Cell

Integrative Genomic Approaches Identify *IKBKE* as a Breast Cancer Oncogene

Jesse S. Boehm,[1,3,4,9,10] Jean J. Zhao,[2,7,10,*] Jun Yao,[1,3,10] So Young Kim,[1,3,4,5,9] Ron Firestein,[1,3,4,8,9] Ian F. Dunn,[1,3,4,6,9] Sarah K. Sjostrom,[1,3,4,9] Levi A. Garraway,[1,3,5,9] Stanislawa Weremowicz,[8] Andrea L. Richardson,[8] Heidi Greulich,[1,5,9] Carly J. Stewart,[1,3,4,9] Laura A. Mulvey,[1,3] Rhine R. Shen,[1,3,4,9] Lauren Ambrogio,[1,9] Tomoko Hirozane-Kishikawa,[2,4] David E. Hill,[2,4] Marc Vidal,[2,4] Matthew Meyerson,[1,8,9] Jennifer K. Grenier,[9] Greg Hinkle,[1,4,9] David E. Root,[9] Thomas M. Roberts,[2,11] Eric S. Lander,[9,11] Kornelia Polyak,[1,3,5,11] and William C. Hahn[1,3,4,5,9,11,*]

[1] Department of Medical Oncology
[2] Department of Cancer Biology
[3] Center for Cancer Genome Discovery
[4] Center for Cancer Systems Biology
Dana-Farber Cancer Institute, Boston, MA 02115, USA
[5] Department of Medicine
[6] Department of Neurosurgery
[7] Department of Surgery
[8] Department of Pathology
Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA
[9] Broad Institute of Harvard and MIT, Cambridge, MA 02142, USA
[10] These authors contributed equally to this work.
[11] These senior authors contributed equally to this work.
*Correspondence: william_hahn@dfci.harvard.edu (W.C.H.), jean_zhao@dfci.harvard.edu (J.J.Z.)
DOI 10.1016/j.cell.2007.03.052

SUMMARY

The karyotypic chaos exhibited by human epithelial cancers complicates efforts to identify mutations critical for malignant transformation. Here we integrate complementary genomic approaches to identify human oncogenes. We show that activation of the ERK and phosphatidylinositol 3-kinase (PI3K) signaling pathways cooperate to transform human cells. Using a library of activated kinases, we identify several kinases that replace PI3K signaling and render cells tumorigenic. Whole genome structural analyses reveal that one of these kinases, *IKBKE* (IKKε), is amplified and overexpressed in breast cancer cell lines and patient-derived tumors. Suppression of IKKε expression in breast cancer cell lines that harbor *IKBKE* amplifications induces cell death. IKKε activates the nuclear factor-kappaB (NF-κB) pathway in both cell lines and breast cancers. These observations suggest a mechanism for NF-κB activation in breast cancer, implicate the NF-κB pathway as a downstream mediator of PI3K, and provide a framework for integrated genomic approaches in oncogene discovery.

INTRODUCTION

Nearly all epithelial malignancies exhibit numerous karyotypic abnormalities and abundant evidence of widespread genetic alterations. This complexity of genome structure complicates efforts to delineate the roles of these mutations in cancer initiation and maintenance. However, the recent development of several complementary experimental approaches now provides the tools necessary to systematically deconvolute the complexity of somatic cancer genetics.

One experimental approach is to identify genes and proteins that drive cellular transformation through the construction of experimental models of cancer. Together with increasingly sophisticated genetically engineered murine cancer models, human cell-based systems enable the examination of the roles of specific mutations implicated in cell transformation. Recent work indicates that a limited set of genetic changes suffice to transform human cells (Hahn et al., 1999). Specifically, the coexpression of the telomerase catalytic subunit (*hTERT*), the SV40 Large T (LT) and small t (ST) oncoproteins, and an activated allele of *H-RAS* (H-RAS^{V12}) render a wide range of human cells tumorigenic (Zhao et al., 2004). These models have facilitated the identification of candidate oncogenes and tumor suppressor genes (Rowland et al., 2005; Westbrook et al., 2005). These approaches facilitate the identification of genes that have the potential to drive tumorigenesis but do not

alone establish whether these genes truly play a role in human cancer.

A second approach is to characterize the genomic alterations seen in cancer specimens using increasingly powerful tools to identify amplifications, deletions, karyotypic rearrangements, and point mutations. Some recurrent amplifications identified by genome scale approaches such as high-density single-nucleotide polymorphism (SNP) arrays and array comparative genomic hybridization (aCGH) contain well-characterized oncogenes such as *EGFR* (Zhao et al., 2005) and *c-MYC* (Morrison et al., 2005). These observations suggest other regions recurrently amplified in human tumors contain other yet-to-be-characterized oncogenes. Indeed, recent applications of these technologies led to the identification of *MITF*, *c-IAP1*, and *YAP* as human oncogenes (Garraway et al., 2005; Zender et al., 2006). However, amplified regions are often large, spanning several megabases to entire chromosome arms. Therefore, functional tools are needed to pinpoint specific genes that drive transformation targeted by copy-number alterations.

A third approach is the systematic modulation of the expression of genes encoded in regions of chromosomal imbalance. Although such studies have previously required painstaking gene-by-gene analyses, RNA interference (RNAi) now provides the means to define the functional consequences of gene copy-number changes in a rapid and comprehensive manner. Indeed, several large RNAi libraries now exist and have been used to examine the function of genes in a high-throughput manner (Berns et al., 2004; Paddison et al., 2004), including those necessary for cancer cell viability (Moffat et al., 2006; Ngo et al., 2006).

In principle, it should be possible to integrate such approaches to develop a framework to identify and validate genes critical to cancer development. Here we have applied such an integrative genomic approach to the Ras signaling pathway. The Ras signaling pathway plays a critical role in coupling inputs from cell-surface receptors to intracellular signaling pathways. Mutations of receptor tyrosine kinases such as *EGFR* (Lynch et al., 2004; Paez et al., 2004), of Ras proteins themselves (Downward, 2003) or of proteins in Ras effector pathways including those regulated by Raf/ERK/MAPK (Davies et al., 2002) or PI3K (Samuels et al., 2004) occur in most, if not all, epithelial cancers. Using these integrated approaches, we identify a kinase oncogene, *IKBKE*, which acts downstream of AKT and is amplified in a substantial percentage of human breast tumors.

RESULTS

Contributions of Ras Effector Pathways to Human Cell Transformation

We previously showed that immortalized human embryonic kidney (HEK) epithelial cells expressing *hTERT*, LT, and ST (HA1E), are rendered tumorigenic by the introduction of H-RAS^{V12} (Hahn et al., 1999). Activation of Ras stimulates many pathways including, but not limited to, the MAPK, PI3K, and RALGDS pathways (Downward, 2003). However, it is likely that only a subset of these pathways participate directly in H-RAS^{V12}-induced transformation. Since activating mutations are found in several members of the MAPK and PI3K pathways in many human cancers, we reasoned that the coactivation of these two pathways might replace H-RAS^{V12} in transformation. To test this hypothesis, we manipulated the MAPK and PI3K pathways in HA1E cells.

To activate the PI3K pathway, we expressed one of three mutant alleles: myristoylated (myr) and therefore constitutively active alleles of *PIK3CA* p110α (Klippel et al., 1996) (myr-110α) or *AKT1* (myr-AKT) (Kohn et al., 1996) or a short hairpin RNA (shRNA) targeting *PTEN* (Boehm et al., 2005). To activate the MAPK pathway, we expressed one of three mutant alleles: an activated allele of *CRAF* (C-Raf 22W) (Stanton et al., 1989), the *BRAF*^{E600} allele (Davies et al., 2002), or a constitutively active *MEK1*^{D218,D222} allele (MEK^{DD}) (Brunet et al., 1994). Using these reagents, we created 16 cell lines expressing all possible combinations of these mutant alleles. We confirmed that these cell lines stably expressed each of these activated alleles either singly or in pairwise combinations (data not shown) and analyzed the ability of these cells to grow in an anchorage-independent (AI) manner or to form tumors in immunodeficient animals (Table 1).

When introduced singly, none of these mutant alleles promoted AI colony formation (Table 1). However, we found that certain combinations of activated alleles substituted for H-RAS^{V12} to render these immortalized HEK cells tumorigenic (Table 1). Specifically, we found that activation of the MAPK pathway by either *BRAF*^{E600} or MEK^{DD} expression together with activation of the PI3K pathway by myr-AKT expression induced AI growth indistinguishable from that induced by H-RAS^{V12} (Table 1, upper panel, and Figure 1). Surprisingly, while either *BRAF*^{E600} or MEK^{DD} cooperated with myr-AKT to induce AI growth, only cells expressing the MEK^{DD} + myr-AKT combination induced tumor growth comparable to that observed in cells expressing H-RAS^{V12} (Table 1, lower panel, and Figure 1). These observations indicated that coactivation of specific members of the MAPK and PI3K pathways suffices to replace H-RAS^{V12} in human cell transformation. In these HEK cells, coexpression of MEK^{DD} and myr-AKT most closely recapitulated the tumorigenic phenotype induced by H-RAS^{V12} (Figure 1).

Identification of Transforming Kinases

This approach not only identified specific combinations of activated members of the MAPK and PI3K pathways that replace H-RAS^{V12} in HEK cell transformation but also established an experimental platform to discover other transforming genes. We extended this work by screening a large collection of genes to identify those that substitute for myr-AKT. Because phosphorylation plays a key role in Ras signalling, we hypothesized that other kinases may participate in PI3K-induced transformation.

