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14. ABSTRACT

Specifically, our hypothesis is that IKK and a form of NF-κB are activated in certain breast tumors (including the majority of basal-like tumors and in Her2+ cancers), leading to the expression of genes which promote oncogenesis and which lead to resistance to therapy. Additionally, we hypothesize that these tumors will respond to inhibitors of this pathway, either alone or in combination with chemotherapy. Based on our findings, we hypothesize that IKK/NF-κB and Bcl2A1 (a key gene regulated by NF-κB that is found upregulated in basal-like breast cancer) are key determinants of cancer therapy resistance in certain breast tumors. In a new direction, we propose that the IKK/NF-κB pathway drives invasion and proliferation of Her2+ breast cancer. Our aims are to: (i) Generate a tumor bank archive for the analysis of NF-κB/IKK activation and associated gene expression, and correlate the findings derived from this analysis to breast tumor subtypes, (ii) Determine the mechanism of activation of Bcl2A1 and other NF-κB-dependent genes in basal-like cells; identify signaling components required for NF-κB activation in basal-like cancer cells; examine inhibitors of the NF-κB/IKK pathway in vitro, and (iii) Characterize animal models of breast cancer for activation of NF-κB and for potential therapeutic responses to NF-κB inhibitors. In new directions, characterize the role of NF-κB downstream of Her2 in breast cancer, determine if Parp1 is a positive regulator of NF-κB in basal-like breast cancer and analyze effects of Parp inhibitors, and study tumor breast tumor initiating cells for activation of NF-κB.

15. SUBJECT TERMS
Breast cancer, NF-kappaB, IKK, animal models, drug studies

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INTRODUCTION:

The goals of this grant are to determine if the NF-κB pathway is active in the basal-like breast cancer subtype and in Her2+ breast cancer (a new extension of the previous aims) and if this pathway can be targeted by small molecule inhibitors in a manner that is therapeutic. Patients with basal-like breast cancer and with Her2+ breast cancer typically exhibit poor outcomes, thus new therapies are required [refs. 1 – 5]. Our evidence is that a set of genes, known to be regulated by NF-κB, is upregulated in basal-like tumors and, interestingly, in cell lines that are basal-like. Some of the basal-like cell lines exhibit phosphorylated IKK, a key upstream regulator of the NF-κB pathway [6]. The NF-κB pathway is known to be involved in oncogenesis, but its role in basal-like breast cancer is unclear [refs. 7, 8]. Animal models of basal-like breast cancer also exhibit upregulation of some of these NF-κB-dependent genes (our data). Additionally, we have found that NF-κB is activated by Her2 (an EGFR family member that is upregulated in approximately 25% of breast cancer), although expression of NF-kB-dependent genes is not identical between the two types of breast cancer. Our goals are to analyze basal-like cell lines, human tumors, and animal models of basal-like cancer to further validate our hypothesis that NF-κB is active in these tumors and may, therefore, represent a new therapeutic target for this breast cancer subtype with poor prognosis. Similar approaches will be performed on Her2+ breast cancer.

Aims ____ of the proposal are to: (i) analyze extracts of human breast cancer for phosphorylated IKK and other markers of NF-κB activation. Determine if these markers correlate with expression of Bcl2A1 and other NF-κB-dependent genes., (ii) determine the mechanism of activation of Bcl2A1 and other NF-κB-dependent genes in basal-like cancer cells, and compare this mechanism with pathways operative in distinct breast cancer subtypes (i.e., Her2+ cells. Thus a new addition to the aims is to analyze Her2+ breast tumors and cell lines to analyze a role for NF-κB in this cancer, and to determine how NF-κB is activated in these cells. Analyze inhibitors of the NF-κB pathway for effects on growth and survival of basal-like, and Her2+ breast cancer cells. (iii). Analyze experimental tumors for markers outlined in Aims 1 and 2. Using animal models represent ative of basal-like and Her2 breast cancer, determine if inhibitors analyzed in Aim 2 will suppress growth of the tumors, and/or sensitize the tumors to chemotherapy. Additionally, we have added 2 new aims (as part of an extended statement of work – we will determine (i) if Parp1 is a regulator of NF-κB in basal-like and other breast cancers and, if so, determine if Parp 1 inhibitors suppress growth/survival of these cancer cells and (ii) analyze tumor initiating cells from breast cancer cell lines and experimental tumors for the activation of NF-κB.

BODY (end of 2nd year report):

Regarding Aim 1 goals:

--(as noted in the last report) we have analyzed extracts of a number of breast tumors (7). We detected phosphorylated p65/RelA in samples 3, 4, 5, 6, and 7 (see Fig. 1). Bcl2A1 expression was found in tumor samples 2, 4 and 5. Tumors 2 and 4 are basal-like and 5 is luminal A/IIE subset (a tumor subtype that is known to express Bcl2A1). Thus, these results show that phospho-p65 ser536 is not directly correlated with Bcl2A1 expression, but that Bcl2A1 is expressed in 2/2 basal-like tumors (consistent with our hypothesis) but not in Her2+, luminal B, or luminal A (not IIE subtype). We are now testing a larger number of tumor samples.

Regarding Aim 2 goals:

--(as noted in the last report) we have performed analysis of basal-like breast cancer cell lines and found the upregulation of the NF-κB subunit c-Rel (see Figs. 2 and 3). cRel is known to regulate Bcl2A1 in other cells. We are testing a larger number of tumors, and animal tumors, to expand this study.

--we performed analysis of Her2+ breast cancer cell lines which indicates that p65/RelA is phosphorylated, and that certain NF-κB-dependent genes are upregulated. Note that Bcl2A1 was not found in the Her2+ breast cancer cell lines, suggesting that either a different NF-κB subunit is involved in control of Bcl2A1 expression in basal-like cancer, or that a different cofactor is involved. IKKα and IKK β are both important in controlling gene expression and in activating NF-κB in these cells. IKKα drives invasion of these cells (these studies are published – Merkhofer et al., 2010 – see ref. 10, and see attached).

--(as noted previously) treatment of basal-like breast cancer cell lines with the Bayer IKK β inhibitor reduces expression of the associated NF-κB-dependent gene set and induces growth arrest (see Table 1 below). Note that another group has now published that NF-κB is important for growth/proliferation of basal-like cells (Yamaguchi et al., 2009). This may preclude our ability to publish these results alone, but we are looking at other aspects to include in a new publication.

--In order to analyze tumor initiating cells from breast tumor cell lines and from murine breast tumors, we have cultured these cells and have shown that they form mammospheres (consistent with a stem-cell like component) (see Fig. 4).

Regarding Aim 3 goals, we have:

--Crossed the RelA fl/fl animal with Her2+ animals, along with expression of cre recombinase in the breast. This will test the role of the p65/RelA subunit in progression of Her2+ breast cancer.

--We are breeding the C3Tag animals for analysis

Regarding new aim of analyzing Parp1 inhibitor:

--(preliminary data – just performed once) we have shown that Parp 1 inhibitor blocks NF-kB activation in basal-like cancer cells.

Regarding new direction of analyzing tumor iniating cells:

--We have isolated previously characterized tumor initiating cells from basal-like and Her2+ breast cancer cell lines and from a primary Her2+ animal tumor, and have shown mammasphere growth (indicative of the presence of tumor initiating cells – see Fig. 4 below).

KEY RESEARCH ACCOMPLISHMENTS:

--Contrasted Her2+ positive breast cancer cells with basal-like cells, indicating differential gene expression (Merkhofer et al., 2010; see attached).

--Demonstrated that IKKα and NF-κB promote invasion in Her2+ breast cancer cells (Merkhofer et al., 2010).

