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KEAP1 E3 Ligase-Mediated Down-Regulation of NF-κB Signaling by Targeting IKKβ

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SUMMARY

IκB kinase β (IKKβ) is involved in tumor development and progression through activation of the nuclear factor (NF)–κB pathway. However, the molecular mechanism that regulates IKKβ degradation remains largely unknown. Here, we show that a Cullin 3 (CUL3)–based ubiquitin ligase, Kelch-like ECH-associated protein 1 (KEAP1), is responsible for IKKβ ubiquitination. Depletion of KEAP1 led to the accumulation and stabilization of IKKβ and to up-regulation of NF-κB–derived tumor angiogenic factors. A systematic analysis of the *CUL3*, *KEAP1*, and *RBX1* genomic loci revealed a high percentage of genome loss and missense mutations in human cancers that failed to facilitate IKKβ degradation. Our results suggest that the dysregulation of KEAP1-mediated IKKβ ubiquitination may contribute to tumorigenesis.

INTRODUCTION

The ubiquitin-proteasome pathway is crucial for controlling the abundance of several proteins, and it plays an essential role in maintaining normal cellular functions. Kelch-like ECH-

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associated protein 1 (KEAP1) is a Bric-a-Brac (BTB)–Kelch protein that functions as a substrate adaptor protein for a Cullin 3 (CUL3)/ Ring-Box 1 (RBX1)–dependent E3 ubiquitin ligase complex (Kobayashi et al., 2004; Zhang et al., 2004). The well-studied substrate for the KEAP1-CUL3 ubiquitin E3 ligase complex is nuclear factor erythroid-2–related factor 2 (NRF2) (Motohashi et al., 2004). The KEAP1-CUL3 ubiquitin E3 ligase complex interacts with and conjugates ubiquitin onto the N-terminal Neh2 domain of NRF2 and promotes NRF2 degradation through the 26S ubiquitin proteasome pathway (Itoh et al., 1999). While studying the role of KEAP1-NRF2 signaling pathway in human cancers, it was noticed that numerous different kinds of KEAP1 somatic mutations were detected in both lung cancer and gallbladder cancer (Ohta et al., 2008; Padmanabhan et al., 2006; Shibata et al., 2008; Singh et al., 2006). Moreover, loss of heterozygosity of KEAP1 genome regions (Singh et al., 2006) were frequently found in both lung cancer cell lines and lung cancer tissues. These genomic alternations result in constitutive NRF2 activation which induces a couple of antioxidant-related enzyme to provide advantages of cancer cells for resistance to chemotherapeutic drugs (Shibata et al., 2008; Singh et al., 2006), implicating that KEAP1 might function as a tumor suppressor and loss of KEAP1 function confers tumorigenesis. Given that KEAP1 might function as a tumor suppressor protein to negatively regulate tumor progression and thus provide an advantage for chemotherapy, it is important to understand the KEAP1-involved signaling cascades.

IκB kinase β (IKKβ) functions as a fine-tuning controller of the nuclear factor (NF)–κB pathway. NF-κB activation governs the expression of myriad genes involved in the immune response, cell proliferation, angiogenesis, cell survival, tumor invasion, metastasis, and the epithelial-mesenchymal transition (Karin and Greten, 2005; Perkins, 2007). Additionally, IKKβ has been shown to promote tumorigenicity through phosphorylation-mediated inhibition of tumor suppressors and thus is considered an oncogenic kinase (Hu et al., 2004; Lee and Hung, 2007; Lee et al., 2007). These findings strongly suggested that IKKβ-mediated signaling cascades link inflammation to cancer pathogenesis.

In the current study, we demonstrated that KEAP1 functions as an IKKβ E3 ubiquitin ligase. Depletion of KEAP1 led to the accumulation and stabilization of IKKβ and up-regulation of NF-κB–derived tumor angiogenic factors. A systematic analysis of *CUL3*, *KEAP1*, and *RBX1* (E3 ubiquitin ligase complex) genomic loci revealed a high percentage of genome loss and missense mutations in human cancers that failed to facilitate IKKβ degradation. Thus, our results suggest that inactivation of the KEAP1-CUL3-RBX1 complex in human cancers may prevent IKKβ degradation and result in NF-κB activation and that induction of KEAP1 expression may have vital clinical implications for the treatment or prevention of inflammation-associated cancers.

RESULTS AND DISCUSSION

KEAP1 Inhibits the NF-κB Signaling Pathway

Given that the genomic alternation of KEAP1 increases tumorigenicity and numerous NF-κB downstream targets benefit the hallmark capabilities of multiple cancers, we sought to determine whether NF-κB is involved in loss-of-KEAP1–induced tumor progression. To address this issue, we increased KEAP1 by ectopic expression or knocked down KEAP1 by siRNA to study the effects of each process on the NF-κB signaling pathway. Compared with vector control, ectopic expression of KEAP1 suppressed tumor necrosis factor (TNF) α–induced NF-κB transcriptional activity as determined by a reporter assay (Figure S1A) and RELA nuclear translocation in cancer cells (from 90% to 36% in Hs578T cells and from 92% to 48% in MDA-MB-435 cells) (Figures 1A and S1B). Consistently, knockdown of KEAP1 increased nuclear location of RELA (Figure 1B and S2) and enhanced NF-κB transcriptional

activity (Figure 1C and S3A) but not SMAD transcriptional activity (Figure S3B). These results suggested that KEAP1 might specifically block the TNFα–NF-κB signaling pathway.

Furthermore, we explored the expression of a broad spectrum of NF-κB–responsive genes in four breast cancer cell lines using quantitative reverse transcription polymerase chain reaction (qRT-PCR). Several NF-κB–regulated genes, including IL-8, CCL2, BIRC3, and PLAU, were up-regulated in multiple human breast cancer cell lines when KEAP1 was depleted, as shown in heat maps (Figure 1D and Table S1). Because IL-8 is a potent angiogenic factor, this result raises an interesting possibility that KEAP1 might inhibit tumor angiogenesis through the down-regulation of IL-8. Indeed, *in vitro* angiogenesis assays revealed that siRNA-mediated inhibition of KEAP1 expression increased human umbilical vein endothelial cell (HUVEC) migration (Figure 1E) and tube formation (Figure 1F). Neutralizing IL-8 using anti–IL-8 antibodies repressed the HUVEC migration and tube formation induced by KEAP1 siRNA knockdown cells to a level similar to that induced by control cells (Figures 1E and 1F).