Table 1. Dissection of H-RAS^{V12} Signaling in Transformation

Anchorage-Independent Growth

	Vector	C-Raf 22W	B-Raf^{E600}	MEK^{DD}
Vector	–	–	–	±
PTEN-shRNA	–	+	–	–
myr-110α	–	–	–	+
myr-AKT	±	+	+++	+++
H-RAS^{V12}	+++	ND	ND	ND

Tumor Formation

	Vector	C-Raf 22W	B-Raf^{E600}	MEK^{DD}
Vector	–	–	–	–
PTEN-shRNA	–	–	–	–
myr-110α	–	–	–	++
myr-AKT	–	–	–	+++
H-RAS^{V12}	+++	ND	ND	ND

Transformation of HEK cells expressing *hTERT*, the SV40ER plus the indicated constructs. Transformation was assessed by both anchorage-independent growth (upper) and the capacity for tumor formation in immunodeficient mice (lower). Colony formation was scored as follows: (–): no colonies, (±): few microscopic (100–200 μm) colonies, (+): many microscopic colonies, (++): microscopic- and small macroscopic (200 μm–1 mm) colonies, (+++): small macroscopic and large macroscopic (>1 mm) colonies. Tumors (3 injections per cell line) were scored as follows: (–): no tumors, (+): single tumor >8 week latency, (++): single tumor <8 week latency or multiple tumors >8 week latency, (+++): multiple tumors <8 week latency. ND, not determined.

To identify such kinases, we created a library of 354 human kinases and kinase-related open reading frames (ORFs), including 256 kinases (Table S1), cloned into Gateway-compatible entry vectors (Rual et al., 2004). Because membrane recruitment via the addition of a myr sequence activates both the PI3K p110α subunit and *AKT* (Klippel et al., 1996; Kohn et al., 1996), we created a Gateway-compatible retroviral destination vector, pWN-MF-DEST, which adds a myr sequence and a FLAG-epitope tag (MF) to each introduced ORF. We confirmed that this destination vector and myr tag were functional (Figure S1). We then transferred each ORF individually into pWN-MF-DEST (Figure 2A).

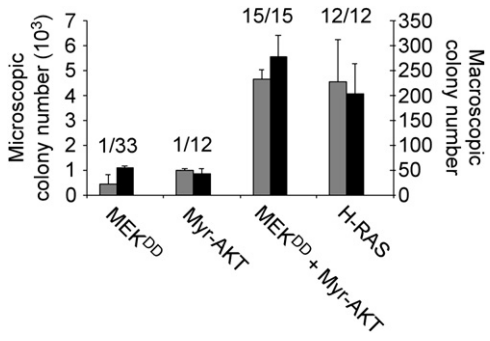

Figure 1. Activated *MEK1* and *AKT1* Cooperate to Replace *H-RAS^{V12}*

AI microscopic (100–200 μm, gray), macroscopic (>200 μm, black) colonies, and tumor formation (number of tumors formed/tumor sites injected) are shown for HEK cells expressing *hTERT*, LT, ST, and the indicated genes. Error bars represent mean ±SD for three independent experiments.

Using this library of 354 myr ORFs, we performed a screen for genes that could replace myr-AKT and induce transformation. We introduced pools consisting of 10–12 unique ORFs into immortal but nontumorigenic HEK cells expressing MEK^{DD} (HA1E-M). Several pools reproducibly induced colony formation more than two standard deviations greater than the median number of colonies (Figure 2B). To determine the identity of the kinases in these pools responsible for AI growth, we then created cell lines expressing individual kinases from each of such pools (Figure 2C). Of the four pools analyzed in this manner, three contained one transforming kinase gene and one pool contained two transforming kinase genes. Each of these kinase genes (*AKT1*, *IKBKE*, *DAK*, *TSSK6*, and *CSNK1E)* also induced tumor formation (Figure 2D and data not shown). Since we had previously shown that myr-AKT cooperates with MEK^{DD} to transform these immortalized cells, the identification of *AKT1* validated this screening approach. Thus, using a library of myr kinases in retroviral vectors, we identified four kinases that cooperate with activated *MEK1* to replace H-RAS^{V12} in human cell transformation.

Amplification of *IKBKE* in Breast Cancer Cell Lines and Patient-Derived Tumor Samples

We next explored whether any of these kinases are altered in human cancers. First, we used high-density SNP arrays to identify regions of copy-number gain or loss in 179 cell lines

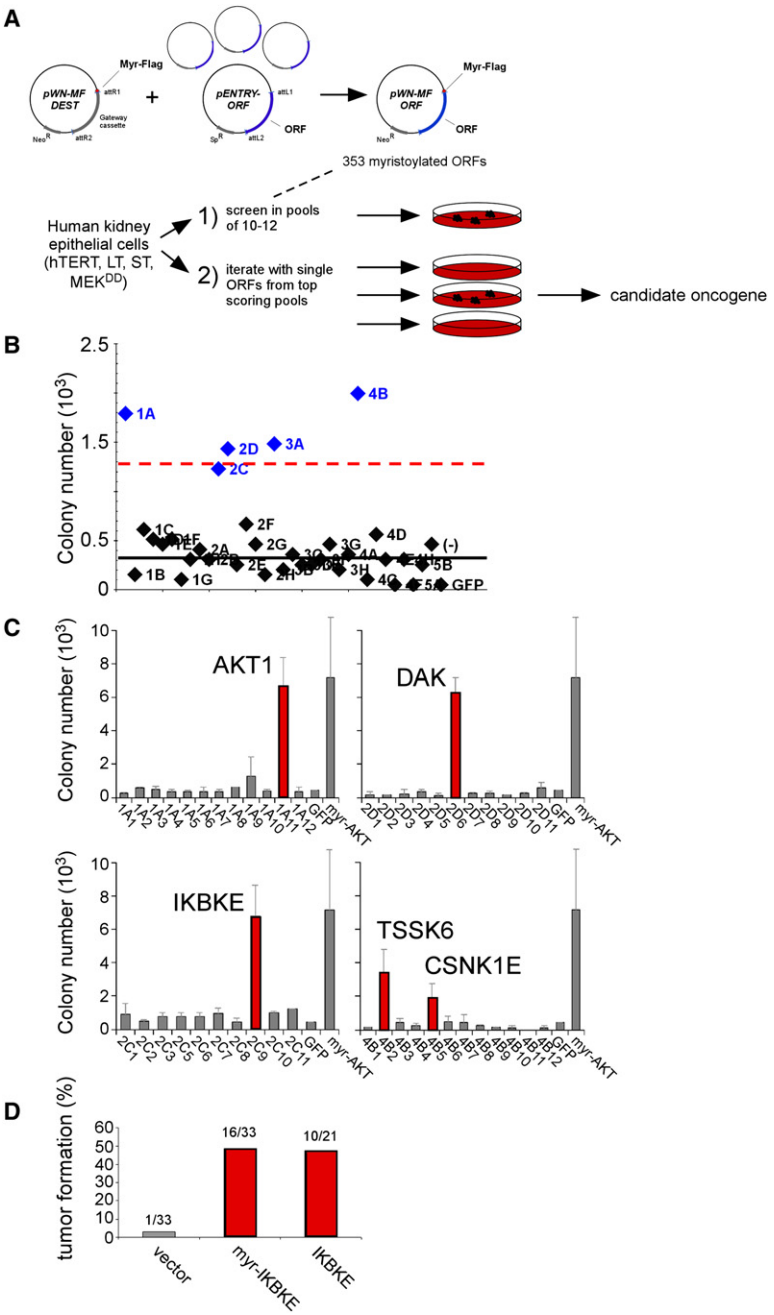

Figure 2. Identification of Oncogenes Using a Myristoylated Kinase Library

(A) Experimental schema.

(B) Al growth of pools named by their plate number (1–5) and row (A–H). (–) and GFP indicate the number of colonies produced by mock- and GFP-vector- infected cells, respectively. Black line and red dotted line indicate the median and 2 SD above the median, respectively.

(C) Identification of putative kinase oncogenes from top scoring pools. Error bars indicate mean ± SD for three independent experiments.

(D) Tumor formation by *IKBKE* in HA1E-M cells. Tumorigenicity is indicated as the number of tumors formed/number of tumor sites injected and shown above the bars for each cell line.

representing a wide spectrum of cancer types (Garraway et al., 2005). We failed to find clear evidence of amplifications of the loci encoding *DAK*, *TSSK6*, or *CSNK1E* in any of these cell lines (data not shown). In contrast, we found copy-number gain or amplification of 1q32 involving the *IKBKE* locus in 8 of 49 (16.3%) breast cancer cell lines (Figure 3A). Indeed, copy-number gain of 1q is the most common alteration in breast cancers (Rennstam et al., 2003).

We next investigated whether *IKBKE* was similarly amplified in primary human breast cancer specimens by performing aCGH on 30 breast tumors representing different clinicopathological stages (Yao et al., 2006). These studies also revealed a region of recurrent copy-number gain involving the *IKBKE* locus on chromosome 1q in 10 of 30 breast tumors (Figure 3B). We note that four of the ten ductal carcinoma in situ (DCIS) cases analyzed showed copy-number gain of *IKBKE*, suggesting that amplification of this locus is an early event in the development of some breast cancers. We failed to find an association of *IKBKE* copy-number gain with estrogen receptor or Her2/neu status or with the presence of lymph node involvement at diagnosis. In addition, we sequenced the

IKBKE gene in 28 breast tumors and 19 breast cancer cell lines and failed to find evidence of somatic mutations, corroborating other sequencing efforts (Bamford et al., 2004).

To confirm that the region of copy-number gain at 1q32 involves *IKBKE*, we performed fluorescence in situ hybridization (FISH) on several breast cancer cell lines and a primary breast tumor containing an amplification of 1q32 (Figure 3C). We detected up to ten copies of *IKBKE* in the UACC-812 and ZR-75-1 breast cancer cell lines and five copies of *IKBKE* in MCF-7 cells. One of the DCIS breast tumors, BWH-T18, harbored five copies of the *IKBKE* locus in the majority of nuclei (Figure 3C).

We also investigated whether these DNA copy-number changes corresponded to increases in *IKBKE* transcript and protein levels. Indeed, serial analysis of gene expression (SAGE), quantitative RT-PCR, and immunoblotting analyses confirmed that *IKBKE* (IKKε) is overexpressed in cell lines and breast tumors that exhibited 1q32 gain, suggesting that *IKBKE* is one target of this amplicon (Figures 3D and 3E and data not shown). In addition, we found that an additional 13 of 17 cell lines (76.5%) and 10 of 21 breast cancers (47.6%) showed increased expression (>2-fold) of *IKBKE* transcripts in the absence of 1q32 copy-number changes. Utilizing two different IKKε antibodies, the observed protein levels correlated with the transcript levels (Figures 3D and 3E) and UACC-812 and ZR-75-1 cells, which harbor 1q32 amplifications (Figures 3A and 3C), showed the highest levels of IKKε of any cell line analyzed (Figure 3E).