--Treatment of basal-like breast cancer cells with an IKKβ inhibitor suppresses expression of the NF-κB-dependent gene set and induces growth arrest/apoptosis.

--Treatment of Her2+ breast cancer cells with the NBD peptide (an inhibitor of total IKK activity) suppressed growth/survival (described in Merkhofer et al., 2010).

REPORTABLE OUTCOMES:

--Manuscript published regarding studies on Her2+ breast cancer cells and the involvement of IKK/NF-κB in controlling gene expression and invasion. This study provides interesting parallels and differences with basal-like cancer.

--Evidence that c-Rel is upregulated in basal-like breast cancer cell lines.

CONCLUSIONS: Both basal-like breast cancer cells and Her2+ breast cancer cells exhibit activation of NF-kB, but the genes that are regulated in these cells by NF-κB appear to be different. Analysis of human breast tumor extracts confirms prediction that Bcl2A1 is active in basal-like cancers and in the luminal A IIE group. Analysis of basal-like breast cancer cell lines indicates that c-Rel is upregulated in these cells, which is a potential link with control of Bcl2A1 gene expression. Comparison of Her2+ breast cancer cells with basal-like cells indicates that NF-κB is active in both types of breast cancer. We have begun the proposed therapy studies in the model for basal-like cancer, using an IKKβ inhibitor which shows growth suppressive activity on basal-like breast cancer cell lines.

REFERENCES:

1. Brenton, J.D., L. Carey, A. Ahmed, and C. Caldes. Molecular classification and molecular forecasting of breast cancer: ready for clinical application? *J. Clin. Oncol*. 29, 7350-7360 [2005].

2. Sorlie, T., C. Perou, T. Aas, S. Geisler, H. Johnsen, T. Hastie, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc. Nat. Acad. Sci. U.S.A*. 100, 8418-8423 [2003].

3. Hu, Z, C Fan, D. Oh, J. Marron, X. He et al. and C. Perou. The molecular portraits of breast tumors are conserved across microarray platforms. *BMC Genomics* 7, 96-103 [2006].

4. Carey, L., C. Perou, L. Livasy, L. Dressler, D. Cowan et al. and R. Millikan. Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. *JAMA* 295, 2492-2502 [2006].

5. Oh, D.S., M. Troester, J. Usary, Z. Hu, X. He, C. Fan, J. Lua, L. Carey, and C. Perou. Estrogen-Regulated Genes Predict Survival in Hormone Receptor-Positive Breast Cancers. *J Clin Oncol* 24, 1656-1664 [2006].

6. Hayden, M. and S. Ghosh. Signaling to NF-κB. *Genes and Dev*. 18, 2195-2224 [2004].

7. Karin, M. NF-κB in cancer development and progression. *Nature* 441, 431-436 [2006].

8. Basseres, D. and A. Baldwin. NF-κB and IKK pathways in oncogenic initiation and progression. *Oncogene* 25, 6817-6830 [2006].

9. Yamaguchi, N., T. Ito, S. Azuma, E. Ito, R. Honma et al. Constitutive NF-κB activation is preferentially involved in the proliferation of basal-like subtype breast cancer cell lines. *Cancer Sci.* 100, 1668-1674 [2009].

10. Merkhofer, E., P. Cogswell, and A. Baldwin. Her2 activates NF-κB and induces invasion through the canonical pathway involving IKKa. *Oncogene* 29, 1238-1248 [2010].

APPENDIX

Figure Legends:

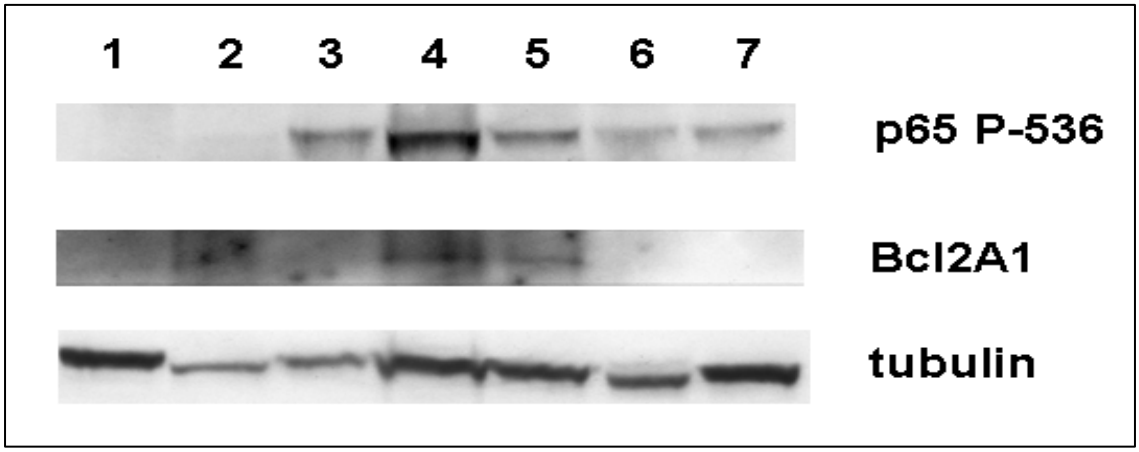
Fig. 1 (see below). Immunoblotting of whole cell extracts of 7 breast tumors stained with antibodies that recognize p65 phosphorylated at ser536, Bcl2A1 and tubulin. Tumors: 1 (Her2+), 2 and 4 (basal), 3 (luminal B), 5 (luminal A – IIE subset), 6 and 7 (luminal A). The results show that Bcl2A1 expression is detected in extracts of tumors from basal and IIE subsets. Phospho-p65 is detected but does not correlate with Bcl2A1 expression.

Fig. 2 (see below). Nuclear extracts were generated from 3 basal-like breast cancer cell lines (Sum102, Sum149, MDA-MB-231), from a luminal-like breast cancer cell (MCF), and from a Her2+ breast cancer cell line (BT474). The nuclear extracts were used with a commercial gel shift/ELISA to determine nuclear levels of the 5 different NF-kB subunits. Results show that c-Rel and RelB are elevated in the basal-like cell lines.

Fig. 3 (see below). Nuclear extracts from the Sum102 basal-like cells were treated with the IKKβ inhibitor (Bay 65) and levels of c-Rel and RelB are diminished with treatment of Bay65.

Fig. 4 (see below). Figure legend is included within the figure.

Fig. 1:



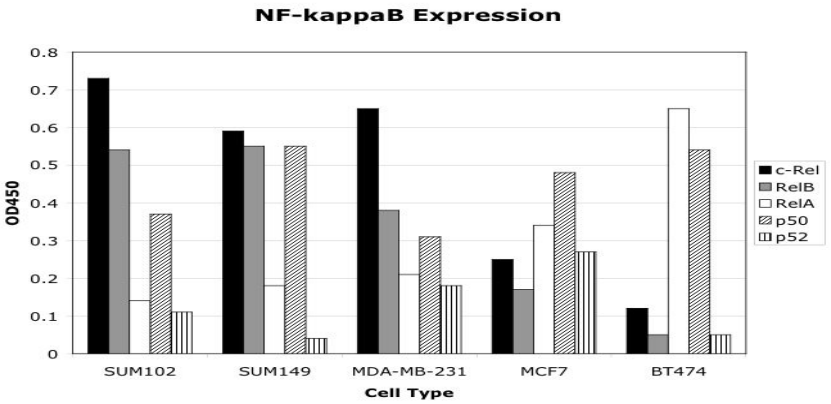


Fig. 6. "Gel shift" ELISA using 5 μg of nuclear extract from the indicated cell line. The NF-κB subunit tested is shown as different color bar graphs.

