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IKKε-Mediated Tumorigenesis Requires K63-Linked Polyubiquitination by a cIAP1/cIAP2/TRAF2 E3 Ubiquitin Ligase Complex

Alicia Y. Zhou,[1,2,3] Rhine R. Shen,[1,2,3] Eejung Kim,[1,2,3] Ying J. Lock,[1,2,3] Ming Xu,[4] Zhijian J. Chen,[4,5] and William C. Hahn[1,2,3,*]
[1]Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA 02115, USA
[2]Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA
[3]Broad Institute of Harvard and MIT, Cambridge, MA 02142, USA
[4]Department of Molecular Biology
[5]Howard Hughes Medical Institute
University of Texas Southwestern Medical Center, Dallas, TX 75390-9148, USA
*Correspondence: william_hahn@dfci.harvard.edu
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SUMMARY

IκB kinase ε (IKKε, *IKBKE*) is a key regulator of innate immunity and a breast cancer oncogene, amplified in ~30% of breast cancers, that promotes malignant transformation through NF-κB activation. Here, we show that IKKε is modified and regulated by K63-linked polyubiquitination at lysine 30 and lysine 401. Tumor necrosis factor alpha and interleukin-1β stimulation induces IKKε K63-linked polyubiquitination over baseline levels in both macrophages and breast cancer cell lines, and this modification is essential for IKKε kinase activity, IKKε-mediated NF-κB activation, and IKKε-induced malignant transformation. Disruption of K63-linked ubiquitination of IKKε does not affect its overall structure but impairs the recruitment of canonical NF-κB proteins. A cIAP1/cIAP2/TRAF2 E3 ligase complex binds to and ubiquitinates IKKε. Altogether, these observations demonstrate that K63-linked polyubiquitination regulates IKKε activity in both inflammatory and oncogenic contexts and suggests an alternative approach to targeting this breast cancer oncogene.

INTRODUCTION

Nuclear factor κB (NF-κB) signaling plays a critical role in innate immunity, and inflammation has been implicated in cancer development (Arkan and Greten, 2011; Bassères and Baldwin, 2006) where aberrant NF-κB signaling in the tumor microenvironment contributes to tumor growth (Karin, 2006). In addition, dysregulation of specific NF-κB proteins can contribute to cell transformation in a cell-autonomous manner. For example, deletion of the tumor suppressor *CYLD* leads to familial cylindromatosis (Brummelkamp et al., 2003; Kovalenko et al., 2003; Trompouki et al., 2003) and mutations in *NFKB1* and *NFKB2* play a role in multiple myeloma (Annunziata et al., 2007).

The canonical NF-κB pathway is activated by proinflammatory signals and converges on the activation of the IκB kinase (IKK) complex by the TRAF E3 ligase family (Perkins, 2007). The IKK complex consists of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ/NEMO (Hayden and Ghosh, 2004; Israël, 2010). Proteasome-dependent and proteasome-independent forms of ubiquitination are required to activate NF-κB signaling (Skaug et al., 2009). Several groups have shown that proteasome-independent Lysine 63 (K63)-linked IKKγ ubiquitination is a key step in IKK complex activation (Tang et al., 2003; Zhou et al., 2004). Linear (Met1) IKKγ ubiquitination also leads to IKK complex activation (Bianchi and Meier, 2009; Iwai and Tokunaga, 2009). IKK activation by nondegradative ubiquitination leads to phosphorylation of inhibitor of κB (IκB) proteins (Baldwin, 1996). This phosphorylation triggers the K48-linked ubiquitination and subsequent proteasome-mediated degradation of the IκB proteins, which allows for the nuclear translocation of NF-κB dimers and activation of proinflammatory NF-κB response genes (Karin and Ben-Neriah, 2000).