Although our initial experiments were performed in immortalized HEK cells, we also found that the introduction of *IKBKE* transforms human mammary epithelial cells (HMEC) expressing *hTERT*, the SV40ER, and MEK^{DD} (HMEC-M) (Figure 3F). In addition, since 1q32 amplifications result in increased levels of wild-type (WT) *IKBKE*, we examined whether forced expression of the WT, nonmyr *IKBKE* allele also rendered cells tumorigenic. We found that cells expressing WT *IKBKE* at levels found in human cancer cells exhibited a similar capacity for tumorigenicity (Figures 2D and 3G), confirming that the allele amplified in breast cancer specimens is transforming. Together, these observations identified *IKBKE* as a transforming kinase that is amplified and overexpressed in a substantial fraction of breast cancer cell lines and primary breast tumors.

Consequences of Suppressing *IKBKE* in Breast Cancer Cells

The findings described above implicate *IKBKE* as breast cancer oncogene. We next sought to explore whether breast cancer cells are functionally dependent on *IKBKE* expression. The unregulated expression of many oncogenes renders cancer cells unusually dependent upon the continued function of the oncogene for cell proliferation or viability. This property of oncogenes, termed oncogene addiction, provides a rationale for targeting oncogenes therapeutically. To determine whether the presence of *IKBKE* amplifications and overexpression induces oncogene addiction, we examined the effects of RNAi targeting *IKBKE* and other genes.

As part of a separate project to systematically discover genes essential for cancer cell viability we have used the shRNA library developed by the RNAi Consortium (TRC) (Moffat et al., 2006) to screen a large collection of genes required for the proliferation of cancer cell lines. As part of this effort, we have screened the MCF-7 breast cancer cell line with 6144 shRNA targeting 1200 genes, including 93% of the human kinome. The results from the screening of MCF-7 cells are of particular relevance to the present study, because this breast cancer cell line harbors a 1q32 copy-number gain (Figures 3A and 3C) that results in a modest 2.5-fold increase in *IKBKE* transcript levels compared to immortalized MCF10A cells (Figure 3D). Examining the data from this screen, we found that three of five shRNA targeting *IKBKE* compromised cell proliferation and viability (Figures 4A, 4C, and 4D). We found that the level of IKKε suppression by these five shRNA correlated with the observed decrease in cell proliferation and viability in MCF-7 cells (Figures 4A, 4C, and 4D). These observations indicate that *IKBKE* is required for the proliferation and survival of MCF-7 breast cancer cells.

We note that this RNAi screen also included constructs targeting three of the other four genes that emerged from our gain-of-function screen (*AKT1, CSNK1E*, and *TSSK6*). Similar to the results from shRNA targeting *IKBKE* we observed that shRNA targeting *AKT1* or *CSNK1E* also inhibited MCF-7 cell proliferation (data not shown).

We next examined the data generated in the high-throughput RNAi screen to determine whether any other kinase encoded within the 1q32 amplicon was similarly required for MCF-7 viability. We identified a minimally amplified region (5.6 Mb) containing *IKBKE* in the UACC-812 breast cancer cell line (Figure 3A). This minimally amplified region contains nine additional kinase genes. However, RNAi-mediated suppression of these other kinase genes failed to induce a statistically significant decrease in viability of MCF-7 cells (Figure 4B). We note that three of four shRNA targeting *NUAK2* partially inhibited the proliferation of MCF-7 cells but failed to satisfy the criteria employed to classify significantly decreased proliferation in our high-throughput RNAi screen (See Experimental Procedures). Although these observations do not eliminate the possibility that other genes within this 1q32 amplicon may cooperate with *IKBKE* to induce transformation, these findings suggest that *IKBKE* is a key target of the 1q32 amplification in breast cancer cell lines and tumors.

To confirm that these observations in MCF-7 cells applied to other breast cancer cell lines that show *IKBKE* amplifications, we next examined the ZR-75-1 breast cancer cell line, which exhibits high-level amplification of *IKBKE* (Figures 3A and 3C) and found that inhibition of *IKBKE* expression in this cell line also compromised viability (Figure 4C). In contrast, shRNA-mediated suppression of *IKBKE* failed to affect viability in PC3 prostate cancer cells, *hTERT*-expressing HMECs, or the MCF10A.DCIS.com breast cancer cell line, all of which do not harbor

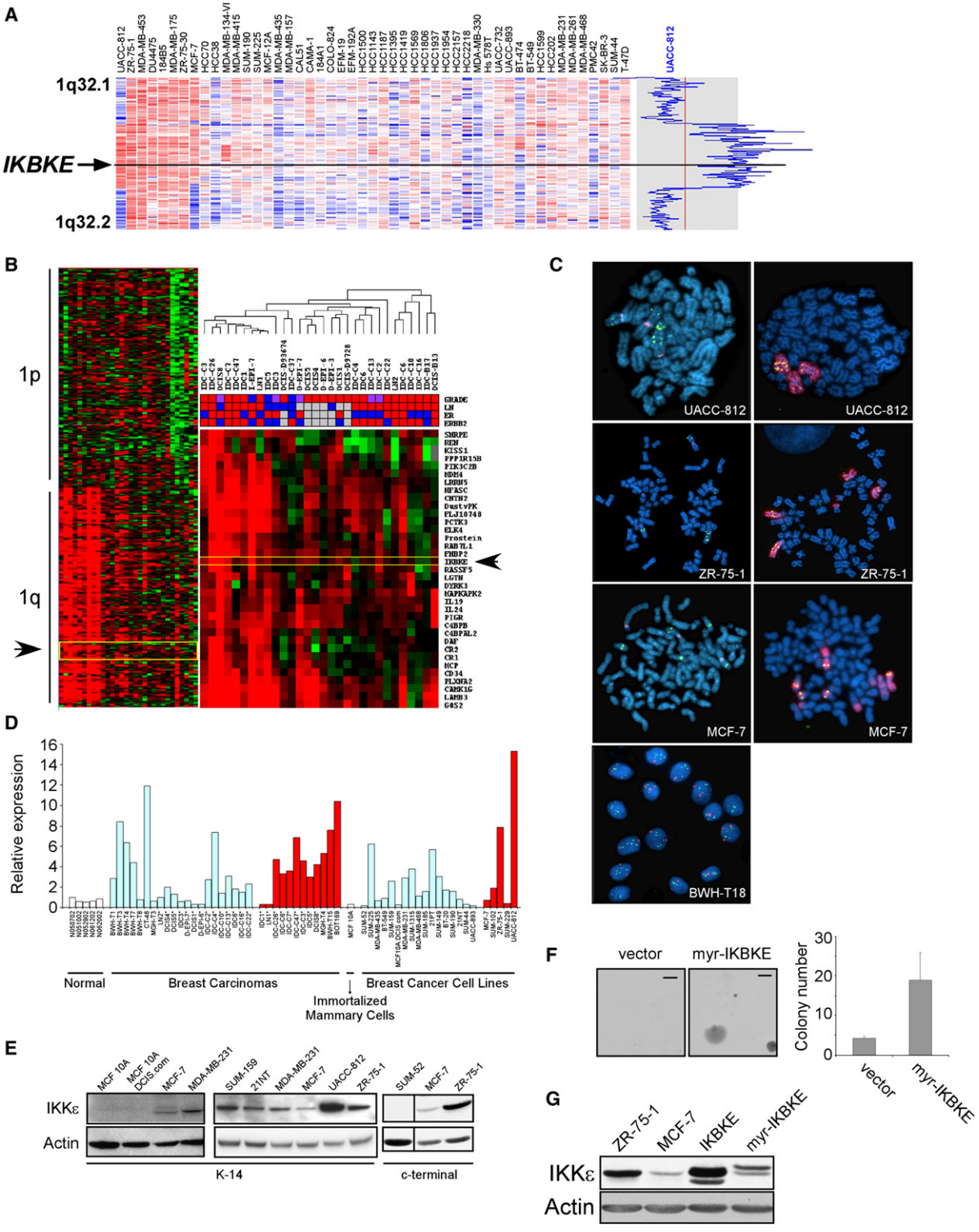

Figure 3. *IKBKE* Is Amplified in Breast Cancer Cell Lines and Tumor Samples

(A) Analysis of the *IKBKE* locus by SNP arrays in 49 breast cancer cell lines. Colorgrams representing SNP copy number at 1q32 are shown. Red indicates allelic gain, while blue denotes allelic loss.

(B) Clustering of aCGH data based on chromosome 1 copy-number gain of 30 breast primary tumors. Colored rectangles indicate tumor grade (red, high; purple, intermediate; and blue, low grade) and lymph node (LN), estrogen receptor (ER), or HER2 status (red, positive; blue, negative; and gray, unknown).

amplifications of *IKBKE* (Figure 4C). Corroborating these qualitative observations, we found that suppression of *IKBKE* in MCF10A.DCIS.com or PC3 cancer cells induced only a small increase in doubling time (9.1%–28.0%) over 11 days (Figure 4D), while suppression of *IKBKE* in MCF-7 and ZR-75-1 cells increased doubling time by 80.6%–114.5% (Figure 4D). These observations suggested that *IKBKE* is essential for proliferation in breast cancer cell lines that show increased *IKBKE* copy number.

IKKε is a member of a family of five distinct but closely related proteins, the IκB kinase (IKK) family, consisting of IKKα, IKKβ, IKKγ, IKKε, and TBK1. Since IKKε function partly overlaps with other IKKs (Fitzgerald et al., 2003; Peters et al., 2000; Sharma et al., 2003), we next determined whether the proliferation of ZR-75-1, MCF-7, or PC3 cells depended upon expression of any of the other IKK family members. We introduced five shRNA targeting each of these IKK family members into MCF-7, ZR-75-1, and PC3 cells. We required more than one shRNA construct targeting the gene to decrease proliferation (see Experimental Procedures) and found that IKKε was the only IKK family member required for proliferation of ZR-75-1 cells, while both IKKε and TBK1 were required for the proliferation of MCF-7 cells (Figure S2). We note that suppression of TBK1 but not IKKε inhibited the proliferation of PC3 cells.