Fig. 2

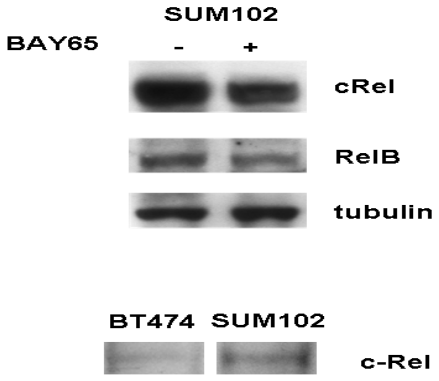


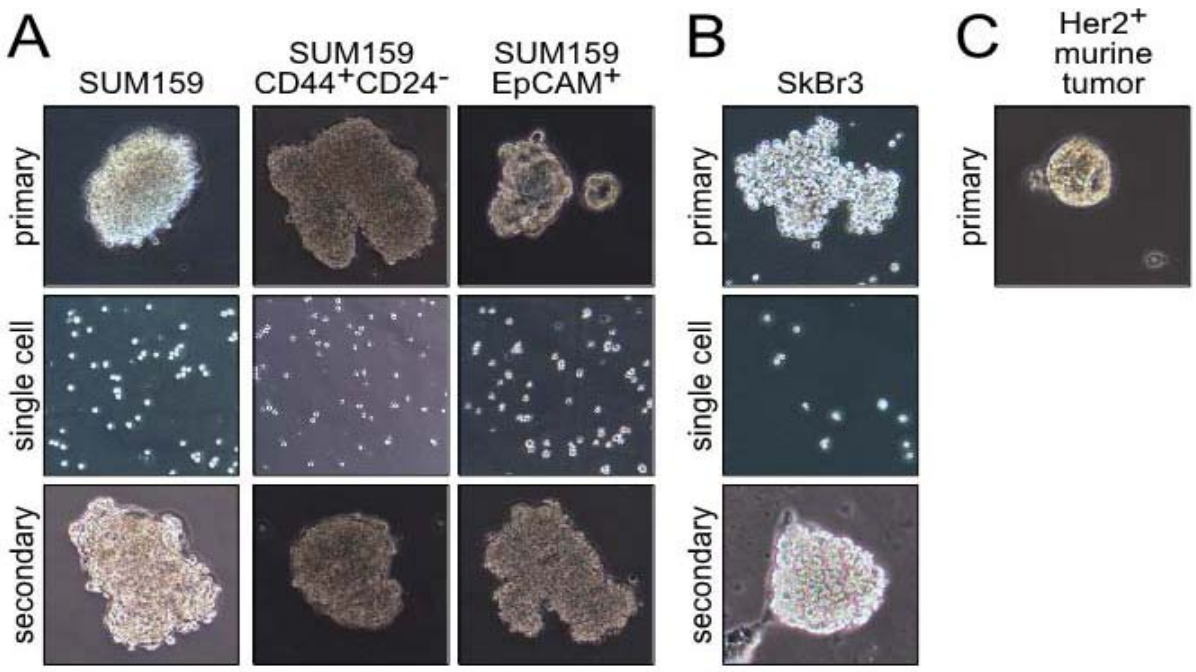
Fig. 7. Western blotting of nuclear extracts from SUM102 (top) with indicated antibodies (right), without or with Bay65 (1 μM for 4 hours). Bottom: c-Rel antibody on nuclear extracts from BT474 and SUM102 (light exposure).

Fig. 3

Table 1. Cell-Cycle Distribution of SUM102 cells treated with Bay65 (2.5 μM), a control (inactive) compound Bay 60, or with DMSO control. Results are an average of two experiments (less than 20% variation within the different phases).

	Cell-Cycle Phases		
	G0/G1	S	G2/M
Bay 65-1942	87	6	7
Control Bay cmpd	58	26	16
DMSO control	55	21	24

Fig. 4



Mammosphere formation from human cell lines and murine mammary tumors

Visualization of mammospheres formed in low-adhesion, serum-free cell culture from the bulk population or TIC sub-population (as indicated) of the human cells lines SUM159 (A) or SkBr3 (B) cells, or from cells derived from murine Her2+ murine mammary tumor tissue (C). Where indicated, cells were dispersed into single cell suspension and formation of secondary mammospheres was observed.

Data not shown: Tertiary mamospheres formed from SUM159 bulk and TIC sub-populations exhibited identical morphology as secondary mammospheres. SkBr3 cells were largely unable to form tertiary mammospheres. Primary and secondary mammospheres were also produced from the $CD44^{+}CD24^{-}$ (TIC) sub-population of SkBr3 cells, resulting in a similar pattern and morphology as for the bulk population.

Oncogene (2010) 29, 1238–1248

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ORIGINAL ARTICLE

Her2 activates NF-κB and induces invasion through the canonical pathway involving IKKα

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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. In addition, 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. In addition this work indicates the importance of IKKα as a mediator of Her2-induced tumor progression.
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Keywords: Her2; IKKalpha; NF-KappaB

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, 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 that NF-κB has in development and progression of Her2-overexpressing breast cancer is still poorly understood. In addition, 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 of 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, 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 domain (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 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.

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Using an siRNA approach, we set out to determine how NF-κB is activated downstream of Her2, and what role the IKK complex has in this signaling cascade, as well as how the activation of the IKK kinases may lead to a malignant state. Although the classical pathway has long been thought to require IKKβ, here we show that IKKα has 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.

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 (Galang *et al*., 1996; Biswas *et al*., 2004). 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 lapatinib for 12 h and whole-cell extracts were analysed 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, whereas non-Her2-overexpressing tumor cell lines (MCF7 and MDA-MB-231) showed no change (Figure 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 (Supplementary Figure 1). 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 h and whole-cell extracts were analysed for levels of phosphorylated IκBα. Phosphorylation of IκBα at serines 32 and 36 was inhibited within 3 h of lapatinib treatment (Figure 1b). Stabilization of IκBα was also observed, consistent with the 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 (Figure 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.

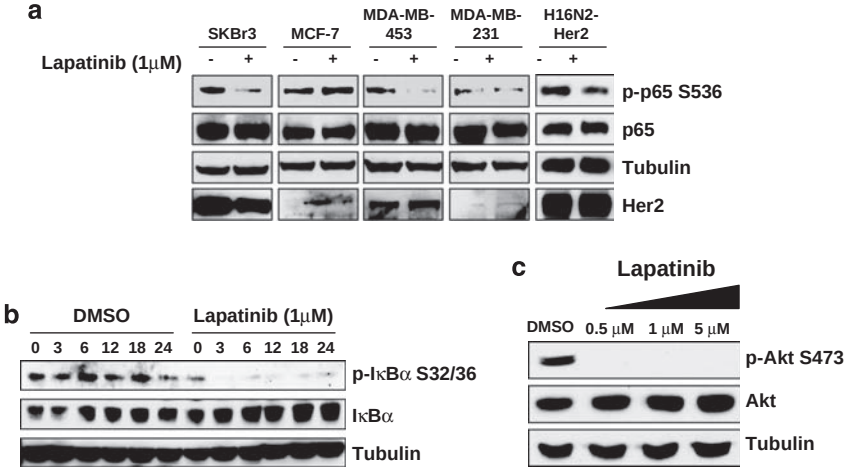

Figure 1 Lapatinib treatment inhibits the NF-κB and PI3K pathways in Her2-overexpressing cells. (**a**) Western blot of phospho-p65^{S536} in multiple breast cancer cell lines treated with lapatinib. Breast cancer cell lines were treated with 1 μM dual EGFR/Her2 inhibitor lapatinib or DMSO vehicle control for 12 h. 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 h 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 h 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.