Inhibitor of κB kinase ε (IKKε, IKK-i, *IKBKE*) is a noncanonical IKK that activates interferon, NF-κB, and STAT signaling (Fitzgerald et al., 2003; Ng et al., 2011; Peters et al., 2000; Shimada et al., 1999). With its structurally related binding partner TBK1, IKKε regulates interferon responses by phosphorylation of IRF3 and IRF7 (Chau et al., 2008; Fitzgerald et al., 2003; Tenoever et al., 2007), which induces nuclear translocation of IRF3/7 and activation of type I interferon genes (Fitzgerald et al., 2003). IKKε is also an oncogene that is amplified and overexpressed in ~30% of breast cancers (Boehm et al., 2007). *IKBKE* induces malignant transformation in an NF-κB-dependent manner, and suppression of IKKε in cancer cells that harbor *IKBKE* amplifications induces cell death. Recent studies demonstrated that STAT3 activates *IKBKE* transcription (Guo et al., 2013) and have identified AKT as one target of TBK1 and IKKε (Guo et al., 2011; Xie et al., 2011). We have identified CYLD as one substrate of IKKε and effector of IKKε-mediated transformation (Hutti et al., 2009). However, the mechanism(s) that regulate IKKε remain poorly understood.

Here, we show that IKKε is K63 ubiquitinated and investigate the role of this modification in IKKε-mediated NF-κB activation and cell transformation.

RESULTS

IKKε Is Ubiquitinated

To determine whether IKKε is ubiquitinated, we introduced hemagglutinin (HA)-tagged ubiquitin (HA-Ub) and either FLAG-tagged or myristolated-FLAG-tagged IKKε (F-IKKε or MF-IKKε) into HEK293T cells. We purified HA immune complexes and found that both F-IKKε and MF-IKKε are ubiquitinated (Figure 1A).

We previously showed that IKKε confers tumorigenicity in human embryonic kidney (HEK) epithelial and mammary epithelial cells (HMEC) expressing the SV40 Early Region (SV40ER), the telomerase catalytic subunit (hTERT), and a constitutively active form of MEK (MEK^{DD}) (Boehm et al., 2007). To test whether IKKε ubiquitination occurs when IKKε is expressed at levels found in cancer cells, we isolated IKKε immune complexes from transformed HEK (HA1EM F-IKKε) and HMEC (HMLEM MF-IKKε) cells and found that IKKε is polyubiquitinated (Figure 1B; Figure S1). We then examined whether IKKε is ubiquitinated in breast cancer cell lines (MCF-7 and ZR-75-1) that harbor an *IKBKE* amplification and found endogenous polyubiquitinated species of IKKε (Figure 1C). These observations demonstrate that IKKε is ubiquitinated in the setting of IKKε-mediated cell transformation.

We next assessed if IKKε is ubiquitinated in response to inflammatory stimuli. We stimulated RAW 264.7 gamma NO(−) macrophages with lipopolysaccharide (LPS) to initiate an innate immunity response. We found LPS stimulation induced both IKKε expression and ubiquitination in these macrophages (Figure 1D). In addition, we treated MCF-7 and ZR-75-1 cells with the inflammatory cytokines, tumor necrosis factor alpha (TNF-α), or interleukin-1β (IL-1β) and found increased IKKε ubiquitination over baseline levels (Figures 1E and 1F). Together, these observations show that IKKε ubiquitination occurs in the context of IKKε-induced transformation and inflammatory stimulation.

IKKε Undergoes K63-Linkage-Specific Ubiquitination

Whereas K48-linked polyubiquitination usually target substrates for proteasome-mediated degradation, modification by K63-linked, K11-linked, and linear ubiquitin chains leads to proteasome-independent changes in protein function (Pickart and Eddins, 2004). To assess if IKKε ubiquitination is proteasome dependent, we treated transformed HA1EM MF-IKKε, MCF-7, and ZR-75-1 cells with two proteasome inhibitors, MG-132 and bortezomib. The overall level of ubiquitination was increased in the presence of proteasome inhibitors. However, we failed to observe differences in the level of IKKε, suggesting that ubiquitination does not regulate IKKε stability (Figure 2A).