NRF2 transcription factor is a major target degraded by KEAP1, so we questioned whether KEAP1-mediated down-regulation of IL-8 expression might be related to NRF2. However, this possibility was ruled out because silencing NRF2 had no effect on IL-8 mRNA or on protein expression induced by KEAP1 knockdown, as determined by qRT-PCR and an enzyme-linked immunosorbent assay (ELISA), respectively (Figures 1G and 1H). Together, these results suggest that KEAP1 down-regulates IKKβ and suppresses NF-κB–mediated IL-8 expression, which culminates in inhibition of *in vitro* angiogenesis.

KEAP1 Directly Interacts with IKKβ

These observations raised an intriguing question of how KEAP1 negatively modulates the NF-κB signaling. Because KEAP1 is an E3 ubiquitin ligase, we hypothesized that KEAP1 suppresses NF-κB activity through down-regulation of an essential component in the TNFα–NF-κB signaling pathway. siRNA-mediated knockdown of KEAP1 caused a significant increase in the amount of IKKβ but did not alter the amounts of other related molecules, which have been suggested to be required for NF-κB activation (Figure 2A); KEAP1-mediated IKKβ down-regulation was rescued by MG132 (Figure S4), suggesting that KEAP1 mediates IKKβ degradation via the ubiquitin proteasome process. To investigate whether IKKβ is required for KEAP1-regulated NF-κB activity, the effect of KEAP1 knockdown was examined on IKKβ-depleted cells, and the result showed that silencing KEAP1 does not up-regulate NF-κB activity (Figure S5), which further supports the notion that KEAP1 represses NF-κB function by inducing IKKβ degradation.

Furthermore, coimmunoprecipitation assays demonstrated that IKKβ physically interacted with KEAP1 *in vivo* (Figure 2B). This interaction was also observed with endogenous IKKβ and KEAP1 when specific antibodies to IKKβ were used (Figure 2C). An *in vitro* pull-down assay further suggested a direct association between IKKβ and KEAP1 (Figure 2D). In addition, the endogenous binding between KEAP1 and IKKβ was not affected by TNFα (Figure 2C), and an increase in IKKβ by KEAP1 depletion did not change after TNFα treatment (Figure 2E), indicating that the regulation of IKKβ by KEAP1 is independent on IKKβ activity. To define the region of KEAP1 required for IKKβ interaction, we used coimmunoprecipitation and mammalian two-hybrid assays to examine the binding abilities of different KEAP1 domain truncation mutants to IKKβ. We found that IKKβ strongly interacted with the Kelch domain of KEAP1 (Figures 2F and 2G). To further examine whether other cancer-related E3 ligases (SMURF2, MDM2, and SKP2) or Kelch domain–containing proteins (ND1L, GIGAXONIN, and ACTINFILIN) could also interact with IKKβ, we performed coimmunoprecipitation assays and found that these well-characterized E3 ligases were unable to coimmunoprecipitate with IKKβ, indicating that the interaction between KEAP1 and IKKβ is specific (Figures S6A and S6B).

KEAP1 Functions as a CUL3-Based E3 Ligase of IKKβ

Given that depletion of KEAP1 leads to IKKβ accumulation and that the Kelch domain is required for KEAP1 substrate recognition and binding, we further investigated whether IKKβ is a physical substrate of KEAP1. Coexpression of KEAP1 resulted in a marked increase in IKKβ ubiquitination *in vivo* compared with that in the cells without KEAP1 cotransfection in the presence of MG132, and KEAP1 did not utilize the ubiquitin K48R mutant to induce IKKβ ubiquitination (Figure 3A), which suggests that the polyubiquitin chains attached to IKKβ is K48-linked polyubiquitination. Consistent with the interaction results (Figure S6A), KEAP1 was much more efficient than other BTB/Kelch domain proteins were in promoting IKKβ ubiquitination (Figure S7). Depletion of KEAP1 decreased endogenous IKKβ ubiquitination (Figure S8A), and the IKKβ ubiquitination status was not altered under TNFα stimulation (Figure S8B), indicating that KEAP1-mediated IKKβ ubiquitination is independent on IKKβ activity. Because KEAP1 functions as an adaptor to recruit its substrates into a CUL3-based ubiquitin ligase complex, we attempted to determine whether IKKβ is associated with and ubiquitinated by the KEAP1-CUL3-RBX1 complex. In CUL3 immunoprecipitates, the IKKβ level was markedly increased in the presence of coexpressed KEAP1 (Figure 3B), which is in agreement with the notion that KEAP1 functions as a bridge for IKKβ and CUL3-RBX1 complex. Additionally, coexpression of CUL3 and RBX1 facilitated KEAP1-mediated IKKβ ubiquitination (Figure S9), indicating that CUL3 and RBX1 are involved in KEAP1-derived ubiquitin conjugation onto IKKβ. Consistently, we reconstituted the ubiquitination of IKKβ *in vitro* and showed that IKKβ was efficiently ubiquitinated when KEAP1, CUL3, and RBX1 all participated in the reaction (Figure 3C). No IKKβ ubiquitination was found when the reaction mixture lacked the E1 ubiquitin-activating enzyme, supporting the evidence that IKKβ ubiquitination occurred *in vitro*.