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 analysed 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 (Figure 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 (Supplementary Figure 2). A similar result was obtained using IKKβ −/− cells (data not shown), indicating that both IKKα and IKKβ are important for Her2 to activate NF-κB in murine fibroblasts. To further investigate the role IKKα may have in the activation of classical NF-κB complexes downstream of Her2, siRNA was again used to target IKK in SKBr3 cells stably expressing a 3× κB luciferase reporter construct, as well as in H16N2–

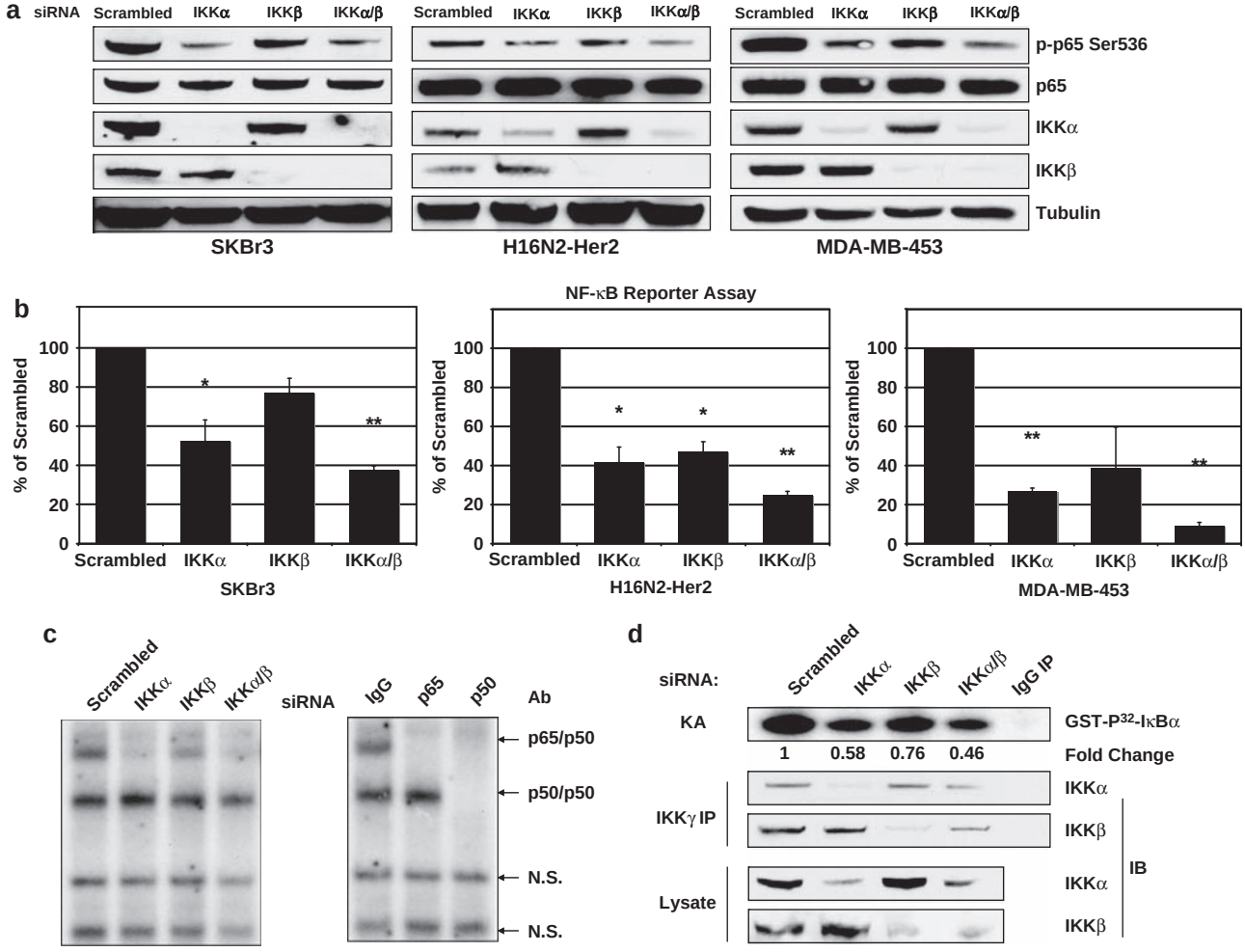

Figure 2 Her2 activation of NF-κB via IKKα and IKKβ involves the canonical pathway. (**a**) Western blot of phospho-p65^{S536} in Her2-overexpressing breast cancer cells transfected with siRNA to IKK catalytic subunits. SKBr3 (left), H16N2-Her2 (center) and MDA-MB-453 (right) cells were transfected with 100 nM siRNA to IKKα and IKKβ and whole-cell extracts were prepared after 72 h 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 h post-siRNA transfection and luciferase levels were measured. Statistically significant differences were determined by Student's *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 three 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 of SKBr3 cells transfected with IKK siRNA. Nuclear extracts were prepared after 72 h. Identities of the bound complexes were determined by super-shift with antibodies to p65 and p50. Non-specific binding complexes are noted with as NS (**d**) Kinase assay measuring IKK *in vitro* phosphorylation of IκBα. SKBr3 cells were transfected with IKK siRNA for 72 h 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 with scrambled siRNA-transfected cells.

Her2 and MDA-MB-453 cells transiently transfected with the 3× 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), whereas knockdown of IKKβ did not show a significant decrease in luciferase reporter activity in two of the three cell lines (Figure 2b). An electrophoretic mobility shift assay 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 (Figure 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 (Figure 2d), further indicating IKKα has a prominent role in the canonical pathway in Her2-overexpressing cells. Taken together, these results show that IKKα has a more significant role than IKKβ in the 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 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 and the serine-protease uPA, was greater upon siRNA knockdown of IKKα than IKKβ in both SKBr3 and H16N2-Her2 breast cancer cell lines (Figure 3a). To show 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 (Figure 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 h post treatment (Figure 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 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 (Figure 4a). NF-κB luciferase reporter activity was also significantly decreased in these cell lines upon siRNA knockdown of NEMO (Figure 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 (Figure 4c). To rule out any effect loss of IKKα could have on non-classical activation of NF-κB, we analysed 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 (Figure 4d). These results suggest that NF-κB activation in $Her2^+$ cells occurs through IKKα and this requires the NEMO subunit. In addition, 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 was reported earlier that expression of dominant-negative PI3K and Akt plasmids can block NF-κB DNA binding downstream of Her2 (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 (Figure 1a). 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 (Figures 5a–c). Furthermore, treatment of SKBr3 cells stably expressing the 3× κB luciferase reporter with LY294002 had no