Together, these observations implicate *IKBKE* as an essential oncogene in breast cancer cell lines harboring amplifications of 1q32 and are reminiscent of similar findings made in cell lines harboring other oncogenes such as *BCR-ABL* and *EGFR*, which also appear to render oncogene-expressing cells particularly dependent upon their expression (Weinstein and Joe, 2006).

Consequences of IKBKE Amplification and Overexpression

Recent work implicates *IKBKE* in the innate immune response to viral infections, as it activates the interferon pathway in part by stimulating the activity of the transcription factor IRF3 (Fitzgerald et al., 2003; Sharma et al., 2003). We tested whether cells expressing *IKBKE* displayed evidence of increased IRF3 transcriptional activity and found a 3- to 5-fold activation of the IRF3-responsive interferon-stimulated regulatory element (ISRE) of the *IFIT2* gene in HA1E-M cells expressing either the myr or WT allele of *IKBKE* (Figure S3A). HA1E-M and HMEC-M cells expressing these *IKBKE* alleles also showed increased transcription of two known interferon-responsive genes, *IFNB1* and *CCL5* (encoding RANTES) (Figures S3B and S3C).

To determine whether activation of interferon-responsive promoters was required for transformation induced by *IKBKE*, we introduced *IRF3*-specific shRNA constructs into transformed HA1E-M cells expressing myr-*IKBKE* (Figure S3D). The suppression of *IRF3* failed to suppress AI growth (Figure S3E). These observations suggested that, although the physiologic function of *IKBKE* involves activation of interferon responses, this activity of *IKBKE* may not be essential for transformation.

IKBKE also has been reported to activate the NF-κB pathway (Peters et al., 2000). Thus, we next determined whether the transforming activity of *IKBKE* is mediated by NF-κB. We examined whether the expression of *IKBKE* destabilized the cytoplasmic pool of IκBα. HA1E-M cells expressing myr-*IKBKE* displayed a 43% reduction in cytoplasmic IκBα compared to levels observed in cells expressing a control vector (Figures 5A and 5B). Moreover, even when we overexpressed IκBα in these *IKBKE*-expressing cells, we failed to observe increased IκBα protein expression (Figure 5C), indicating that IKKε effectively drives IκBα degradation. One consequence of IκBα degradation is the translocation of NF-κB transcription factors from the cytoplasm to the nucleus. Consistent with this expectation, we found that HA1E-M cells expressing myr-*IKBKE* displayed a 32% reduction in cytoplasmic NF-κB p50 (Figures 5A and 5B), and 71% of these cells displayed nuclear NF-κB p50 compared to 8% in cells harboring a control vector (Figures 5D and 5E).

We also tested whether overexpression of *IKBKE* induced NF-κB transcriptional activity. HA1E-M cells expressing myr-*IKBKE* exhibited a 5-fold higher level of NF-κB activity compared to cells expressing a control vector, as assessed by measuring the activity of a synthetic NF-κB reporter gene (Figure 5F). To confirm that *IKBKE* activated the expression of endogenous NF-κB target genes, we measured the expression levels of nine NF-κB target genes in HA1E-M or HMEC-M cells expressing myr- or WT *IKBKE*, respectively. *IKBKE* induced increased expression of all of these transcripts compared

(C) FISH analysis of *IKBKE* amplification in UACC-812, ZR-75-1, and MCF-7 breast cancer cell lines and BWH-T18 primary breast tumor. Green signal indicates hybridization using an *IKBKE*-specific BAC probe, while red color indicates hybridization using a chromosome 1 specific α satellite DNA probe (left panels) or chromosome 1 painting probe (right panels).

(D) Quantitative RT-PCR analysis of *IKBKE* expression in breast cancer cell lines and patient samples. Carcinomas and cell lines are color-coded to indicate presence of amplified 1q32 (blue, nonamplified and red, amplified). Asterisks (*) indicate patient samples with corresponding aCGH data in (B). Raw values were normalized to *RPL39* levels. Fold changes are referenced to values obtained with the normal sample with the highest *IKBKE* expression, N050702.

(E) Immunoblotting for IKKε. Using the K-14 anti-IKKε antibody (Santa Cruz, CA), immunoblot in left panel compares breast cancer and immortalized breast cell lines lacking *IKBKE* copy-number gain (MCF10A, MCF10A.DCIS.COM) to breast cancer cell lines with low levels of *IKBKE* (MCF-7, MDA-MB-231) and the middle immunoblot compares breast cancer cell lines harboring different levels of *IKBKE* copy-number gain. The immunoblot on the right was performed with a different anti-IKKε antibody (Sigma C-terminal).

(F) AI growth of HMEC-M cells harboring either a control vector (vector) or myr-*IKBKE*. Bar represents 2 mm. Colony number reflects large macroscopic colonies (>1 mm). Error bars represent mean ± standard deviation (SD) for three independent experiments.

(G) Immunblotting for IKKε in the indicated breast cancer cell lines or HMEC-M cells expressing myr- or wild-type *IKBKE*.

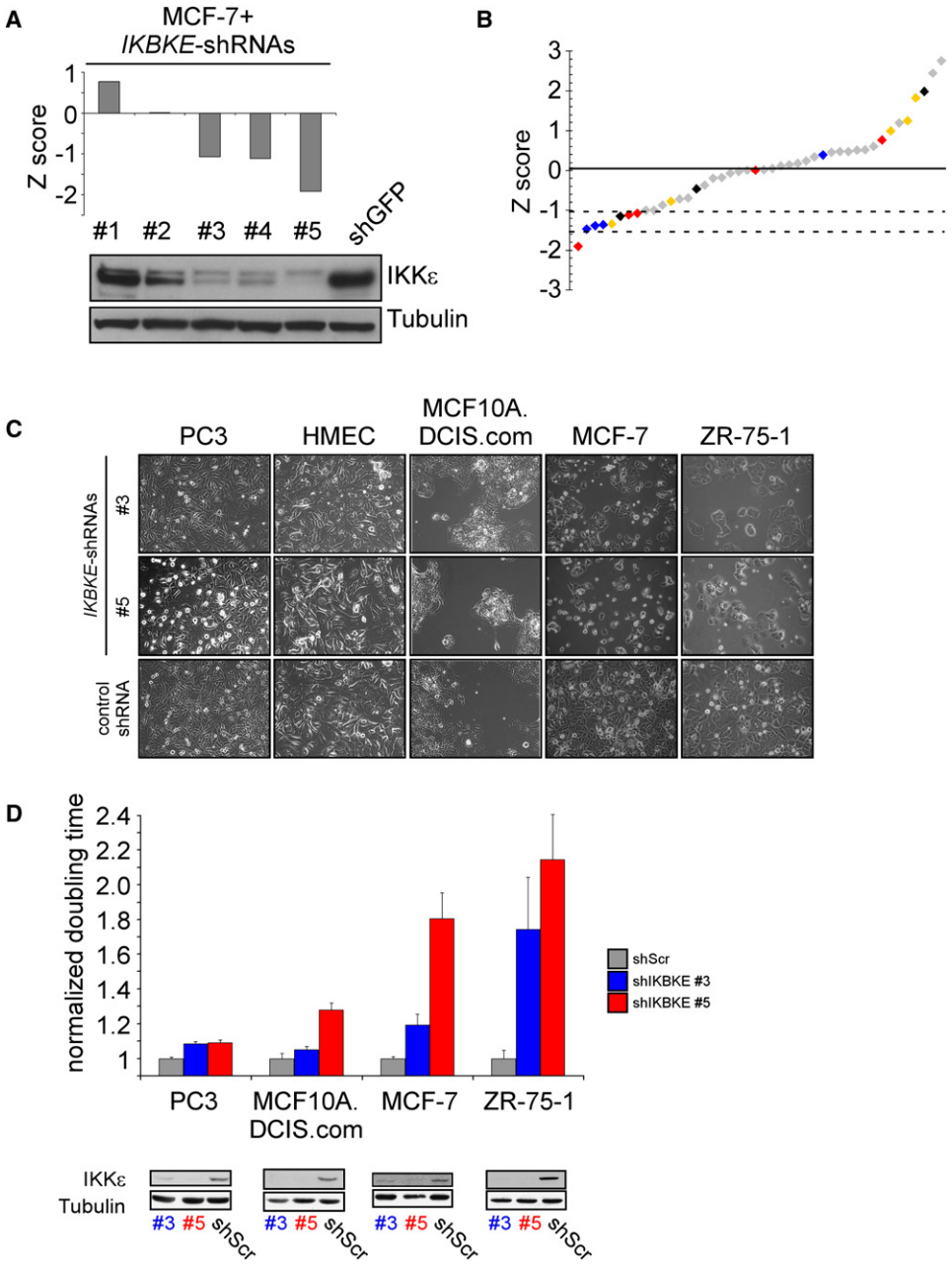

Figure 4. *IKBKE* Is an Essential Kinase in Breast Cancer Cells

(A) *IKBKE* is essential for MCF-7 cell proliferation. Lower panels show IKKε expression in MCF-7 cells expressing *IKBKE*-specific shRNAs or a control shRNA targeting *GFP* (shGFP).

(B) Effects of suppressing kinase genes located at 1q32. MCF-7 cells were infected with shRNA constructs (five per gene) targeting ten kinases (*ETNK2*, *PIK3C2B*, *RIPK5*, *DYRK3*, *IKBKE*, *MAPKAPK2*, *PCTK3*, *PFKFB2*, *NUCKS1*, and *NUAK2*) located at 1q32. Solid line represents the median and dotted lines indicate 1 and 1.5 SD below the median. shRNA targeting *IKBKE* (red), *NUAK2* (blue), *PFKFB2* (yellow), and *ETNK2* (black) or the other genes (gray) are shown.

(C) Effects of IKKε suppression on viability. Representative micrographs obtained five days after infection with the indicated shRNA are shown.

(D) Effects of IKKε suppression on proliferation. Cells were plated on day 0 and infected with *IKBKE* shRNA 24 hr later and cumulative cell numbers were determined for 11 days. The doubling time for each cell line was determined and then normalized to cells expressing a control, scrambled shRNA (shScr). Expression of IKKε is shown in lower panels for the indicated cell lines. Error bars represent mean ± SD for three independent experiments.