It has been shown that KEAP1's substrates have a (D/N)XE(T/S)GE motif that is required for KEAP1 binding (Kobayashi et al., 2004; Lo and Hannink, 2006). We noticed that IKKβ also contains this motif ($NQE^{36}TGE^{39}$) at its kinase domain (Figure 3D). Compared with the IKKβ wild type, IKKβ mutants (E36A and E39A) were resistant to down-regulation by KEAP1 (Figure 3E). Consistently, the IKKβ ETGE motif mutants had decreased ability to bind to KEAP1 (Figure 3F) and were resistant to KEAP1-mediated ubiquitination (Figure 3G). We further applied mass spectrometric analysis to identify the ubiquitination site in IKKβ and found that K555 is the site of KEAP1-mediated IKKβ ubiquitination (Figure 3H). These results suggest that the KEAP1-CUL3-RBX1 ubiquitin proteasome complex is responsible for ubiquitin-mediated proteolysis of the IKKβ and IKKβ ETGE motifs and is essential for KEAP1 recognition and binding.

Of interest, this motif exists only in human, chimpanzee, and dog and not in rat and mouse, suggesting that Keap1/Ikkβ regulation may not exist in the rodent cells. To support this notion, we examined whether Keap1 negatively regulates Ikkβ in mouse cells. We found that knockdown of Keap1 did not affect endogenous Ikkβ expression in mouse mammary tumor 4T1 cells (Figure S10A). In addition, mouse Keap1 did not associate with Ikkβ wild type and E39A mutant (Figure S10B) or induce Ikkβ ubiquitination (Figure S10C). Taken together, our data suggest that KEAP1-mediated IKKβ ubiquitination and down-regulation may be specific to advanced mammals.

Genomic Losses of KEAP1, CUL3, and RBX1 Loci in Human Cancers

In the early stages of cancer development, the loss of genomic stability is considered an imperative molecular and genetic step toward providing a permissive environment for the occurrence of alterations in oncogenes and tumor-suppressor genes. Progressive accumulation of genetic and epigenetic alterations leads to the transformation of normal cells into malignant tumors. Using comparative genomic approaches, we investigated whether the KEAP1-CUL3-

RBX1-IKKβ axis is dysregulated in human breast, esophageal, and liver cancers, which generally show abnormal up-regulation of NF-κB activity. In 42 breast cancer cell lines, an evaluation using single-nucleotide polymorphism (SNP) arrays of copy numbers at the *CUL3* (between rs2216460 and rs1368884), *KEAP1* (between rs2112527 and rs726789), and *RBX1* (between rs4266112 and rs139480) loci revealed that recurrent copy-number loss involving both the upstream and downstream regions of the *CUL3* (25 of 42), *KEAP1* (10 of 42), and *RBX1* (23 of 42) loci was frequent in breast cancer cell lines (Figure 4A). The average amount of copy-number loss was greater at the *CUL3* (0.85 for rs2216460 and 0.86 for rs1368884) and *RBX1* (0.83 for rs426612 and 0.92 for rs139480) loci than at the KEAP1 locus (1.08 for rs2112527 and 0.98 for rs726789), implying that genomic losses of *CUL3* and *RBX1* might lead to up-regulated NF-κB signaling through dysregulation of the IKKβ ubiquitin proteolysis mechanism in breast cancers. In addition, British Columbia Cancer Research Center (BCCRC) submegabase resolution tiling (SMRT) arrays of 17 breast cancer cell lines analyzed using System for Integrative Genomic Microarray Analysis (SIGMA) software (Chari et al., 2006) supported the notion that allelic loss of the *CUL3* and *RBX1* loci occurs in breast cancer (Figure 4B). Similarly, regular copy-number loss of the *KEAP1* (0.85 for rs426612 and 0.90 for rs139480 in 9 liver cancer cell lines; Figure S11A) and *CUL3* (0.71 for rs2216460 and 0.80 for rs1368884 in 28 esophageal cancer cell lines; Figure S11B) loci occurred. High percentages of genome copy number variations were found in liver, colorectal, and lung cancer tissues, but almost no variations were found in their adjacent normal tissues (Table S2), indicating that genome alternation at the *CUL3*, *KEAP1*, and *RBX1* loci might contribute to several types of cancer development. In summary, these genomic studies found that different rates of loss at the *CUL3*, *KEAP1*, and *RBX1* loci occurs in multiple human cancers, which results in an incomplete KEAP1-CUL3-RBX1 ubiquitin proteasome complex and may culminate in NF-κB activation through impairment of IKKβ degradation.

Clinical Association between IKKβ Expression, KEAP1 and CUL3 Expression, and Survival in Breast Cancer Patients

To justify the pathological relevance of the relationship between IKKβ and the KEAP1-CUL3-RBX1 ubiquitin complex, we analyzed IKKβ, KEAP1, and CUL3 protein expression in 119 human breast cancer specimens using immunohistochemical (IHC) staining. After hierarchical clustering, the results showed that negative IKKβ expression was detected in 26 (66.7%) of 39 specimens with high KEAP1/CUL3 expression and that IKKβ expression was inversely correlated with KEAP1/CUL3 expression ($P < 0.034$; Figure 4C and Table S3). This correlation was also observed between the KEAP1/CUL3 complex and NRF2 ($P < 0.007$; Table S4). Figure 4D shows the representative IHC-stained tumor specimens from consecutive sections in two patients demonstrating the inverse correlation between IKKβ expression and KEAP1/CUL3 expression.

We next analyzed KEAP1 and CUL3 expression in breast tumor tissues and correlated the findings with patient survival data. The Kaplan-Meier overall survival curves showed that low KEAP1 levels were associated with poor survival ($P < 0.014$; Figure S12). Moreover, the combination of low KEAP1/CUL3 expression and high IKKβ expression was a better predictor of survival than either factor alone was ($P < 0.006$ versus $P < 0.006$; Figure 4E). Consistently, cDNA microarray data from 286 lymph-node–negative patients (Wang et al., 2005), identified in the Integrated Tumor Transcriptome Array and Clinical data Analysis database (Elfilali et al., 2006), showed that KEAP1 expression in breast cancer patients with a relapse-free survival duration of at least 5 years was higher than that in patients with a relapse-free survival of less than 5 years (Figure S13). Taken together, the tumor IHC staining data and cDNA microarray data further strengthened the notion that the KEAP1-CUL3-RBX1 ubiquitin complex promotes oncogenic kinase IKKβ degradation and that down-regulation of KEAP1 and/or CUL3 expression is associated with poor clinical outcome in patients with breast cancer.