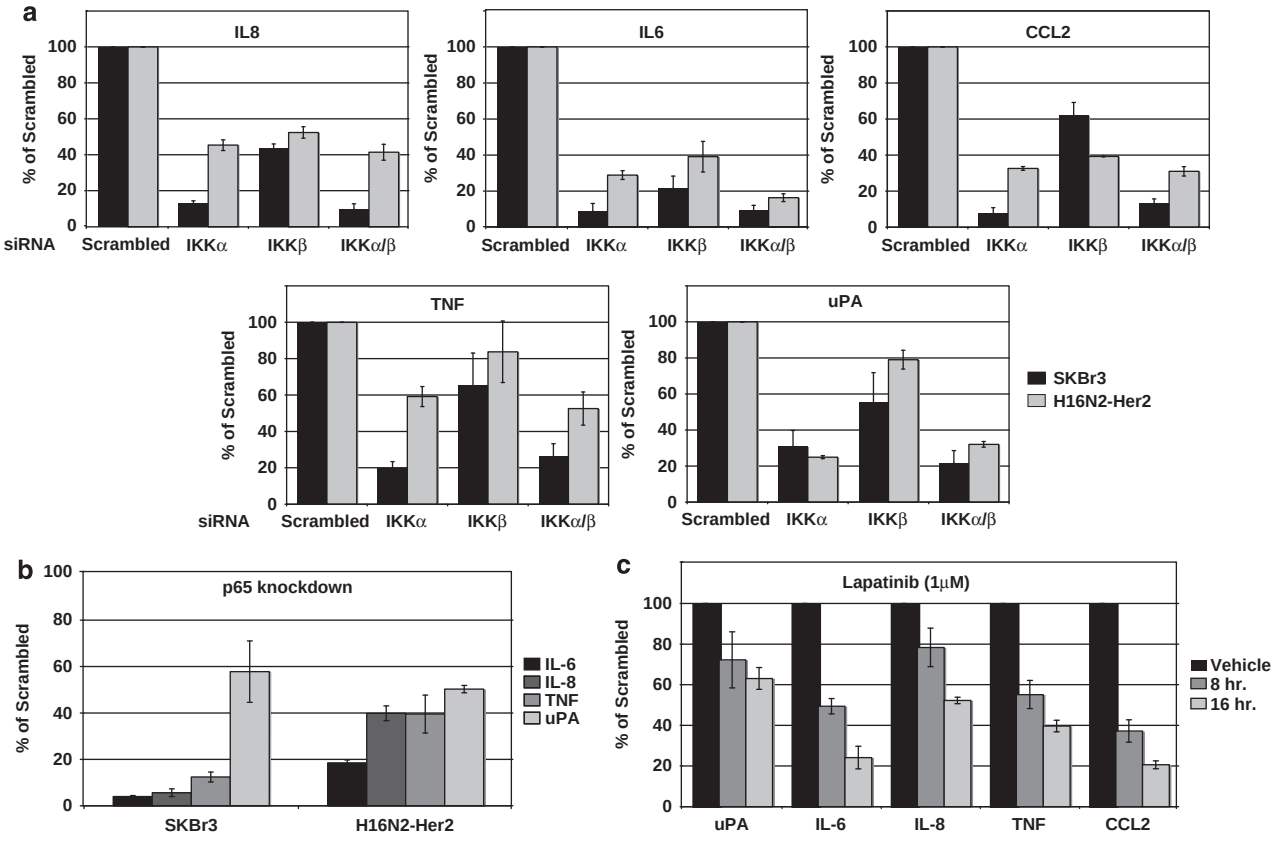

Figure 3 Her2 induces NF-κB-regulated gene expression through IKKα and IKKβ. (**a**) Quantitative real-time PCR of multiple genes shows different gene expression profiles upon IKKα or IKKβ knockdown. qRT–PCR was performed on extracts from SKBr3 (black bars) and H16N2-Her2 (gray bars) cells transfected with 100 nM IKKα or IKKβ siRNA for 72 h. 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 three 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 h 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 three 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 h and gene expression levels of uPA, IL-6, IL-8, TNF and CCL2 were compared with DMSO-treated cells. Fold change of transcript levels is shown relative to scrambled siRNA-treated cells. Error bars are ± 1 s.e.

effect on NF-κB transcriptional activity (Figure 5d). These results show 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 had no inhibitory effect on cell proliferation (Figure 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 (Figure 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 6c). This suggests that Her2 overexpression results in the 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 (Figure 6d).

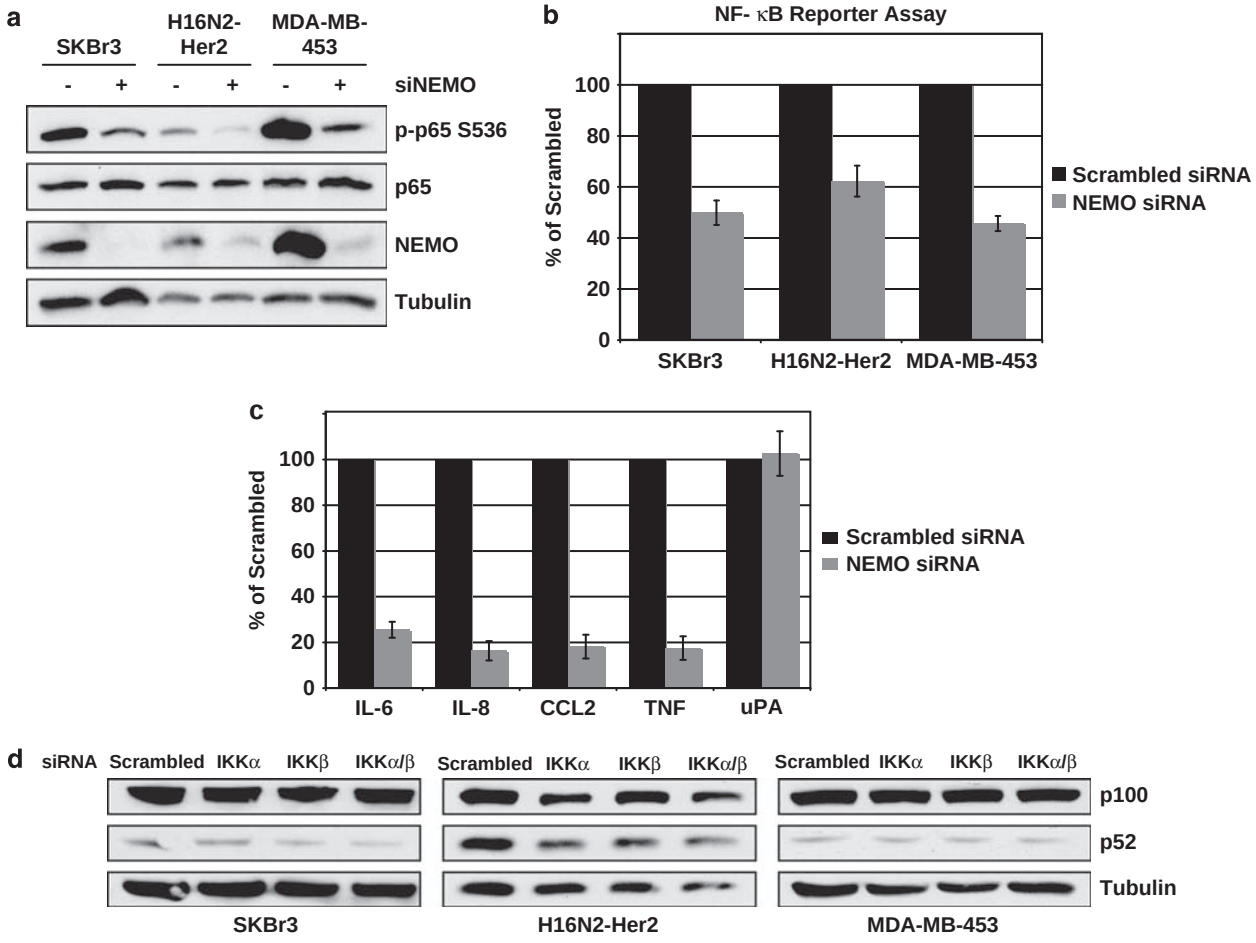

Figure 4 Knockdown of NEMO blocks NF-κB activation through the canonical pathway. (**a**) Her2$^+$ breast cancer cells were transfected with 100 nM NEMO siRNA and whole-cell lysates were collected 72 h post transfection and western blot analysis of phosphorylated p65 was performed using 25 μg total protein. (**b**) Her2$^+$ cell lines were transfected with 100 nM NEMO siRNA and whole-cell extracts were prepared 72 h 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 three 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 h 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 h post transfection. Levels of p100 and p52 were measured by western blot analysis using 25 μg of total protein.