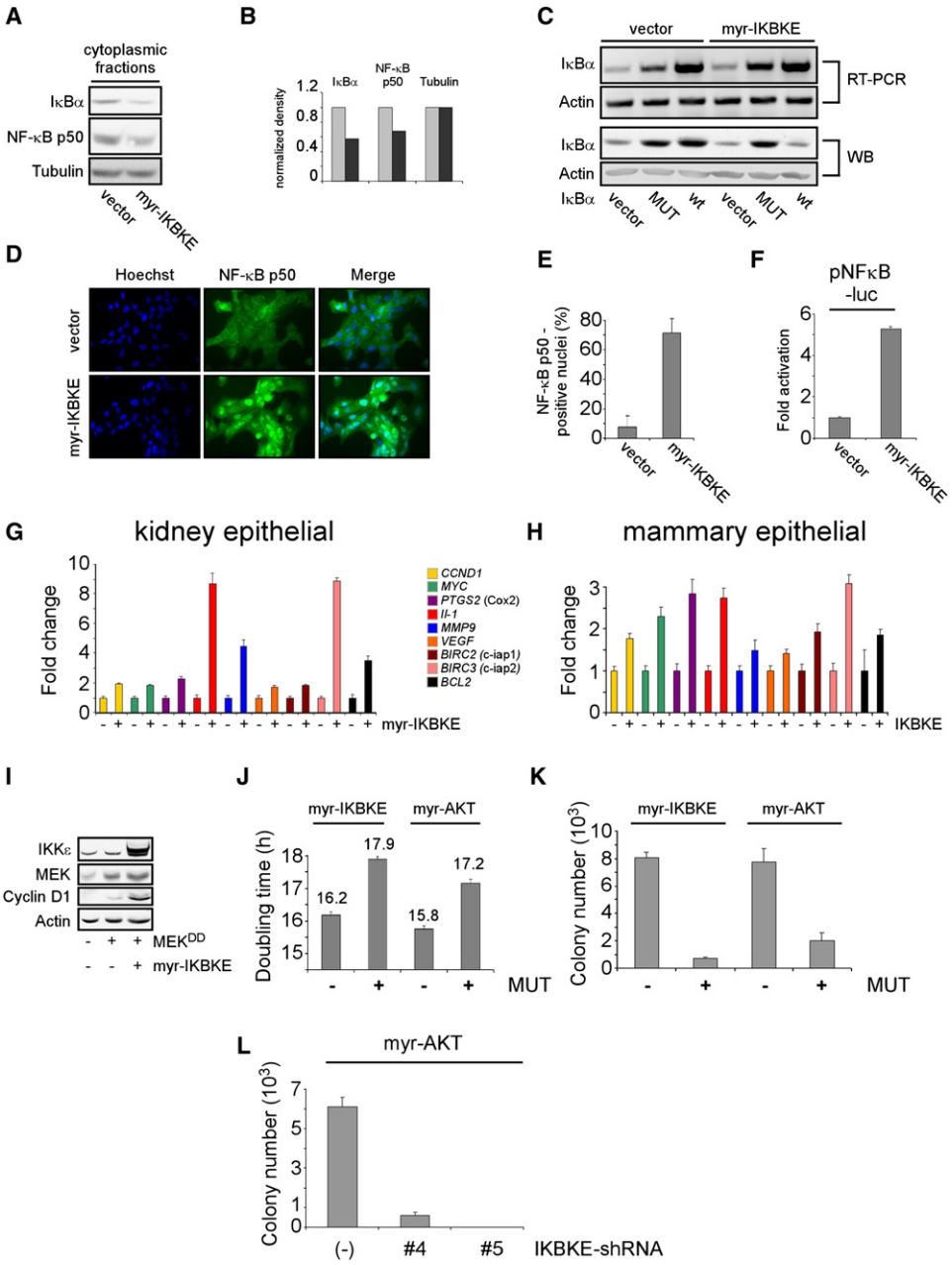

Figure 5. *IKBKE* Activates the NF-κB Pathway in Transformation Models

(A and B) Effects of IKKε expression on IκBα stability. Cytoplasmic IκBα levels in HA1E-M cells and quantification.

(C) RT-PCR and immunoblot (WB) analysis of IκBα overexpression. MUT: IκBα super-repressor, wt: wt IκBα allele.

(D and E) Localization of NF-κB p50. The antibody used here detects both NF-κB p50 and the NF-κB p50 precursor (p105).

(F) NF-κB reporter activity in the indicated cell lines.

(G) Change in expression of selected NF-κB-regulated genes in HA1E-M cells harboring either a control vector (–) or myr-*IKBKE* (+).

(H) Change in expression of selected NF-κB-regulated genes in HMEC-M cells harboring either a control vector (–) or WT *IKBKE* (+).

(I) Analysis of cyclin D1 protein levels in HA1E cells expressing MEK^{DD} and/or the indicated genes.

(J and K) Effects of expressing MUT IκBα on the doubling time (J) and AI growth (K) of HA1E-M cells expressing either myr-*IKBKE* or myr-AKT.

(L) Effects of suppressing *IKBKE* on AI growth of AKT-transformed cells. (–) indicates control cells. Error bars in (E)–(H) and (J)–(L) represent mean ± SD for three independent experiments.

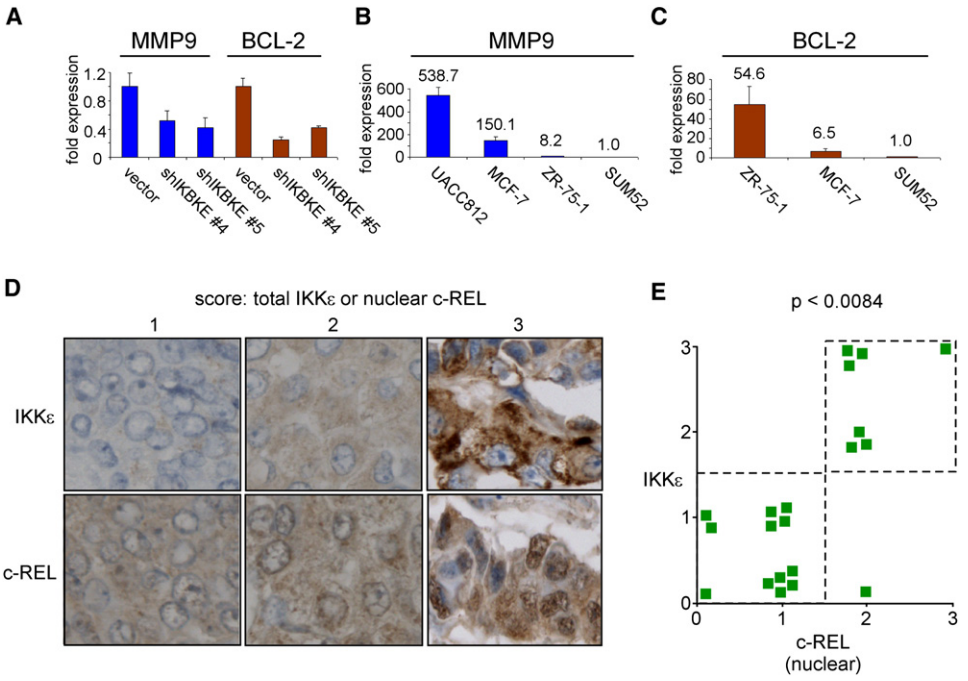

Figure 6. *IKBKE* Activates the NF-κB Pathway in Breast Cancer

(A) Downregulation of NF-κB target genes in ZR-75-1 breast cancer cells after *IKBKE* suppression as determined by quantitative RT-PCR.

(B and C) Relative expression of *MMP9* (B) and *BCL2* (C) in breast cancer cell lines that harbor (UACC-812, ZR-75-1, MCF-7) or lack (SUM52) IKKε gain or amplification. UACC-812 cells do not express *BCL2*. Error bars in (A)–(C) represent mean ± SD for three independent experiments.

(D and E) Examination of IKKε overexpression and NF-κB pathway activation in primary breast cancer specimens. In (D), representative IHC staining and scoring were used to assess IKKε and nuclear c-REL expression. 0, no staining; 1, low staining; 2, moderate staining; 3, high staining.

(E) Coexpression of IKKε and nuclear c-REL. Each point represents one breast cancer specimen. The p value determined by Fisher's exact test.

to cells expressing a control vector (Figures 6G and 6H). Since cyclin D1 is overexpressed in over 50% of breast cancers (Bartkova et al., 1994), we examined whether the observed increases in *CCND1* mRNA levels corresponded to increased cyclin D1 protein levels. Indeed, both HA1E-M or HMEC-M cells expressing myr-*IKBKE* showed substantially elevated levels of cyclin D1 (Figure 5I and data not shown). These data demonstrated that *IKBKE* activates the NF-κB pathway in two epithelial cell types.

To determine whether activation of the NF-κB pathway is necessary for transformation induced by *IKBKE*, we inhibited NF-κB activity in *IKBKE*-transformed HEK cells by expressing a mutant (MUT) "super-repressor" allele of IκBα harboring two amino acid substitutions (S32A/S36A), which renders this MUT IκBα resistant to phosphorylation and degradation (Brown et al., 1995). Expression of MUT IκBα in *IKBKE*-transformed HEK cells increased doubling times (Figure 5J) and suppressed AI colony formation by 90.7% compared to cells infected with a control vector (Figure 5K). In summary, these results implicate the activation of the NF-κB pathway in *IKBKE*-mediated cell transformation.

We initially identified *IKBKE* as a kinase that replaced AKT in transformation (Figure 2A). Since AKT has also been reported to activate the NF-κB pathway (Ozes et al., 1999; Romashkova and Makarov, 1999), we determined if the NF-κB pathway is similarly required for AKT-dependent transformation. Introduction of the MUT IκBα allele increased the doubling time of HEK cells transformed by AKT (Figure 5J) and suppressed AI growth by 73.9% (Figure 5K). We next examined whether *IKBKE* contributed to cell transformation induced by AKT. Indeed, we found that suppression of *IKBKE* by two different shRNA constructs increased doubling times by 20.0%–59.0% and inhibited the capacity of AKT-transformed HEK to proliferate and grow in an AI manner (Figure 5L and data not shown). In aggregate, these observations implicate aberrant activation of the NF-κB pathway by *IKBKE* as a critical step in cell transformation induced by AKT.