Human Cancer Somatic Mutations Significantly Reduced KEAP1-Mediated IKKβ Ubiquitination

To further determine whether deficient functioning of the *KEAP1* mutation may negate its ability to degrade IKKβ in human cancers, we sequenced KEAP1 protein-coding exons (E2–E6) in a set of 26 cancer cell lines (18 from breast, 4 from liver, and 4 from lung) and 119 primary tumors (17 from breast, 78 from liver, 13 from lung, and 11 from colon). One new SNP (G9R) and three nonsynonymous somatic mutations (H274L, S404X, and D479G) were identified in the liver cancer specimens (Figure S14 and Table S5). We then investigated the functional effects of these KEAP1 mutations and found that the S404X and D479G mutants, but not the G9R and H274L mutants, exhibited reduced IKKβ binding potential compared with wild-type KEAP1 and thus were unable to promote IKKβ ubiquitination (Figure 4F). Consistently, S404X and D479G mutants were not able to regulate NRF2 ubiquitination and degradation (Figures S15A and S15B). To further validate these cancer-associated mutations with regard to NF-κB activation, we knocked down endogenous KEAP1 by KEAP1 siRNA targeting the *KEAP1* mRNA 3′ untranslated region (UTR), re-expressing the KEAP1 wild type and mutants into cells, and studying their effects on NF-κB pathway by examining a panel of NF-κB–targeted mRNA expressions by qRT-PCR. The results showed that wild-type KEAP1 decreased several NF-κB–targeted mRNA expressions in comparison to vector control and that S404X and D479G mutants function as dominant-negative forms as shown by the significant increase in NF-κB downstream gene expression via re-expressing these mutants (Figure 4G). These data suggest that cancer-associated mutations fail to facilitate IKKβ degradation and further contribute to cancer development via the up-regulation of NF-κB activity.

Moreover, several KEAP1 mutants have recently been identified in lung and breast cancers (Ohta et al., 2008; Padmanabhan et al., 2006; Singh et al., 2006; Wood et al., 2007). Some mutations on the N-terminal and BTB domains of KEAP1 were found to interfere with the KEAP1-CUL3-RBX1 ubiquitin complex formation, and other mutations on Kelch domains to reduce KEAP1 binding affinity for NRF2, which results in stabilizing NRF (Nioi and Nguyen, 2007; Ohta et al., 2008; Padmanabhan et al., 2006; Singh et al., 2006). We sought to determine whether these previously identified KEAP1 mutants (C23Y, G333C, G364C, R413L, and G430C) were unable to catalyze IKKβ ubiquitination. We found that four of the five KEAP1 mutants (G333C, G364C, R413L, and G430C) were not able to associate with and trigger IKKβ ubiquitination (Figure S16), which was consistent with the results on decreased E3 ligase activity on NRF2 (Padmanabhan et al., 2006; Singh et al., 2006). However, there was no difference between wild-type KEAP1 and C23Y mutant–mediated IKKβ ubiquitination. It is worthwhile to mention that these six functionally deficient KEAP1 mutants (G333C, G364C, S404X, R413L, G430C, and D479G) are all mutated in the Kelch domain (amino acids 315–598, the IKKβ interaction region), which is consistent with our finding of the importance of the KEAP1 Kelch domain for IKKβ binding (Figures 2F and 2G). Taken together, these results suggest a possibility that allelic loss of the *KEAP1*, *CUL3*, and *RBX1* genes and/or missense mutations on *KEAP1* may increase IKKβ stability, which then results in activation of the NF-κB pathway in human cancers

We also investigated whether mutations exist in the IKKβ NQETGE region, which might prevent binding to KEAP1 and result in increased IKKβ expression, but we did not find any somatic mutations in this region in a set of 119 primary tumors (17 from breast, 78 from liver, 13 from lung, and 11 from colon) and 26 cancer cell lines (18 from breast, 4 from liver, and 4 from lung) (data not shown). Recently, a cancer-derived IKKβ mutant (A360S) was identified in a human breast cancer cell line (Wood et al., 2007). We examined whether this mutation is resistant to KEAP1-mediated ubiquitination and found that the breast cancer–derived IKKβ A360S mutant could still be ubiquitinated by KEAP1 (Figure S17), suggesting that

dysregulation of KEAP1-mediated IKKβ degradation might result primarily from low activity of the KEAP1-CUL3-RBX1 ubiquitin complex (as a result of somatic mutations or allelic loss) but not from somatic *IKKβ* mutations. In summary, the loss of KEAP1-CUL3-RBX1 function is a frequent event in human cancers that prevents IKKβ from degrading, leading to activation of that NF-κB pathway and angiogenesis.

In summary, we discovered that KEAP1 down-regulates the NF-κB signaling pathway by way of functioning as an IKKβ E3 ligase (Figure S18). Our study links cancer genomic alternations and the aberrant activation of oncogenic signaling pathways and suggests that ubiquitin-mediated oncoprotein proteolysis contributes to inflammation-associated tumor development. Given that the induction of KEAP1 expression will kill two birds (IKKβ and NRF2) with one stone (KEAP1), further investigations to identify the KEAP1 activation mechanism and development of drugs targeting KEAP1 expression may have vital clinical implications for the treatment or prevention of cancers.

EXPERIMENTAL PROCEDURES

Supplemental Experimental Procedures are provided in the Supplemental Data.

Quantitative RT-PCR

We extracted total RNA from the cells using TRIzol (Invitrogen) and quantified the total RNA using the SmartSpec Plus Spectrophotometer (BIORAD). We performed reverse transcription using the Superscript III first-stand synthesis system (Invitrogen) according to the manufacturer's instructions. We performed qRT-PCR analyses of human *BIRC4*, *PTGS2*, *NFKBIA*, *PLAU*, *BIRC3*, *IL-8*, *GADD45B*, *SOD2*, *CCND1*, *CCL2*, *IL-1A*, *BCL2L1*, and *KEAP1* using designed primers (Table S6) and iQ SYBR Green Supermix (BIORAD). We then performed assays using the ABI StepOne Plus Real-Time PCR system (Applied Biosystems). We used *GAPDH* for normalization.

