.

Many of the current methods commonly used to identify such genes limit the depth of analysis of gene expression in cancer. A candidate gene approach is restricted to analysis of genes previously known be regulated by NF-κB, and is tedious. Array-based technologies allow greater coverage of the transcriptome, but these methods are still inherently biased by oligo selection. The development of high-throughput deep sequencing technologies offers tremendous promise in the analysis of transcription factor mediated gene expression dysregulation. Chromatin-immunoprecipitation followed by sequencing (ChIP-Seq) is a newer technology that will be indispensable for analysis of global transcription factor binding. ChIP-Seq provides researchers with greater resolution and higher genome coverage, with less noise, than previous platforms such as array-based ChIP-CHIP (Park 2009). While this platform has been used to identify genome-wide binding of multiple transcription factors, to our knowledge there are no reports using ChIP-Seq to analyze genome-wide NF-κB binding sites. The development of another high-throughput platform, RNA-Seq, allows analysis of the complete transcriptome of a cell (Wang, Gerstein, and Snyder 2009). A combinatorial approach combining these two high-throughput platforms would therefore allow analysis of genome-wide NF-κB binding and how this change in global binding leads to NF-κB regulated gene expression changes. We are currently optimizing a protocol to use these platforms for analysis of NF-κB regulated gene expression in multiple cancer cell lines.

The Potential Role of NF-κB Signaling Members in Targeted Therapy

The development of resistance to targeted therapies in Her2-overexpressing breast cancer is a problem of utmost importance which needs to be addressed. Unlike with inhibitors targeting other receptor tyrosine kinase where resistance is often due to mutations in the target receptor (Gorre et al. 2001; Pao et al. 2005), the Her2 receptor rarely harbors mutations in this subtype of breast cancer (Stephens et al. 2004). Therefore, downstream signaling pathways are the most likely culprits for development of resistance to these therapeutics. The PI3K pathway has been implicated in this resistance (Eichhorn et al. 2008; Faratian et al. 2009), and recently the NF-κB pathway was suggested to be involved in trastuzamab resistance (Flowers and Thompson 2009). Activation of NF-κB, specifically increased transcription of survivin and increased phosphorylation of p65/RelA at serine 529, has been reported to be involved in resistance to lapatinib (Chen, Xia, and Spector 2008; Xia et al.). The role of IKKα and/or TAK1 in the development of acquired resistance to trastuzamab and lapatinib therefore warrants investigation. Furthermore, this raises the possibility of using NF-κB inhibitors as treatment options in trastuzamab or lapatinib resistance breast cancers.

The Development of NF-κB Mouse Models for Her2⁺ Breast Cancer

The development of mouse models of breast cancer has played a key role in the understanding of this disease. The first Her2 mouse model was introduced 22 years ago (Muller et al. 1988), and since then, this multiple permutations of Her2 mouse models of breast cancer have been developed (Guy et al. 1992; Andrechek et al. 2000). Using these and other mouse models, multiple groups have begun to investigate the role of NF-κB in

Her2-induced tumor formation *in vivo*. Using a mouse with the activation loop serines mutated in IKKα, Cao et al. have shown the importance of IKKα for tumor initiating cell self renewal in Her2 breast cancer (Cao, Luo, and Karin 2007). Recently, another group has shown the importance of NF-κB in Her2-induced tumor growth and vasculogenesis using immune-deficient and immune-competent orthotopic mouse models (Liu et al. 2009). However, the use of genetic NF-κB knockout mouse models in Her2-overexpressing breast cancer has not yet been performed. Genetic knockout of IKKβ or the RelA/p65 subunit of NF-κB is embryonic lethal due to massive liver apoptosis (Tanaka et al. 1999; Beg et al. 1995), while IKKα knockout mice are viable, despite exhibiting defects in morphogenesis (Li et al. 1999; Hu et al. 1999). Our lab, as well as one other lab, has developed a conditional mouse model for RelA/p65 inactivation using the Cre-recombinase system (Steinbrecher et al. 2008; Geisler et al. 2007). We have attempted to develop a tetracycline-induced knockout of p65 in a Her2-overexpressing mouse model of breast cancer; however, these attempts were unsuccessful due to incomplete induction of inactivation of the p65 locus (data not shown). We are currently developing a constitutive tissue-specific p65 knockout Her2-overexpressing mouse using the MMTV-Cre transgene. Development of this mouse model will be extremely useful in delineating the role of this NF-κB subunit in Her2-overexpressing breast cancer *in vivo*. Constitutive knockout of TAK1 in mice also leads to embryonic lethality at E9.5. However, development of Cre-mediated conditional TAK1 knockout mice has allowed for genetic studies of TAK1 *in vivo* (Sato et al. 2005). Considering the data we present here showing the importance of TAK1 for NF-κB activation in Her2-overexpressing breast cancer, development of a TAK1 conditional knockout in a Her2-overexpressing

mouse model of breast cancer would be quite beneficial in understanding the role TAK1 plays in Her2-overexpressing breast cancer *in vivo*.