Finally, we determined whether *IKBKE* regulates the NF-κB pathway in breast cancer cell lines and patient-derived breast cancer tissue samples. We first examined changes in NF-κB gene expression after *IKBKE* suppression in ZR-75-1 and MCF-7 breast cancer cells and found that of the expression of two *IKBKE*-induced NF-κB regulated genes, *MMP9* and *BCL2* (Figures 5G and 5H), decreased upon suppression of *IKBKE* (Figure 6A and data not shown). Breast cancer cell lines harboring the 1q32 amplification showed increased expression of these genes compared to a breast cancer cell line that lacks IKKε expression (SUM52; Figures 3E, right panel, 6B,

and 6C). We next confirmed that these observations in cell lines also extended to primary breast cancer specimens. Since IKKε has recently been shown to induce nuclear accumulation of the NF-κB family member c-REL (Harris et al., 2006), we performed immunohistochemistry (IHC) to detect IKKε and c-REL in an independent set of 20 breast cancer tissue samples. We found that 7 of 20 (35%) of these breast cancer specimens showed increased expression of IKKε (Figures 6D and 6E), confirming our observations in the initial breast cancer specimens (Figures 3B and 3D). In addition, we found that seven of seven (100%) breast tumors that exhibited increased IKKε expression also displayed nuclear c-REL. In contrast, c-REL was expressed at very low levels or was predominantly located in the cytoplasm in 12 of 13 (92%) of breast tumors that do not exhibit increased IKKε expression (Figures 6D and 6E). This correlation between IKKε expression and nuclear localization of c-REL was statistically significant ($p < 0.0084$). Together, these observations demonstrated that IKKε activates the NF-κB pathway in breast cancer.

DISCUSSION

Identification of *IKBKE* as a Human Breast Cancer Oncogene

Mutations of proto-oncogenes and tumor suppressor genes allow incipient cancer cells to escape the normal mechanisms that regulate cell and tissue homeostasis. In particular, kinase oncogenes play essential roles in many, if not all, human cancers and therefore are particularly attractive therapeutic targets. Indeed, several kinase inhibitors are already in clinical use. Based on these examples, several properties identify oncogenes with particular promise for clinical translation including clear evidence that mutation, amplification, or translocation of the putative oncogene occurs in primary tumors and functional confirmation that cancer cells exhibit an unusual dependence upon the oncogene for viability or proliferation.

Here, we identify *IKBKE* as a human breast cancer oncogene. *IKBKE* is amplified and overexpressed in a significant percentage of human breast tumors. In addition, *IKBKE* expression leads to cell transformation when overexpressed in immortalized human cells at levels found in breast cancer specimens, and cancer cell lines that exhibit *IKBKE* copy-number gain or amplification require IKKε expression for viability. Together, these observations strongly support the conclusion that amplifications of *IKBKE* play an important role in the pathogenesis of a subset of breast tumors and identify IKKε as promising cancer target.

IKBKE, NF-κB and Breast Cancer

Prior studies have shown that activation of the NF-κB pathway occurs in a significant percentage of human cancers (Karin et al., 2002) and specifically in breast tumors (Biswas et al., 2004; Sovak et al., 1997). Although members of the NF-κB signaling pathway including *IKBKB, CARD11, MALT1*, and *BCL10* are required for the survival and proliferation of specific diffuse large B cell lymphoma cell lines (Ngo et al., 2006), the mechanism responsible for increased NF-κB activity in human breast tumors remained undefined (Karin et al., 2002). By examining breast cancer cell lines and breast cancer tissue specimens, we found that overexpression of IKKε activates the NF-κB signaling pathway and that this activity is necessary for the transforming potential of IKKε. Furthermore, the observation that IKKε expression strongly correlates with nuclear localization of c-REL in primary breast cancer specimens suggests that a significant fraction of NF-κB activation in breast cancer is driven by aberrant expression of IKKε. These observations corroborate prior reports that showed that IKKε directly activates c-REL (Harris et al., 2006) or other NF-κB family members (Adli and Baldwin, 2006; Eddy et al., 2005) and that c-REL accumulates in nuclei of breast cancer cells (Sovak et al., 1997).

Although IKKε is a member of the IKK family, IKKε phosphorylates only one of two serine residues in IκBα necessary for ubiquitination and degradation of IκBα (Peters et al., 2000). Since IKKε participates in the activation of innate immune responses mediated by IRF3 and IRF7 (Fitzgerald et al., 2003; Sharma et al., 2003; Tenoever et al., 2007), a key physiological function of IKKε and the closely related family member *TBK1* is likely to be related to innate immunity. More recently, the *Drosophila* IKK family member most closely related to IKKε, called DMIKKε, appears to play several roles in development including activation of nonapoptotic caspase function by phosphorylation of DIAP1 (Kuranaga et al., 2006), F actin assembly (Oshima et al., 2006), and mRNA localization during oogenesis (Shapiro and Anderson, 2006). Although the MUT IκBα (super-repressor) construct blocked IKKε-dependent AI growth, further work is necessary to determine whether other pathways regulated by IKKε also contribute to the tumorigenic phenotype in breast and other cancers.

While several breast cancer cells required IKKε for survival, some cancer cells including PC3 cells appear to depend upon the expression of TBK1 for proliferation. Although we failed to observe amplifications involving the TBK1 locus (data not shown), it remains possible that these IKKs interact or functionally substitute for each other since these two noncanonical IKK family members share similar functions in other assays (Fitzgerald et al., 2003; Sharma et al., 2003). A recent report identifies TBK1 as a component of the RalB/Sec5 exocyst complex and indicates that TBK1 is essential for H-RAS^{V12}-induced transformation (Chien et al., 2006). Since several members of the canonical NF-κB signaling pathway are altered in human leukemia and lymphomas (Karin et al., 2002), these findings indicate that NF-κB activation may occur by distinct mechanisms in different tumor types.

We found that breast cancer cell lines harboring 1q32 amplification and expressing high levels of IKKε exhibit exquisite sensitivity to suppression of IKKε expression. In contrast, we observed that suppression of IKKε in other

cancer cell lines lacking significant 1q32 amplifications failed to affect viability and proliferation. We note, however, that a dominantly interfering *IKBKE* allele suppresses AI proliferation of a breast cancer cell line without 1q32 amplification (Hs 578T) (Eddy et al., 2005), and *IKBKE* suppression in HeLa cells leads to apoptosis (Adli and Baldwin, 2006; MacKeigan et al., 2005). Both Hs 587T and HeLa cells express elevated levels of IKKε (Adli and Baldwin, 2006; Eddy et al., 2005). Since we found that several breast tumors overexpress *IKBKE* yet lacked amplifications (Figure 3D), these observations suggest that other alterations beyond copy-number alterations also lead to increased *IKBKE* expression and/or activity in some cancer cell lines and tumors.

IKBKE Replaces *AKT* in Transformation

We found that *IKBKE* replaces AKT in transformation and that AKT-driven cell transformation requires NF-κB activity and *IKBKE*. These observations implicate *IKBKE* and the NF-κB pathway as a key effector pathway triggered by AKT signaling in transformation. Although previous reports demonstrated that AKT activation of NF-κB involves direct activation of the canonical IKK complex (Ozes et al., 1999; Romashkova and Makarov, 1999), our observations suggest that in some cell types AKT and IKKε are functionally related.

In addition to *IKBKE*, we identified three other kinases (*DAK*, *TSSK6*, and *CSNK1E*) that replaced activated *AKT1* in an experimental model of human cell transformation. Further work may determine that *DAK*, *TSSK6*, or *CSNK1E* are altered in human tumors. Furthermore, identifying genetic alterations that cooperate with *IKBKE* amplification in breast cancer may pinpoint other pathways that synergize with this oncogene.

Dissection of Ras Signaling

To identify kinases that replace myr-AKT in human cell transformation, we determined whether activation of the PI3K and MAPK pathways would cooperate to replace H-RAS^{V12} in an experimental model of human cell transformation. Previous studies using similar transformation systems used three mutant H-RAS^{V12} alleles that preferentially activate the PI3K, MAPK, or RALGDS pathways and identified an important role for the pathway regulated by RALGDS in H-RAS^{V12}-induced AI growth in several types of human cells (Hamad et al., 2002; Rangarajan et al., 2004). However, the further activation of the MAPK or PI3K pathway in these experiments was necessary to permit such engineered cells to form tumors.

Although activated MEK and AKT alleles cooperated to replace H-RAS^{V12} in human cell transformation, we also found that the expression of some activated alleles within the PI3K and MAPK pathways failed to cooperate to induce tumorigenic potential. For example, although either *BRAF*^{E600} or MEK^{DD} cooperated with myr-AKT to confer AI growth, *BRAF*^{E600} failed to cooperate with myr-AKT to promote tumor formation (Table 1). Since *BRAF* mutations have not yet been identified in renal cancer but are frequently observed in other malignancies such as melanoma, these observations are consistent with the view that particular combinations of Ras effector pathway mutations cooperate in specific cell types (Rangarajan et al., 2004). Alternatively, since accumulating evidence suggests that activation of specific Ras effector pathways leads to feedback inhibition (Courtois-Cox et al., 2006), these observations may reflect lineage-specific feedback loops.

Functional Genomics and Integrating Complementary Genomic Approaches

The development of methodologies to identify genetic alterations at genome scale promises to provide a comprehensive view of cancer-associated mutations. However, a full appreciation of these genetic alterations will require equally efficient high-throughput methods to assess the functional consequences of these genetic changes and to evaluate them as potential therapeutic targets. The development of genome-scale libraries of RNAi reagents has facilitated loss-of-function approaches in mammalian cells that have identified candidate tumor-suppressor genes (Kolfschoten et al., 2005; Westbrook et al., 2005). In parallel to these loss-of-function approaches, candidate oncogenes have been identified using screening tools such as cDNA libraries derived from cancer cells or the use of viral integration to activate endogenous genes (Dupuy et al., 2006; Peeper et al., 2002).