Genome Structure Analyses

For SNP-based copy number analysis, we obtained Affymetrix 10K SNP microarray genotype data for 42 breast cancer cell lines, 9 liver cancer cell lines, and 27 esophageal cancer cell lines from the Cancer Genome Project of the Wellcome Trust Sanger Institute. We analyzed the SNP data of the *CUL3*, *KEAP1*, and *RBX1* loci, visualized them using the Cluster and TreeView software (Eisen, MB), and presented the results using heat maps. For array comparative genomic hybridization (aCGH) analysis, we used SIGMA software (Chari et al., 2006) to examine the genomic gain and loss of the *CUL3*, *KEAP1*, and *RBX1* loci and obtained results from BCCRC SMRT arrays of 17 breast cancer cell lines with genome karyograms using the March 2006 genomic build.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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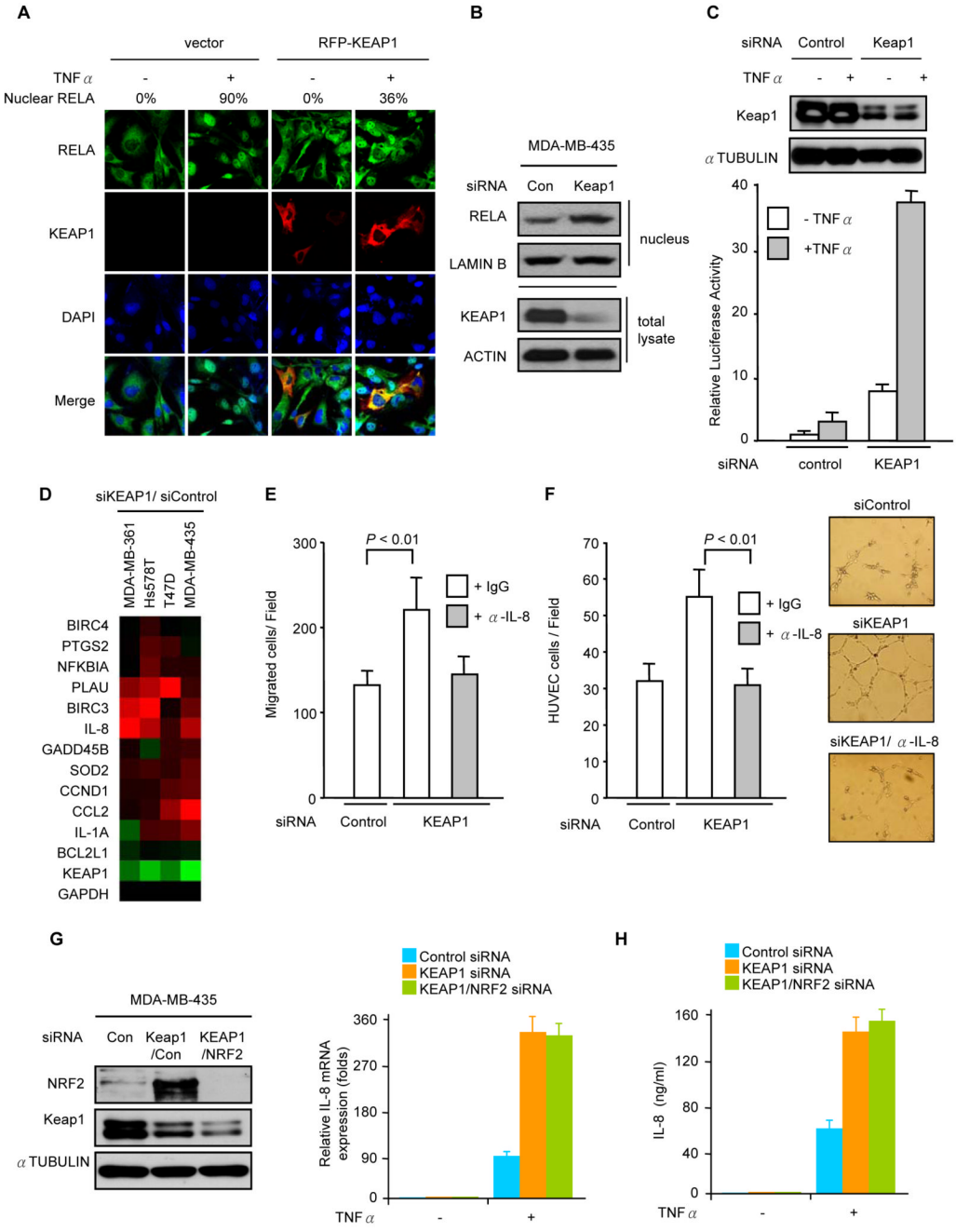

Figure 1. KEAP1 selectively inhibits the NF-κB signaling pathway

(A) Ectopic of KEAP1 suppressed TNFα–mediated RELA nuclear translocation. Hs578T breast cancer cells were transfected with either RFP-KEAP1 or vector, serum-starved overnight, treated with 2 ng/ml TNFα for 30 min, stained with anti-RELA antibodies (green), and examined by confocal microscopy. The nucleus was stained with DAPI (blue). The arrow indicates KEAP1-expressed cells (red).

(B) Depletion of endogenous KEAP1 by KEAP1 siRNA led to accumulation of nuclear RELA.