Discussion

Although Her2-positive breast cancer is known to activate both NF-κB and PI3K/Akt pathways, (Pianetti *et al*., 2001; Knuefermann *et al*., 2003; Biswas *et al*., 2004; She *et al*., 2008), it has been unclear how Her2 induces NF-κB and whether PI3K is involved with this pathway. In addition, potential roles for IKKα and IKKβ in controlling Her2-induced NF-κB have not been addressed. The latter point is of interest because 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 has 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 the invasion of Her2$^+$ cells, with apparently 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 has 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 (Xia *et al*., 2002; Zhou *et al*., 2004), although Herceptin has been shown to inhibit NF-κB activation

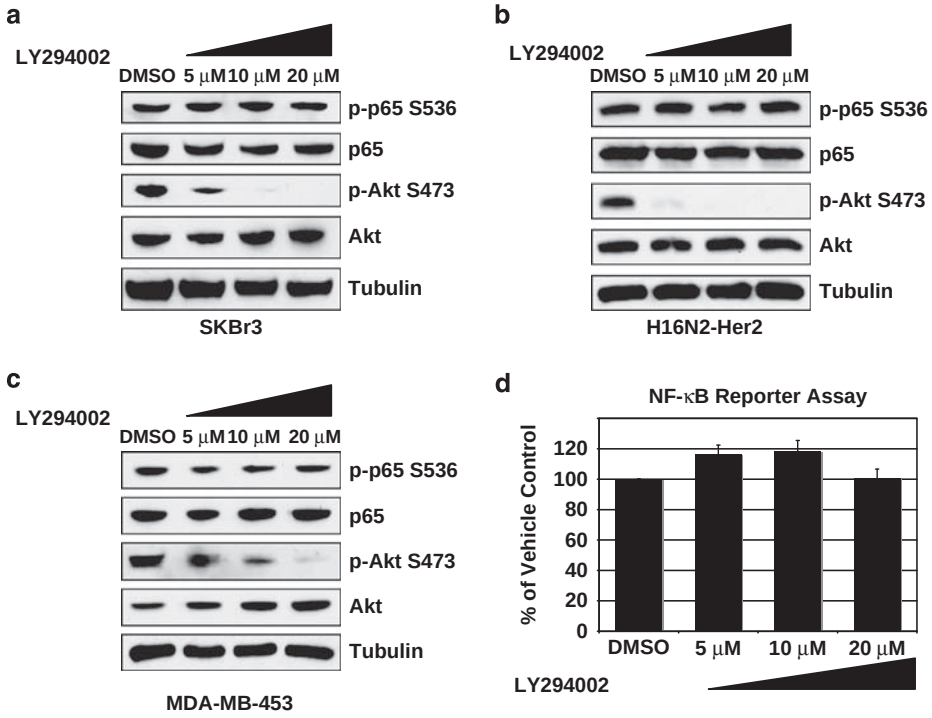

Figure 5 Inhibition of the PI3K-pathway does not block NF-κB activation. Western blot of phospho-p65 serine 536 from SKBr3 (**a**), H16N2-Her2 (**b**) and MDA-MB-453 (**c**) cells treated with PI3K-inhibitor inhibitor LY294002 for 2 h. Western blot analysis was performed with 25 μg whole-cell extracts. (**d**) Luciferase reporter assay of SKBr3 cells were 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 three experiments. Error bars are ± 1 s.e. Samples are normalized by protein concentration.

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 (Figures 1a and b). Lapatinib also blocked NF-κB-induced gene transcription (Figure 3c). Treatment of SKBr3 cells with lapatinib led to complete loss of phosphorylation of Akt at serine 473 (Figure 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 (Figure 2a). Furthermore, knockdown of IKKα strongly reduced NF-κB activation as measured through electrophoretic mobility shift assay and NF-κB-dependent reporter assays, whereas IKKβ knockdown had less of an effect (Figures 2b and c). Similarly, knockdown of IKKα was more effective at blocking IKK activity than knockdown of IKKβ (Figure 2d). SKBr3 cells show 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 the 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 *et al*., 2007) is consistent with our results showing 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 (Figure 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 (Figure 3b). To show that these genes are controlled through Her2, and not through Her2-independent pathways, lapatinib was shown to block target gene expression (Figure 3c). This increase in chemokine and cytokine gene expression by Her2, as well as the increase in the expression of the

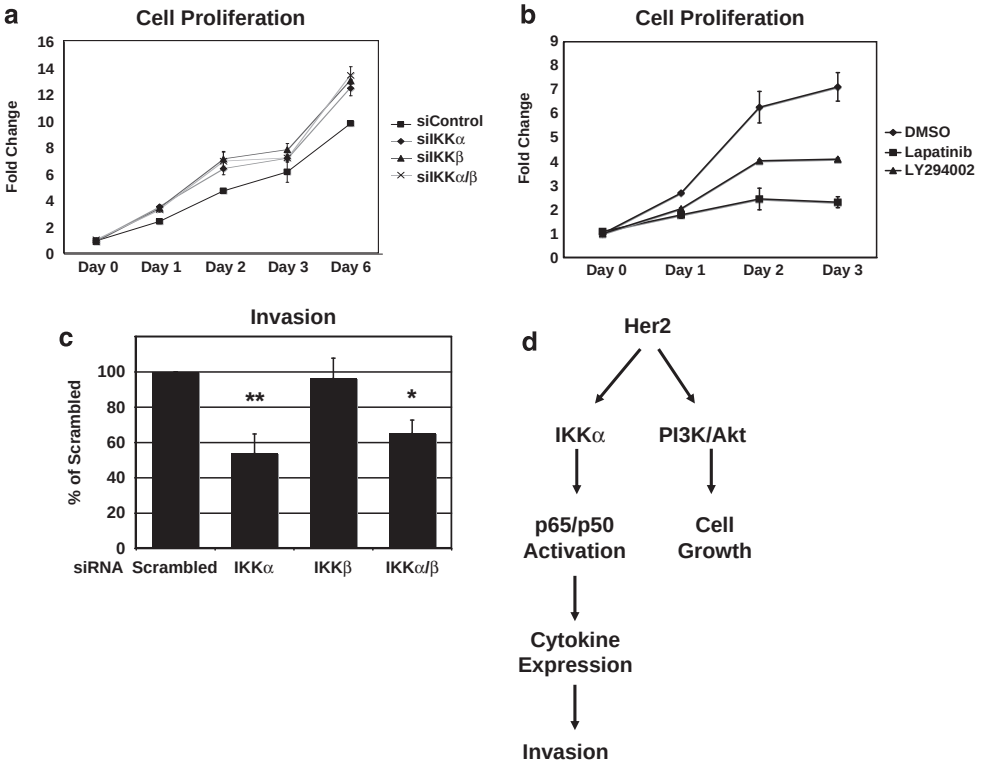

Figure 6 Inhibition of PI3K blocks cell proliferation, knockdown of IKKα blocks cell invasion. (**a**) Cell proliferation of SKBr3 cells transfected with siRNA to IKKα or IKKβ was measured for 6 days post-transfection compared with scrambled siRNA-treated cells using CellTiter cell viability reagent. 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 h fluorometrically. Statistical significance was measured by Student's *t*-test (*<0.01, **<0.001). Error bars represent ±1 s.d. (**d**) Model of activation NF-κB and PI3K/Akt pathways downstream of Her2 overexpression.