The data we have presented represent new insights into the mechanisms and biologicals result of Her2 activation of NF-κB in breast cancer (Fig. 5.1). Ultimately, further research using genetic analysis, high-throughput sequencing and mouse models will allow us to develop an even greater understanding of the role of NF-κB in Her2-overexpressing breast cancer, and may uncover even more possible therapeutic targets in this disease.

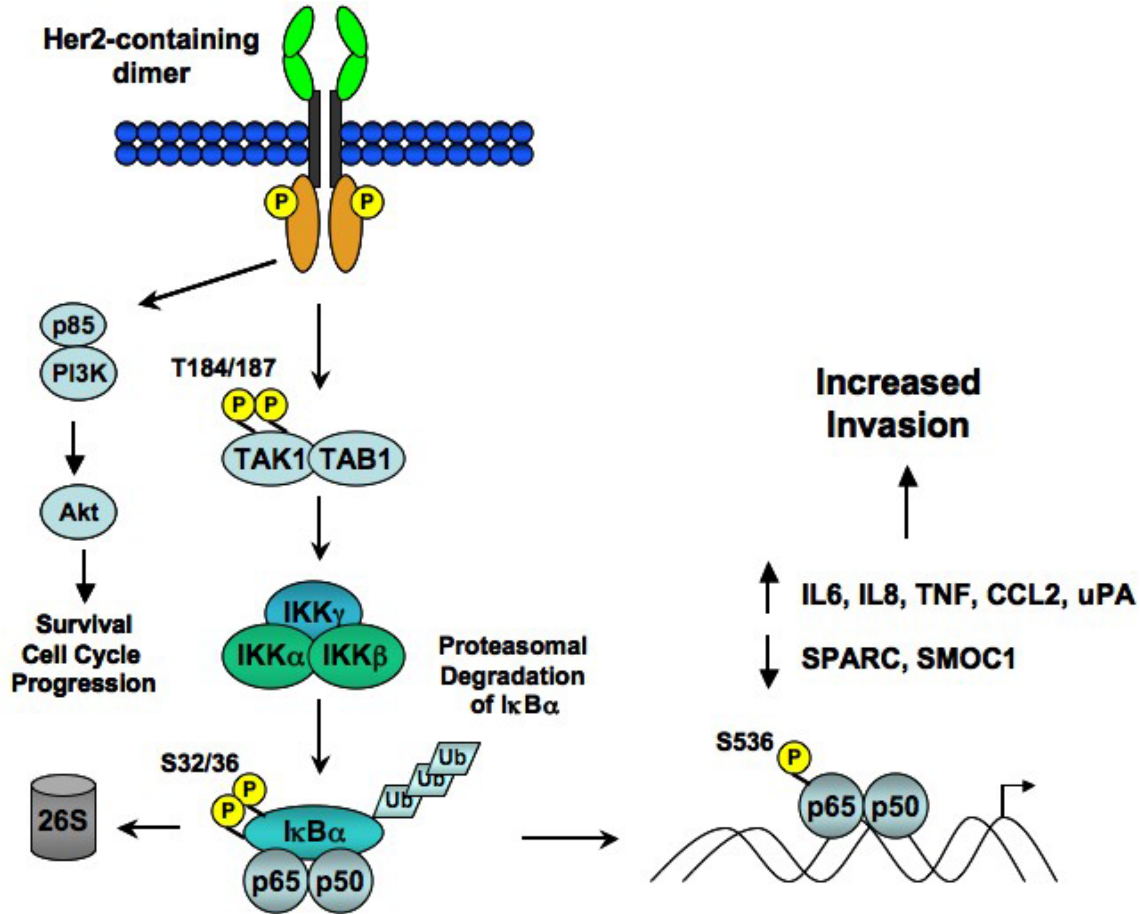

Figure 5.1. Summary of findings elucidating the role of NF-κB in Her2⁺ breast cancer. In this thesis, we present data showing that activation of Her2-containing dimers leads to PI3K/Akt activation resulting in increased cell proliferation, independent of NF-κB activation. Her2 activates TAK1 in a TAB1 dependent manner, leading to autophosphorylation of TAK1 at threonine 184 and 187. TAK1 then activates NF-κB, requiring IKKα, leading to phosphorylation of IκBα and ultimately phosphorylation of p65/RelA at serine 536. Activation of p65 containing NF-κB complexes results in increased transcription of cytokines and chemokines IL-6, IL-8, CCL2 and TNF-α, as well as the serine protease uPA, and downregulation of the BM-40 family genes SPARC and SMOC1. This Her2-activation of NF-κB through IKKα ultimately leads to an increase in invasiveness of Her2⁺ cells.

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