(C) Knockdown of KEAP1 increased TNFα–induced NF-κB activation. The 5IκB-Luc reporter and TK-rLuc (internal control) were transfected with either KEAP1 siRNA or control siRNA into MDA-MB-435 cells. Then, 48 hr post-transfection, cells were serum-starved overnight

and treated with 2 ng/ml TNFα. After 8 hr of TNFα treatment, cells were recovered in 1% serum medium overnight and then lysed for luciferase assays. Error bars represent SDs (n=3).
(D) Silencing KEAP1 by KEAP1 siRNA up-regulated the expression of NF-κB–responsive genes in human breast cancer cells. A heat map depicts the relative expression of 12 NF-κB–dependent genes by qRT-PCR. A nonspecific siRNA was used as the control.
(E–F) *In vitro* angiogenesis assays showed that the silencing of KEAP1 increase HUVEC tube formation and migration in comparison with the control. Depletion of IL-8 by antibodies to IL-8 (α-IL-8) suppressed knockdown of KEAP1-induced HUVEC migration and tube formation. Error bars represent SDs (n=3).
(G–H) Depletion of KEAP1 following TNFα stimulation increased IL-8 mRNA and protein levels as determined by qRT-PCR and ELISA, respectively. Knockdown of NRF2 had no effects on KEAP1 depletion–induced IL-8 expression. Error bars represent SDs (n=3).

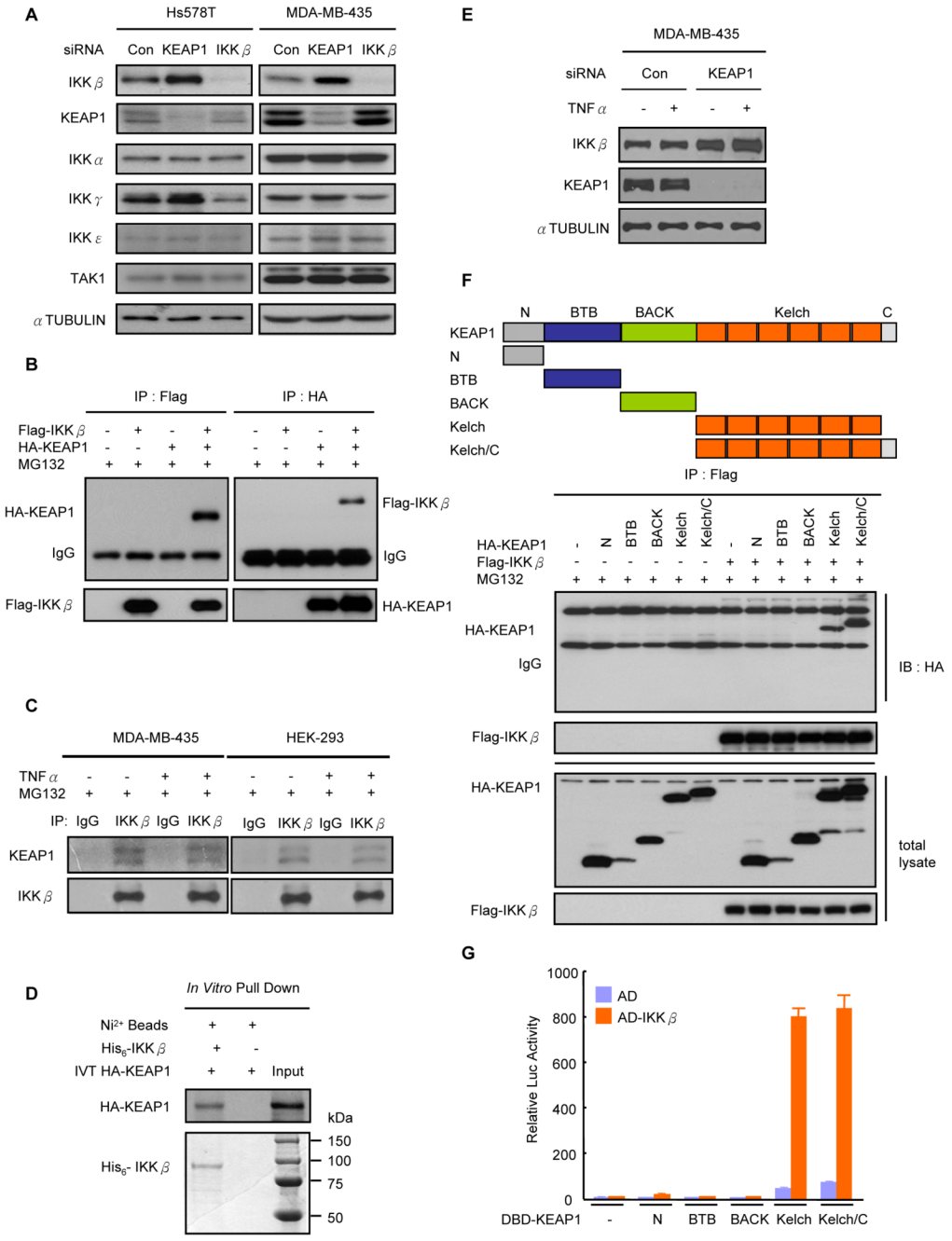

Figure 2. KEAP1 interacts with IKKβ via KEAP1 Kelch domain

(A) Depletion of endogenous KEAP1 by KEAP1 siRNA led to accumulation of IKKβ but not of other components involved in NF-κB activation.

(B) Exogenous interaction between KEAP1 and IKKβ. Lysates of HEK-293T cells cotransfected with HA-tagged KEAP1 and Flag-tagged IKKβ.

(C) Endogenous interaction between KEAP1 and IKKβ in both unstimulated and TNFα–stimulated MDA-MB-435 and HEK-293 cells.

(D) KEAP1 directly interacted with IKKβ. *In vitro* transcribed and translated [35S]methionine-labeled KEAP1 proteins were incubated with recombinant His-tagged IKKβ proteins, pulled

down by Ni^{2+} beads, separated by SDS-PAGE, and visualized by autoradiography. IVT, *in vitro* transcription and translation.

(E) The effect of KEAP1 knockdown on TNFα–mediated IKKβ activation.

(F) Interaction between the KEAP1 Kelch domain and IKKβ as examined by co-IP. Schematic shows five different domains of KEAP1, including an N-terminal region (amino acids 1–60), a BTB domain (amino acids 61–179), a BACK domain (amino acids 180–314), a Kelch domain (amino acids 315–598, six Kelch motifs), and a C-terminal domain (amino acids 599–624).