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 (Wang *et al*., 1999; Arihiro *et al*., 2000; Chavey *et al*., 2007; Vazquez-Martin *et al*., 2008). Therefore, our gene expression data suggests that IKKα has an important role in regulating genes involved in breast cancer progression, and this requires the scaffolding subunit NEMO (Figure 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 (Figure 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. In addition, 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 *et al*., 2007) as well as our own (Figure 6b) show that activation of the PI3K pathway has an important role in cell proliferation/viability. Interestingly, knockdown of IKKα or IKKβ subunits (individually or together) by siRNA has no measurable inhibitory effect on cell proliferation (Figure 6a).

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 *et al*., 2008). Knockdown of IKKα, but not knockdown of IKKβ, significantly blocks the invasive phenotype of SKBr3 cells (Figure 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 *et al*., 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 (Figure 6d). These data suggest 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.

Materials and methods

Cell culture and reagents
The tumor-derived SKBr3 cell lines were maintained in McCoy's 5A medium (Mediatech, Manassas, VA, USA) 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 MEF cell lines, were maintained in Dulbecco's Modified Eagle's Medium (Gibco, Invitrogen, Carlsbad, CA, USA) 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 3 × 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, Indianapolis, IN, USA) and maintained under selection with G418 (Geneticin, Sigma-Aldrich, St Louis, MO, USA). The Her2 wild-type and mutant (V654E) plasmids were constructed previously (Li *et al*., 2004) (Addgene plasmid 16257 and Addgene plasmid 16259, Addgene, Cambridge, MA, USA). The Her2 coding sequences were subcloned into retroviral pLHCX vector (Stratagene, La Jolla, CA, USA) and virus was produced in 293T cells with cotransfection of AmphoPAK. MEFs were transduced with virus with polybrene and lysed 48 h 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 (Beverly, MA, USA); antibodies against Her2, IKKα clone 14A231 and IKKβ clone10AG2 and p100/p52 from Millipore (Billerica, MA, USA), antibodies against p65 and p50 (supershift), β-tubulin and IKKγ from Santa Cruz Biotechnology (Santa Cruz, CA, USA), antibody against total p65 from Rockland (Gilbertsville, PA, USA) LY294002 and Wortmannin were purchased from Cell Signaling Technology. Lapatinib (GW572016; Tykerb, GSK, Brentford, Middlesex, UK) 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, Waltham, MA, USA) according to the manufacturer's instructions supplemented with protease inhibitor mix (Roche) and phosphatase inhibitor mix (Sigma). Nuclear and cytoplasmic extracts were prepared as previously described (Mayo *et al*., 1997). Protein concentrations were determined by Bradford assay (Biorad Laboratories, Hercules, CA, USA) 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 (Lafayette, CO, USA) 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 non-targeting control no. 3 (D001201-03). Cells were grown to approximately 50% confluency and transfected with 100 nmol/l siRNA with Dharmafect 1 reagent according to the manufacturer's instructions.

Quantitative real-time PCR
Total RNA extracts were obtained from cells approximately 72 h post-transfection by Trizol (Invitrogen) extraction. Two micrograms of RNA were reverse transcribed using random primers and MMLV-reverse transcriptase (Invitrogen). Real-time PCR was performed and analysed 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 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 min 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, 1 × protease inhibitor (Roche Applied Science) and 1 × 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 analysed as previously described (Steinbrecher *et al*., 2005) using GST–IκBα as a substrate.

Luciferase assay
SKBr3 cells stably expressing the 3 × κB plasmid were plated in equal number in triplicate in 24-well plates and transfected with siRNA for 72 h or treated overnight with LY294002. Cells were lysed in MPER and luciferase activity was measured with Promega Luciferase Assay System (Promega, Madison, WI, USA). Luciferase levels were normalized by protein concentration using a Bradford assay. H16N2–Her2 and MDA-MB-453 cells were transfected with siRNA 72 h before lysates were obtained, and were transfected with 3 × κB reporter plasmid and pRL–CMV (Promega) renilla plasmid 24 h before 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, CA, USA). Cells were transfected with siRNA for 48 h before seeding. Invasion assay was performed as per the manufacturer's protocol for 48 h. 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.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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References

Arihiro K, Oda H, Kaneko M, Inai K. (2000). Cytokines facilitate chemotactic motility of breast carcinoma cells. *Breast Cancer* **7**: 221–230.

Arora P, Cuevas BD, Russo A, Johnson GL, Trejo J. (2008). Persistent transactivation of EGFR and ErbB2/HER2 by protease-activated receptor-1 promotes breast carcinoma cell invasion. *Oncogene* **27**: 4434–4445.

Baselga J, Tripathy D, Mendelsohn J, Baughman S, Benz CC, Dantis L *et al*. (1999). Phase II study of weekly intravenous trastuzumab (Herceptin) in patients with HER2/neu-overexpressing metastatic breast cancer. *Semin Oncol* **26**: 78–83.

Basseres DS, Baldwin AS. (2006). Nuclear factor-kappaB and inhibitor of kappaB kinase pathways in oncogenic initiation and progression. *Oncogene* **25**: 6817–6830.

Belguise K, Sonenshein GE.. (2007). PKCtheta promotes c-Rel-driven mammary tumorigenesis in mice and humans by repressing estrogen receptor alpha synthesis. *J Clin Invest* **117**: 4009–4021.

Bhaumik D, Scott GK, Schokrpur S, Patil CK, Campisi J, Benz CC. (2008). Expression of microRNA-146 suppresses NF-kappaB activity with reduction of metastatic potential in breast cancer cells. *Oncogene* **27**: 5643–5647.

Biswas DK, Shi Q, Baily S, Strickland I, Ghosh S, Pardee AB *et al*. (2004). NF-kappa B activation in human breast cancer specimens and its role in cell proliferation and apoptosis. *Proc Natl Acad Sci USA* **101**: 10137–10142.

Bonizzi G, Karin M. (2004). The two NF-kappaB activation pathways and their role in innate and adaptive immunity. *Trends Immunol* **25**: 280–288.

Cao Y, Luo JL, Karin M. (2007). I{kappa}B kinase {alpha} kinase activity is required for self-renewal of ErbB2/Her2-transformed mammary tumor-initiating cells. *Proc Natl Acad Sci USA* **104**: 15852–15857.

Chavey C, Bibeau F, Gourgou-Bourgade S, Burlinchon S, Boissiere F, Laune D *et al*. (2007). Oestrogen receptor negative breast cancers exhibit high cytokine content. *Breast Cancer Res* **9**: R15.

Chen D, Xu LG, Chen L, Li L, Zhai Z, Shu HB. (2003). NIK is a component of the EGF/heregulin receptor signaling complexes. *Oncogene* **22**: 4348–4355.

Cogswell PC, Guttridge DC, Funkhouser WK, Baldwin Jr AS. (2000). Selective activation of NF-kappa B subunits in human breast cancer: potential roles for NF-kappa B2/p52 and for Bcl-3. *Oncogene* **19**: 1123–1131.

Dan HC, Cooper MJ, Cogswell PC, Duncan JA, Ting JP, Baldwin AS. (2008). Akt-dependent regulation of NF-{kappa}B is controlled by mTOR and Raptor in association with IKK. *Genes Dev* **22**: 1490–1500.

Dillon RL, White DE, Muller WJ. (2007). The phosphatidyl inositol 3-kinase signaling network: implications for human breast cancer. *Oncogene* **26**: 1338–1345.

Ethier SP, Mahacek ML, Gullick WJ, Frank TS, Weber BL. (1993). Differential isolation of normal luminal mammary epithelial cells and breast cancer cells from primary and metastatic sites using selective media. *Cancer Res* **53**: 627–635.