Here we have combined both gain-of-function and loss-of-function approaches with whole-genome characterization of genetic alterations in cancer cell lines and tumors. This integrated approach permitted us both to identify *IKBKE* as a breast cancer oncogene amplified in a significant percentage of human breast tumors and to verify its functional role in mammary cell transformation. Similarly, other recent reports highlight the power of integrating complementary genomic approaches to identify genes associated with oncogenesis (Garraway et al., 2005; Zender et al., 2006) and metastasis (Kim et al., 2006). Together, these reports suggest a framework for comprehensive approaches to annotate structural and functionally important genetic alterations essential for cancer development while efficiently validating targets of particular promise for clinical translation.

EXPERIMENTAL PROCEDURES

Cell Culture, Vectors, and Retroviral Infection

HEK cells (Hahn et al., 1999) and HMECs expressing *hTERT* (Elenbaas et al., 2001) have been described. To introduce or suppress specific genes, retroviral (pBabe, pWzl, and pMKO) (Boehm et al., 2005; Morgenstern and Land, 1990) or lentiviral (pLKO) (Moffat et al., 2006) vectors were used. Retroviruses and lentiviruses were generated as described (Boehm et al., 2005; Moffat et al., 2006). Details regarding cell-culture conditions and retroviral vector construction are described in the Supplemental Experimental Procedures. Tumor specimens and breast cancer cell lines were obtained as described (Yao et al., 2006).

Myristoylated Kinase Library

ORFs were cloned into Gateway-compatible pEntry vectors by performing site-specific BP recombination reactions as described (Rual et al., 2004). pWzl-Neo-Myr-Flag DEST (pWN-MF-DEST) was created by inserting an N-terminal myr-FLAG tag and the Gateway-selectable cassette into the pWzl-Neo retroviral vector. To create specific pWN-MF-ORF vectors, site-specific LR recombination reactions were performed individually as described (Rual et al., 2004).

AI Growth and Tumorigenicity Assays

Growth of cells in soft agar was determined by plating 5×10^4 cells in triplicate in 0.4% (HEK) or 0.3% (HMEC) Noble agar. Colonies greater than 100 μm in diameter were counted microscopically 8 weeks after plating. Colonies greater than 200 nm in diameter were counted macroscopically 8 weeks after plating. Tumor xenograft experiments were performed as described (Boehm et al., 2005). Tumor injection sites were monitored for 80 days for tumor formation.

Immunoblotting and PCR

Immunoblotting was performed as described (Boehm et al., 2005). The following antibodies were used: anti-IKKε K-14 (Santa Cruz, CA) (Figure 3) or anti-IKKε c-terminal (Sigma) (Figures 3–6), anti-IκBα C-15 (Santa Cruz, CA), anti-NF-κB H-119 (Santa Cruz, CA), anti-MEK1/2 (Cell Signaling), anti-Cyclin D1 (Neomarkers), or anti-IRF3 (Santa Cruz, CA). For RT-PCR, total RNA was isolated using TRIzol (Invitrogen). For quantitative RT-PCR, we used SYBR-Green (Applied Biosystems). A list of primers may be found in Supplemental Experimental Procedures.

IHC

Breast cancer tissue microarray slides (Biomax BR803) were immunostained with anti-IKKε- C-terminal (1:250; Sigma) and anti-c-Rel C terminus (1:200; Chemicon) antibodies using microwave-citrate antigen retrieval followed by standard IHC staining procedures. Arrays were scored in a blinded manner by a pathologist on a scale of 0–3.

Genome Structure Analyses

SNP array, aCGH, and SAGE data were collected and analyzed as described (Garraway et al., 2005).

RNAi

To stably suppress *IKBKE* and other members of the IKK family we used pLKO.1 lentiviral shRNA constructs generated by TRC (Moffat et al., 2006). Lentiviral infections were performed as described (Moffat et al., 2006). Cell Titer Glo (Promega) was used to measure viability 5 days after lentiviral infection. Z scores were calculated by normalizing the viability score of each well to the plate mean viability score and standard deviation (SD) of plate viability scores: $Z = (X-\mu)/\sigma$, where X = assay value (relative luminescence), μ = mean plate viability score, and σ = SD of plate viability scores. Suppression of viability was defined as the capacity for at least one shRNA to decrease viability more than 1.5 SD below the mean Z score and at least one additional shRNA targeting the same kinase to decrease viability more that 1 SD below the mean Z score.

Supplemental Data

Supplemental Data include three figures, one table, and Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://www.cell.com/cgi/content/full/129/6/1065/DC1/.

ACKNOWLEDGMENTS

We thank D. Sabatini, N. Hacohen, and T. Golub for advice. We thank E. Shin for assistance with IHC, L. Prickett for assistance with fluorescence-activated cell sorting, and H. Widlund for reagents and helpful discussions. We thank E. Knipp and J.T. Boehm for assistance in manuscript preparation and members of the Hahn laboratory for helpful discussions and support. This work was supported in part by grants K01 CA94223 (W.C.H.), P50 CA112962 (W.C.H.), CA30002 (T.R.), CA89021 (T.R.), CA015607 (T.R.) from the NIH, the Tisch Family Fund (W.C.H. and K.P.), DFCI High Tech Fund (S. Korsmeyer), the DFCI Strategic Planning Initiative for support (W.H. and M.V.), an Ellison Foundation grant (M.V.), a Breast Cancer Center of Excellence Award (DAMD170210692, K.P.), a Claudia Barr Award (J.J.Z.), a Woman's Cancer Program Award (J.J.Z.), and a Department of Defense Breast Cancer Program Award BC051565 (J.J.Z.). In compliance with Harvard Medical School guidelines, we disclose that W.C.H., T.M.R., M.M., and K.P. are consultants for Novartis Pharmaceuticals, Inc.

Received: September 24, 2006
Revised: January 16, 2007
Accepted: March 22, 2007
Published: June 14, 2007

REFERENCES

Adli, M., and Baldwin, A.S. (2006). IKK-i/IKKepsilon controls constitutive, cancer cell-associated NF-kappaB activity via regulation of Ser-536 p65/RelA phosphorylation. J. Biol. Chem. *281*, 26976–26984.

Bamford, S., Dawson, E., Forbes, S., Clements, J., Pettett, R., Dogan, A., Flanagan, A., Teague, J., Futreal, P.A., Stratton, M.R., et al. (2004). The COSMIC (Catalogue of Somatic Mutations in Cancer) database and website. Br. J. Cancer *91*, 355–358.

Bartkova, J., Lukas, J., Muller, H., Lutzhoft, D., Strauss, M., and Bartek, J. (1994). Cyclin D1 protein expression and function in human breast cancer. Int. J. Cancer *57*, 353–361.

Berns, K., Hijmans, E.M., Mullenders, J., Brummelkamp, T.R., Velds, A., Heimerikx, M., Kerkhoven, R.M., Madiredjo, M., Nijkamp, W., Weigelt, B., et al. (2004). A large-scale RNAi screen in human cells identifies new components of the p53 pathway. Nature *428*, 431–437.

Biswas, D.K., Shi, Q., Baily, S., Strickland, I., Ghosh, S., Pardee, A.B., and Iglehart, J.D. (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.

Boehm, J.S., Hession, M.T., Bulmer, S.E., and Hahn, W.C. (2005). Transformation of human and murine fibroblasts without viral oncoproteins. Mol. Cell. Biol. *25*, 6464–6474.

Brown, K., Gerstberger, S., Carlson, L., Franzoso, G., and Siebenlist, U. (1995). Control of I kappa B-alpha proteolysis by site-specific, signal-induced phosphorylation. Science *267*, 1485–1488.

Brunet, A., Pages, G., and Pouyssegur, J. (1994). Constitutively active mutants of MAP kinase kinase (MEK1) induce growth factor-relaxation and oncogenicity when expressed in fibroblasts. Oncogene *9*, 3379–3387.

Chien, Y., Kim, S., Bumeister, R., Loo, Y.M., Kwon, S.W., Johnson, C.L., Balakireva, M.G., Romeo, Y., Kopelovich, L., Gale, M., Jr., et al. (2006). RalB GTPase-mediated activation of the IkappaB family kinase TBK1 couples innate immune signaling to tumor cell survival. Cell *127*, 157–170.

Courtois-Cox, S., Genther Williams, S.M., Reczek, E.E., Johnson, B.W., McGillicuddy, L.T., Johannessen, C.M., Hollstein, P.E., MacCollin, M., and Cichowski, K. (2006). A negative feedback signaling network underlies oncogene-induced senescence. Cancer Cell *10*, 459–472.

Davies, H., Bignell, G.R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffendin, H., Garnett, M.J., Bottomley, W., et al. (2002). Mutations of the BRAF gene in human cancer. Nature *417*, 949–954.

Downward, J. (2003). Targeting RAS signalling pathways in cancer therapy. Nat. Rev. Cancer *3*, 11–22.

Dupuy, A.J., Jenkins, N.A., and Copeland, N.G. (2006). Sleeping beauty: a novel cancer gene discovery tool. Hum. Mol. Genet. *15 Spec No 1*, R75–R79.

Eddy, S.F., Guo, S., Demicco, E.G., Romieu-Mourez, R., Landesman-Bollag, E., Seldin, D.C., and Sonenshein, G.E. (2005). Inducible IkappaB kinase/IkappaB kinase epsilon expression is induced by CK2 and promotes aberrant nuclear factor-kappaB activation in breast cancer cells. Cancer Res. *65*, 11375–11383.

Elenbaas, B., Spirio, L., Koerner, F., Fleming, M.D., Zimonjic, D.B., Donaher, J.L., Popescu, N.C., Hahn, W.C., and Weinberg, R.A. (2001). Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells. Genes Dev. *15*, 50–65.

Fitzgerald, K.A., McWhirter, S.M., Faia, K.L., Rowe, D.C., Latz, E., Golenbock, D.T., Coyle, A.J., Liao, S.M., and Maniatis, T. (2003). IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. Nat. Immunol. *4*, 491–496.

Garraway, L.A., Widlund, H.R., Rubin, M.A., Getz, G., Berger, A.J., Ramaswamy, S., Beroukhim, R., Milner, D.A., Granter, S.R., Du, J., et al. (2005). Integrative genomic analyses identify MITF as a lineage survival oncogene amplified in malignant melanoma. Nature *436*, 117–122.