(G) Mammalian-two hybrid assay revealed the direct interaction between KEAP1 Kelch domain and IKKβ. The indicated regions of KEAP1 fused to the GAL4 DNA-binding domain (DBD) were cotransfected VP16 activation domain (AD) –fused IKKβ (full length), GAL4 luciferase reporter, and TK-rLuc reporter (internal control). Error bars represent SDs (n=3).

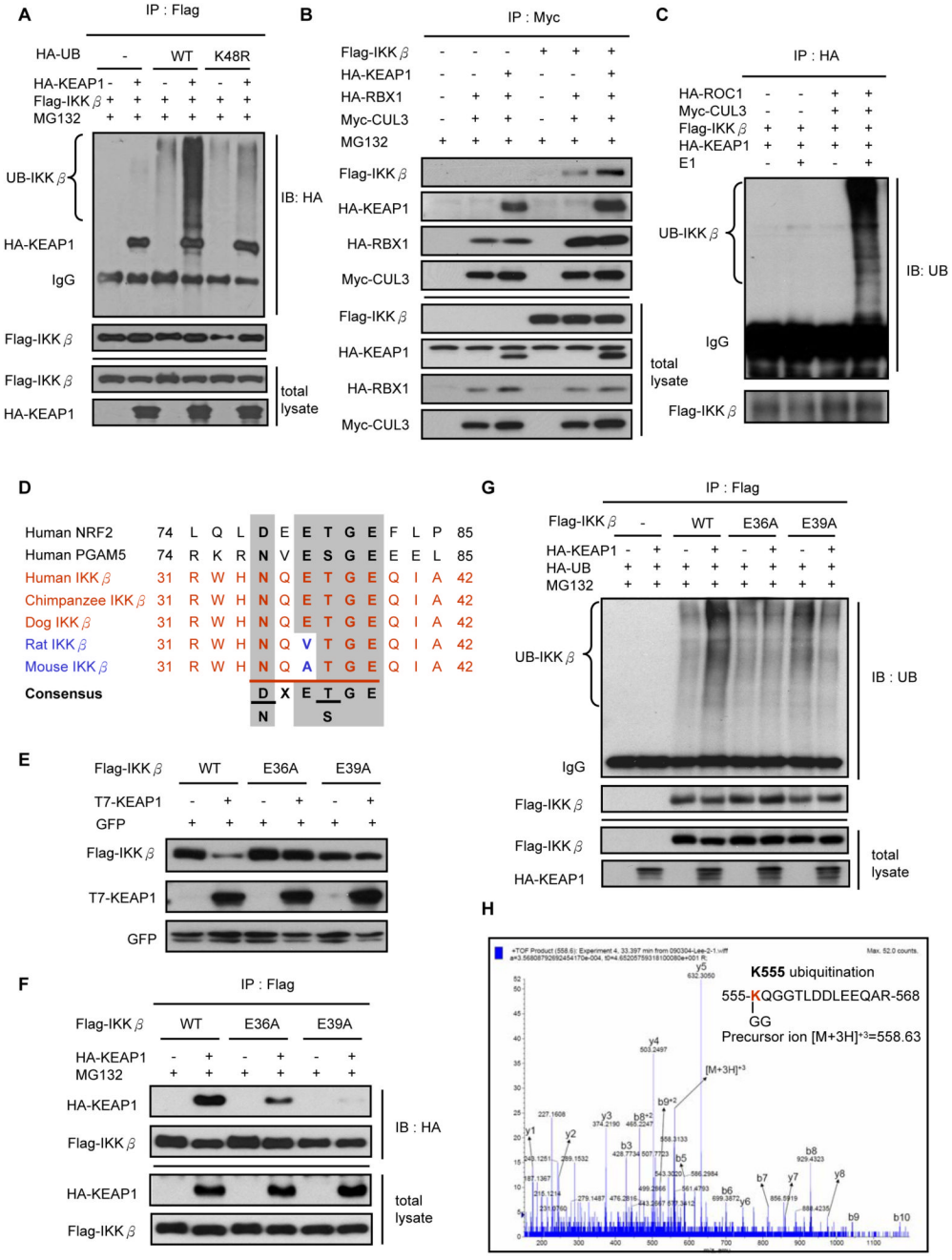

Figure 3. KEAP1 functions as a CUL3-based E3 ligase of IKKβ

(A) KEAP1-dependent K48 ubiquitination of IKKβ *in vivo*. Flag-tagged IKKβ and HA-tagged KEAP1 were cotransfected with either wild-type or K48R mutant ubiquitin into HEK-293T cells. After 24 hr post-transfection, cells were treated with MG132 for 6 hr. Flag-tagged IKKβ was immunoprecipitated, and then ubiquitination was analyzed by blotting with anti-ubiquitin antibody.

(B) KEAP1 bridges the interaction between IKKβ and the CUL3-RBX1 complex. CUL3 immunoprecipitates were analyzed by immunoblot with the indicated antibodies.

(C) *In vitro* ubiquitination of IKKβ by the KEAP1-CUL3-RBX1 complex. Flag-tagged IKKβ was incubated with KEAP1, CUL3, and RBX1 in the presence of E1, E2, His-Ubiquitin, and ATP as indicated.
(D) Human IKKβ contains a KEAP1-binding (D/N)XE(T/S)GE motif (D, aspartic acid; N, asparagine; E, glutamic acid; T, threonine; S, serine; G. glycine; and X, any amino acid).
(E) KEAP1 markedly decreased steady-state levels of wild-type but not E36A and E39A mutant IKKβ. GFP served as an internal control.
(F–G). E36A and E39A mutant IKKβ markedly reduced their binding ability to KEAP1 and were resistant to KEAP1-mediated ubiquitination.
(H) Mass spectrometry analysis revealed that IKKβ K555 is a polyuibiquitination site. Flag-tagged IKKβ, HA-tagged KEAP1, and HA-tagged ubiquitin were cotransfected into HEK-293T cells. After 1 day post-transfection, cells were treated with MG132 for 6hr to prevent the degradation of polyubiquitinated IKKβ. Polyubiquitinated IKKβ were pull down by using antibodies against HA tags and analyzed by μ-LC/MS/MS mass spectrometry.