Galang CK, Garcia-Ramirez J, Solski PA, Westwick JK, Der CJ, Neznanov NN *et al*. (1996). Oncogenic Neu/ErbB-2 increases ets, AP-1, and NF-kappaB-dependent gene expression, and inhibiting ets activation blocks Neu-mediated cellular transformation. *J Biol Chem* **271**: 7992–7998.

Ghosh S, Karin M. (2002). Missing pieces in the NF-kappaB puzzle. *Cell* **109**(Suppl): S81–S96.

Gilmore TD. (2006). Introduction to NF-kappaB: players, pathways, perspectives. *Oncogene* **25**: 6680–6684.

Gum R, Wang SW, Lengyel E, Yu D, Hung MC, Juarez J *et al*. (1995). Upregulation of urokinase-type plasminogen activator expression by the HER2/neu proto-oncogene. *Anticancer Res* **15**: 1167–1172.

Habib AA, Chatterjee S, Park SK, Ratan RR, Lefebvre S, Vartanian T. (2001). The epidermal growth factor receptor engages receptor interacting protein and nuclear factor-kappa B (NF-kappa B)-inducing kinase to activate NF-kappa B. Identification of a novel receptor-tyrosine kinase signalosome. *J Biol Chem* **276**: 8865–8874.

Hayden MS, Ghosh S. (2004). Signaling to NF-kappaB. *Genes Dev* **18**: 2195–2224.

Hegde PS, Rusnak D, Bertiaux M, Alligood K, Strum J, Gagnon R *et al*. (2007). Delineation of molecular mechanisms of sensitivity to lapatinib in breast cancer cell lines using global gene expression profiles. *Mol Cancer Ther* **6**: 1629–1640.

Hynes NE, Stern DF. (1994). The biology of erbB-2/neu/HER-2 and its role in cancer. *Biochim Biophys Acta* **1198**: 165–184.

Klapper LN, Kirschbaum MH, Sela M, Yarden Y. (2000). Biochemical and clinical implications of the ErbB/HER signaling network of growth factor receptors. *Adv Cancer Res* **77**: 25–79.

Knuefermann C, Lu Y, Liu B, Jin W, Liang K, Wu L *et al*. (2003). HER2/PI-3K/Akt activation leads to a multidrug resistance in human breast adenocarcinoma cells. *Oncogene* **22**: 3205–3212.

Lam LT, Davis RE, Ngo VN, Lenz G, Wright G, Xu W *et al*. (2008). Compensatory IKKalpha activation of classical NF-kappaB signaling during IKKbeta inhibition identified by an RNA interference sensitization screen. *Proc Natl Acad Sci USA* **105**: 20798–20803.

Li YM, Pan Y, Wei Y, Cheng X, Zhou BP, Tan M *et al*. (2004). Upregulation of CXCR4 is essential for HER2-mediated tumor metastasis. *Cancer Cell* **6**: 459–469.

Ma L, Teruya-Feldstein J, Weinberg RA. (2007). Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. *Nature* **449**: 682–688.

Makino K, Day CP, Wang SC, Li YM, Hung MC. (2004). Upregulation of IKKalpha/IKKbeta by integrin-linked kinase is required for HER2/neu-induced NF-kappaB antiapoptotic pathway. *Oncogene* **23**: 3883–3887.

Mayo MW, Wang CY, Cogswell PC, Rogers-Graham KS, Lowe SW, Der CJ *et al*. (1997). Requirement of NF-kappaB activation to suppress p53-independent apoptosis induced by oncogenic Ras. *Science* **278**: 1812–1815.

Papa S, Bubici C, Zazzeroni F, Pham CG, Kuntzen C, Knabb JR *et al*. (2006). The NF-kappaB-mediated control of the JNK cascade in the antagonism of programmed cell death in health and disease. *Cell Death Differ* **13**: 712–729.

Pegram MD, Lipton A, Hayes DF, Weber BL, Baselga JM, Tripathy D *et al*. (1998). Phase II study of receptor-enhanced chemosensitivity using recombinant humanized anti-p185HER2/neu monoclonal antibody plus cisplatin in patients with HER2/

neu-overexpressing metastatic breast cancer refractory to chemotherapy treatment. *J Clin Oncol* **16**: 2659–2671.

Pianetti S, Arsura M, Romieu-Mourez R, Coffey RJ, Sonenshein GE. (2001). Her-2/neu overexpression induces NF-kappaB via a PI3-kinase/Akt pathway involving calpain-mediated degradation of IkappaB-alpha that can be inhibited by the tumor suppressor PTEN. *Oncogene* **20**: 1287–1299.

Sakurai H, Chiba H, Miyoshi H, Sugita T, Toriumi W. (1999). IkappaB kinases phosphorylate NF-kappaB p65 subunit on serine 536 in the transactivation domain. *J Biol Chem* **274**: 30353–30356.

She QB, Chandarlapaty S, Ye Q, Lobo J, Haskell KM, Leander KR *et al*. (2008). Breast tumor cells with PI3K mutation or HER2 amplification are selectively addicted to Akt signaling. *PLoS ONE* **3**: e3065.

Singh S, Shi Q, Bailey ST, Palczewski MJ, Pardee AB, Iglehart JD *et al*. (2007). Nuclear factor-kappaB activation: a molecular therapeutic target for estrogen receptor-negative and epidermal growth factor receptor family receptor-positive human breast cancer. *Mol Cancer Ther* **6**: 1973–1982.

Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* **235**: 177–182.

Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE *et al*. (1989). Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* **244**: 707–712.

Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A *et al*. (2001). Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* **344**: 783–792.

Solt LA, May MJ. (2008). The IkappaB kinase complex: master regulator of NF-kappaB signaling. *Immunol Res* **42**: 3–18.

Steinbrecher KA, Wilson III W, Cogswell PC, Baldwin AS. (2005). Glycogen synthase kinase 3beta functions to specify gene-specific, NF-kappaB-dependent transcription. *Mol Cell Biol* **25**: 8444–8455.

Vazquez-Martin A, Colomer R, Menendez JA. (2008). Her-2/neu-induced 'cytokine signature' in breast cancer. *Adv Exp Med Biol* **617**: 311–319.

Verma IM, Stevenson JK, Schwarz EM, Van Antwerp D, Miyamoto S. (1995). Rel/NF-kappa B/I kappa B family: intimate tales of association and dissociation. *Genes Dev* **9**: 2723–2735.

Wang W, Abbruzzese JL, Evans DB, Chiao PJ. (1999). Overexpression of urokinase-type plasminogen activator in pancreatic adenocarcinoma is regulated by constitutively activated RelA. *Oncogene* **18**: 4554–4563.

Weih F, Caamano J. (2003). Regulation of secondary lymphoid organ development by the nuclear factor-kappaB signal transduction pathway. *Immunol Rev* **195**: 91–105.

Wilson III W, Baldwin AS. (2008). Maintenance of constitutive IkappaB kinase activity by glycogen synthase kinase-3alpha/beta in pancreatic cancer. *Cancer Res* **68**: 8156–8163.

Xia W, Mullin RJ, Keith BR, Liu LH, Ma H, Rusnak DW *et al*. (2002). Anti-tumor activity of GW572016: a dual tyrosine kinase inhibitor blocks EGF activation of EGFR/erbB2 and downstream Erk1/2 and AKT pathways. *Oncogene* **21**: 6255–6263.

Zhou H, Kim YS, Peletier A, McCall W, Earp HS, Sartor CI. (2004). Effects of the EGFR/HER2 kinase inhibitor GW572016 on EGFR- and HER2-overexpressing breast cancer cell line proliferation, radiosensitization, and resistance. *Int J Radiat Oncol Biol Phys* **58**: 344–352.

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)