Hahn, W.C., Counter, C.M., Lundberg, A.S., Beijersbergen, R.L., Brooks, M.W., and Weinberg, R.A. (1999). Creation of human tumour cells with defined genetic elements. Nature *400*, 464–468.

Hamad, N.M., Elconin, J.H., Karnoub, A.E., Bai, W., Rich, J.N., Abraham, R.T., Der, C.J., and Counter, C.M. (2002). Distinct requirements for Ras oncogenesis in human versus mouse cells. Genes Dev. *16*, 2045–2057.

Harris, J., Oliere, S., Sharma, S., Sun, Q., Lin, R., Hiscott, J., and Grandvaux, N. (2006). Nuclear accumulation of cRel following C-terminal phosphorylation by TBK1/IKK{epsilon}. J. Immunol. *177*, 2527–2535.

Karin, M., Cao, Y., Greten, F.R., and Li, Z.W. (2002). NF-kappaB in cancer: from innocent bystander to major culprit. Nat. Rev. Cancer *2*, 301–310.

Kim, M., Gans, J.D., Nogueira, C., Wang, A., Paik, J.H., Feng, B., Brennan, C., Hahn, W.C., Cordon-Cardo, C., Wagner, S.N., et al. (2006). Comparative oncogenomics identifies NEDD9 as a melanoma metastasis gene. Cell *125*, 1269–1281.

Klippel, A., Reinhard, C., Kavanaugh, W.M., Apell, G., Escobedo, M.A., and Williams, L.T. (1996). Membrane localization of phosphatidylinositol 3-kinase is sufficient to activate multiple signal-transducing kinase pathways. Mol. Cell. Biol. *16*, 4117–4127.

Kohn, A.D., Takeuchi, F., and Roth, R.A. (1996). Akt, a pleckstrin homology domain containing kinase, is activated primarily by phosphorylation. J. Biol. Chem. *271*, 21920–21926.

Kolfschoten, I.G., van Leeuwen, B., Berns, K., Mullenders, J., Beijersbergen, R.L., Bernards, R., Voorhoeve, P.M., and Agami, R. (2005). A genetic screen identifies PITX1 as a suppressor of RAS activity and tumorigenicity. Cell *121*, 849–858.

Kuranaga, E., Kanuka, H., Tonoki, A., Takemoto, K., Tomioka, T., Kobayashi, M., Hayashi, S., and Miura, M. (2006). Drosophila IKK-related kinase regulates nonapoptotic function of caspases via degradation of IAPs. Cell *126*, 583–596.

Lynch, T.J., Bell, D.W., Sordella, R., Gurubhagavatula, S., Okimoto, R.A., Brannigan, B.W., Harris, P.L., Haserlat, S.M., Supko, J.G., Haluska, F.G., et al. (2004). Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N. Engl. J. Med. *350*, 2129–2139.

MacKeigan, J.P., Murphy, L.O., and Blenis, J. (2005). Sensitized RNAi screen of human kinases and phosphatases identifies new regulators of apoptosis and chemoresistance. Nat. Cell Biol. *7*, 591–600.

Moffat, J., Grueneberg, D.A., Yang, X., Kim, S.Y., Kloepfer, A.M., Hinkle, G., Piqani, B., Eisenhaure, T.M., Luo, B., Grenier, J.K., et al. (2006). A lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. Cell *124*, 1283–1298.

Morgenstern, J.P., and Land, H. (1990). Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res. *18*, 3587–3596.

Morrison, C., Radmacher, M., Mohammed, N., Suster, D., Auer, H., Jones, S., Riggenbach, J., Kelbick, N., Bos, G., and Mayerson, J. (2005). MYC amplification and polysomy 8 in chondrosarcoma: array comparative genomic hybridization, fluorescent in situ hybridization, and association with outcome. J. Clin. Oncol. *23*, 9369–9376.

Ngo, V.N., Davis, R.E., Lamy, L., Yu, X., Zhao, H., Lenz, G., Lam, L.T., Dave, S., Yang, L., Powell, J., et al. (2006). A loss-of-function RNA interference screen for molecular targets in cancer. Nature *441*, 106–110.

Oshima, K., Takeda, M., Kuranaga, E., Ueda, R., Aigaki, T., Miura, M., and Hayashi, S. (2006). IKKvarepsilon regulates F actin assembly and interacts with Drosophila IAP1 in cellular morphogenesis. Curr. Biol. *16*, 1531–1537.

Ozes, O.N., Mayo, L.D., Gustin, J.A., Pfeffer, S.R., Pfeffer, L.M., and Donner, D.B. (1999). NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. Nature *401*, 82–85.

Paddison, P.J., Silva, J.M., Conklin, D.S., Schlabach, M., Li, M., Aruleba, S., Balija, V., O'Shaughnessy, A., Gnoj, L., Scobie, K., et al. (2004). A resource for large-scale RNA-interference-based screens in mammals. Nature *428*, 427–431.

Paez, J.G., Janne, P.A., Lee, J.C., Tracy, S., Greulich, H., Gabriel, S., Herman, P., Kaye, F.J., Lindeman, N., Boggon, T.J., et al. (2004). EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science *304*, 1497–1500.

Peeper, D.S., Shvarts, A., Brummelkamp, T., Douma, S., Koh, E.Y., Daley, G.Q., and Bernards, R. (2002). A functional screen identifies hDRIL1 as an oncogene that rescues RAS-induced senescence. Nat. Cell Biol. *4*, 148–153.

Peters, R.T., Liao, S.M., and Maniatis, T. (2000). IKKepsilon is part of a novel PMA-inducible IkappaB kinase complex. Mol. Cell *5*, 513–522.

Rangarajan, A., Hong, S.J., Gifford, A., and Weinberg, R.A. (2004). Species- and cell type-specific requirements for cellular transformation. Cancer Cell *6*, 171–183.

Rennstam, K., Ahlstedt-Soini, M., Baldetorp, B., Bendahl, P.O., Borg, A., Karhu, R., Tanner, M., Tirkkonen, M., and Isola, J. (2003). Patterns of chromosomal imbalances defines subgroups of breast cancer with distinct clinical features and prognosis. A study of 305 tumors by comparative genomic hybridization. Cancer Res. *63*, 8861–8868.

Romashkova, J.A., and Makarov, S.S. (1999). NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. Nature *401*, 86–90.

Rowland, B.D., Bernards, R., and Peeper, D.S. (2005). The KLF4 tumour suppressor is a transcriptional repressor of p53 that acts as a context-dependent oncogene. Nat. Cell Biol. *7*, 1074–1082.

Rual, J.F., Hirozane-Kishikawa, T., Hao, T., Bertin, N., Li, S., Dricot, A., Li, N., Rosenberg, J., Lamesch, P., Vidalain, P.O., et al. (2004). Human ORFeome version 1.1: a platform for reverse proteomics. Genome Res. *14*, 2128–2135.

Samuels, Y., Wang, Z., Bardelli, A., Silliman, N., Ptak, J., Szabo, S., Yan, H., Gazdar, A., Powell, S.M., Riggins, G.J., et al. (2004). High frequency of mutations of the PIK3CA gene in human cancers. Science *304*, 554.

Shapiro, R.S., and Anderson, K.V. (2006). Drosophila Ik2, a member of the I kappa B kinase family, is required for mRNA localization during oogenesis. Development *133*, 1467–1475.

Sharma, S., tenOever, B.R., Grandvaux, N., Zhou, G.P., Lin, R., and Hiscott, J. (2003). Triggering the interferon antiviral response through an IKK-related pathway. Science *300*, 1148–1151.

Sovak, M.A., Bellas, R.E., Kim, D.W., Zanieski, G.J., Rogers, A.E., Traish, A.M., and Sonenshein, G.E. (1997). Aberrant nuclear factor-kappaB/Rel expression and the pathogenesis of breast cancer. J. Clin. Invest. *100*, 2952–2960.

Stanton, V.P., Jr., Nichols, D.W., Laudano, A.P., and Cooper, G.M. (1989). Definition of the human raf amino-terminal regulatory region by deletion mutagenesis. Mol. Cell. Biol. *9*, 639–647.

Tenoever, B.R., Ng, S.L., Chua, M.A., McWhirter, S.M., Garcia-Sastre, A., and Maniatis, T. (2007). Multiple functions of the IKK-related kinase IKKepsilon in interferon-mediated antiviral immunity. Science *315*, 1274–1278.

Weinstein, I.B., and Joe, A.K. (2006). Mechanisms of disease: Oncogene addiction–a rationale for molecular targeting in cancer therapy. Nat. Clin. Pract. Oncol. *3*, 448–457.

Westbrook, T.F., Martin, E.S., Schlabach, M.R., Leng, Y., Liang, A.C., Feng, B., Zhao, J.J., Roberts, T.M., Mandel, G., Hannon, G.J., et al. (2005). A genetic screen for candidate tumor suppressors identifies REST. Cell *121*, 837–848.

Yao, J., Weremowicz, S., Feng, B., Gentleman, R.C., Marks, J.R., Gelman, R., Brennan, C., and Polyak, K. (2006). Combined cDNA array comparative genomic hybridization and serial analysis of gene expression analysis of breast tumor progression. Cancer Res. *66*, 4065–4078.

Zender, L., Spector, M.S., Xue, W., Flemming, P., Cordon-Cardo, C., Silke, J., Fan, S.T., Luk, J.M., Wigler, M., Hannon, G.J., et al. (2006). Identification and validation of oncogenes in liver cancer using an integrative oncogenomic approach. Cell *125*, 1253–1267.

Zhao, J.J., Roberts, T.M., and Hahn, W.C. (2004). Functional genetics and experimental models of human cancer. Trends Mol. Med. *10*, 344–350.

Zhao, X., Weir, B.A., LaFramboise, T., Lin, M., Beroukhim, R., Garraway, L., Beheshti, J., Lee, J.C., Naoki, K., Richards, W.G., et al. (2005). Homozygous deletions and chromosome amplifications in human lung carcinomas revealed by single nucleotide polymorphism array analysis. Cancer Res. *65*, 5561–5570.