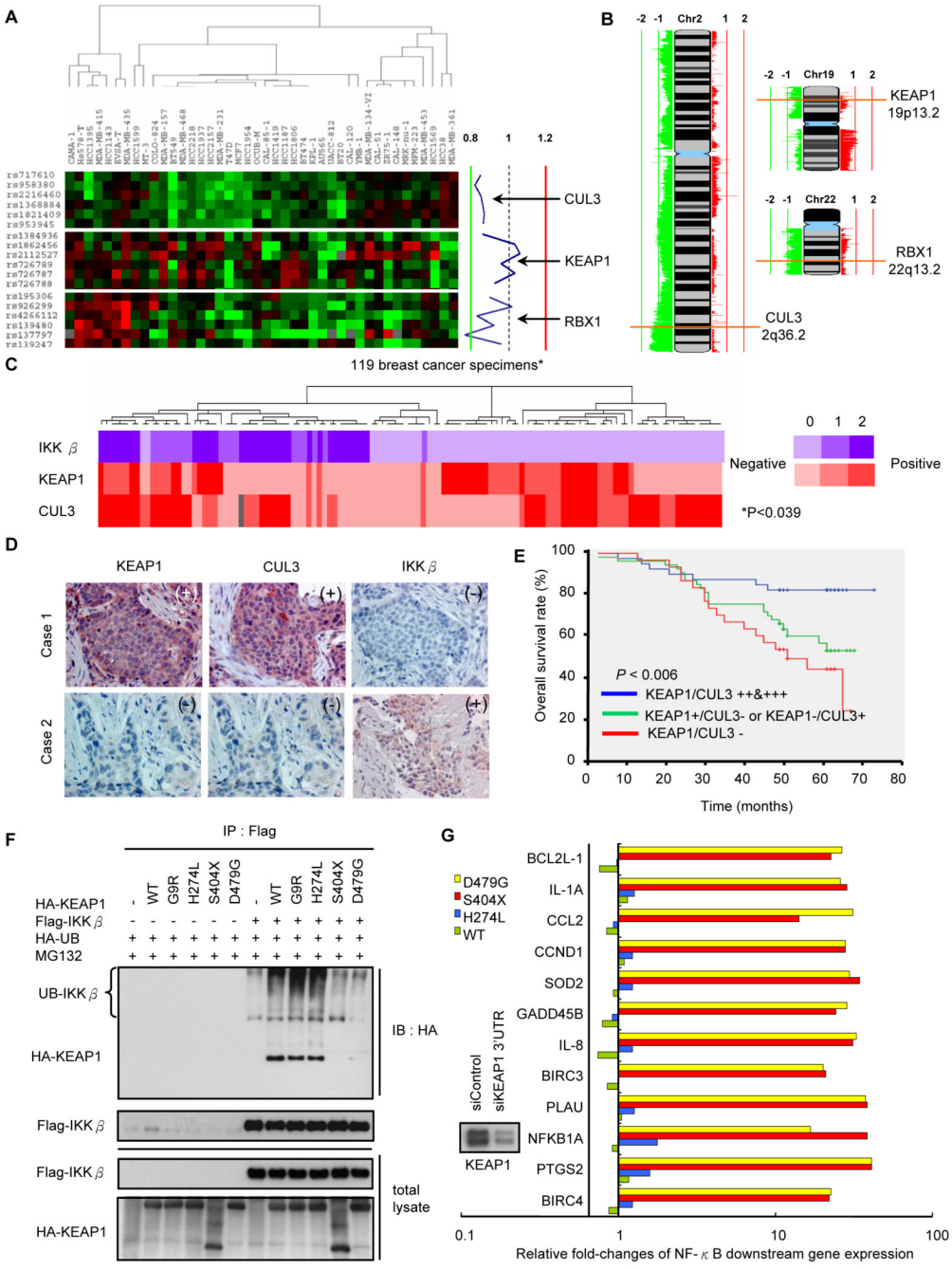

Figure 4. Negative regulation of IKKβ by KEAP1-CUL3-RBX1 ubiquitin complex is altered in human cancers

(A) Analysis of the *CUL3*, *KEAP1*, and *RBX1* loci by 10 K SNP arrays in 42 breast cancer cell lines. Colograms represent SNP copy numbers. *Red* represents allelic gain; *green* indicates allelic loss.

(B) *SIGMA* analysis of the *CUL3*, *KEAP1*, and *RBX1* loci based on aCGH data. *Red* indicates areas of recurrent gain; *green* denotes areas of recurrent loss. The maximum values of 1 and −1 represent gain and loss of that area in all samples, respectively.

(C) A reverse clinical correlation existed between IKKβ, KEAP1, and CUL3 expression in 119 breast cancer specimens ($P < 0.039$). Hierarchical clustering was performed on the CLUSTER

program and constructed using Treeview software to show a cohort of 119 breast cancer samples stained with IKKβ, KEAP1, and CUL3 expressions, and the heat map representation of the data is shown. Detailed information is presented in Table S3.
(D) IKKβ expression levels were negatively associated with KEAP1 and CUL3 expression in 119 primary human breast cancer specimens. Shown are two representative specimens.
(E) The Kaplan-Meier overall survival curves indicate that KEAP1 and CUL3 together are associated with an increase in overall survival in breast cancer patients.
(F) In liver cancer, the KEAP1 Kelch domain mutants (S404X and D479G), but not the N domain mutant (G9R) or BACK domain mutant (H274L), have weaker binding abilities to IKKβ and consequently were unable to catalyze IKKβ ubiquitination.
(G) Wild-type KEAP1 negatively down-regulates NF-κB activity, and KEAP1 Kelch domain mutants (S404X and D479G) serve as dominant-negative forms to regulate NF-κB function. Endogenous KEAP1 was first knocked down by Accell KEAP1 siRNA, which targets the *KEAP1* mRNA 3′UTR region. The KEAP1–knocked-down MDA-MB-435 cells were further transfected with wild-type and mutant KEAP1, and a panel of NF-κB–targeting gene expression was examined by qRT-PCR.