We then used three methods to determine the linkage-type of IKKε ubiquitination. First, we introduced Myc-tagged IKKε and HA-tagged wild-type (WT), K11-only, K48-only, or K63-only ubiquitin mutants into HEK293T cells. We note that the HA-epitope tag directly interferes with the formation of head-to-tail ubiquitin chains and renders these constructs as Met1-linkage-deficient mutants. We isolated IKKε immune complexes and found that IKKε is robustly ubiquitinated by WT and K63-only ubiquitin (Figure 2B). In contrast, IKKε was not ubiquitinated by the K11-only and K48-only ubiquitin mutants (Figure 2B).

To confirm these observations, we used linkage-specific ubiquitin antibodies. In MCF-7 and ZR-75-1 cells, we isolated K48- or K63-linkage-specific immune complexes and found that IKKε was present only in the immune complexes formed by the K63-linkage-specific antibody (Figure 2C).

Finally, we used a genetic system in which endogenous ubiquitin is inducibly suppressed by ubiquitin-specific small hairpin RNAs (shRNAs) in parallel to inducible expression of WT or mutant ubiquitin (Xu et al., 2009). In U2OS shUb-Ub(WT) cells, a shRNA-insensitive WT ubiquitin is expressed. In U2OS shUb-Ub(K63R) cells, a shRNA-insensitive K63R mutant form of ubiquitin is expressed, which is unable to form K63-linkage-specific chains. We isolated IKKε immune complexes from U2OS shUb-Ub(WT) and shUb-Ub(K63R) cells in the presence or absence of tetracycline and assessed these complexes for ubiquitin. We confirmed that IKKε is modified by WT ubiquitin chains but is not modified by the K63R chains (Figure 2D). In aggregate, we concluded that IKKε is modified by K63-linked ubiquitin chains in breast cancer cells.

IKKε Is Ubiquitinated at K30, K401, and K416

To determine the lysine residues on which IKKε is ubiquitinated, we expressed glutathione S-transferase (GST)-tagged IKKε and HA-tagged ubiquitin in HEK293T cells, separated GST immune complexes by electrophoresis, and submitted four bands for mass spectrometry analysis (Figure 3A). We identified IKKε K30, K401, and K416 as polyubiquitinated (Figure 3B; Table S1).

To confirm these observations, we generated site-specific lysine-to-alanine (K30A, K401A, K416A) and lysine-to-arginine (K30R, K401R, K416R) IKKε mutants. After expressing WT and mutant IKKε and HA-ubiquitin in HEK293T cells, we isolated IKKε immune complexes and found that the K30 and K401 mutants exhibited decreased IKKε ubiquitination but saw no changes in ubiquitinated species of the IKKε K416 mutant (Figure 3C). We noted that the lysine-to-arginine and lysine-to-alanine IKKε mutants behaved identically in all assays.

We then created stable lines expressing each IKKε mutant and determined if they exhibited differential levels of IKKε ubiquitination. We found that the K30 and K401 IKKε mutants exhibited a significant decrease in ubiquitinated IKKε species while the ubiquitination of the K416 mutant was unchanged (Figure 3D; Figure S2). These observations suggested that the K30 and K401 residues of IKKε are essential for IKKε ubiquitination.

IKKε Ubiquitination at K30 and K401 and IKKε Activity

We previously identified CYLD as an IKKε substrate (Hutti et al., 2009). To determine the role of IKKε ubiquitination on IKKε function, we isolated CYLD immune complexes from U2OS shUb-Ub(WT) and shUb-Ub(K63R) cells (Figure 2D) cultured in the presence or absence of tetracycline and assessed the levels of both phospho-CYLD (pCYLD) and total CYLD. We found that under conditions where IKKε was not K63-linked ubiquitinated, IKKε exhibited impaired kinase activity (Figure 4A). Specifically,

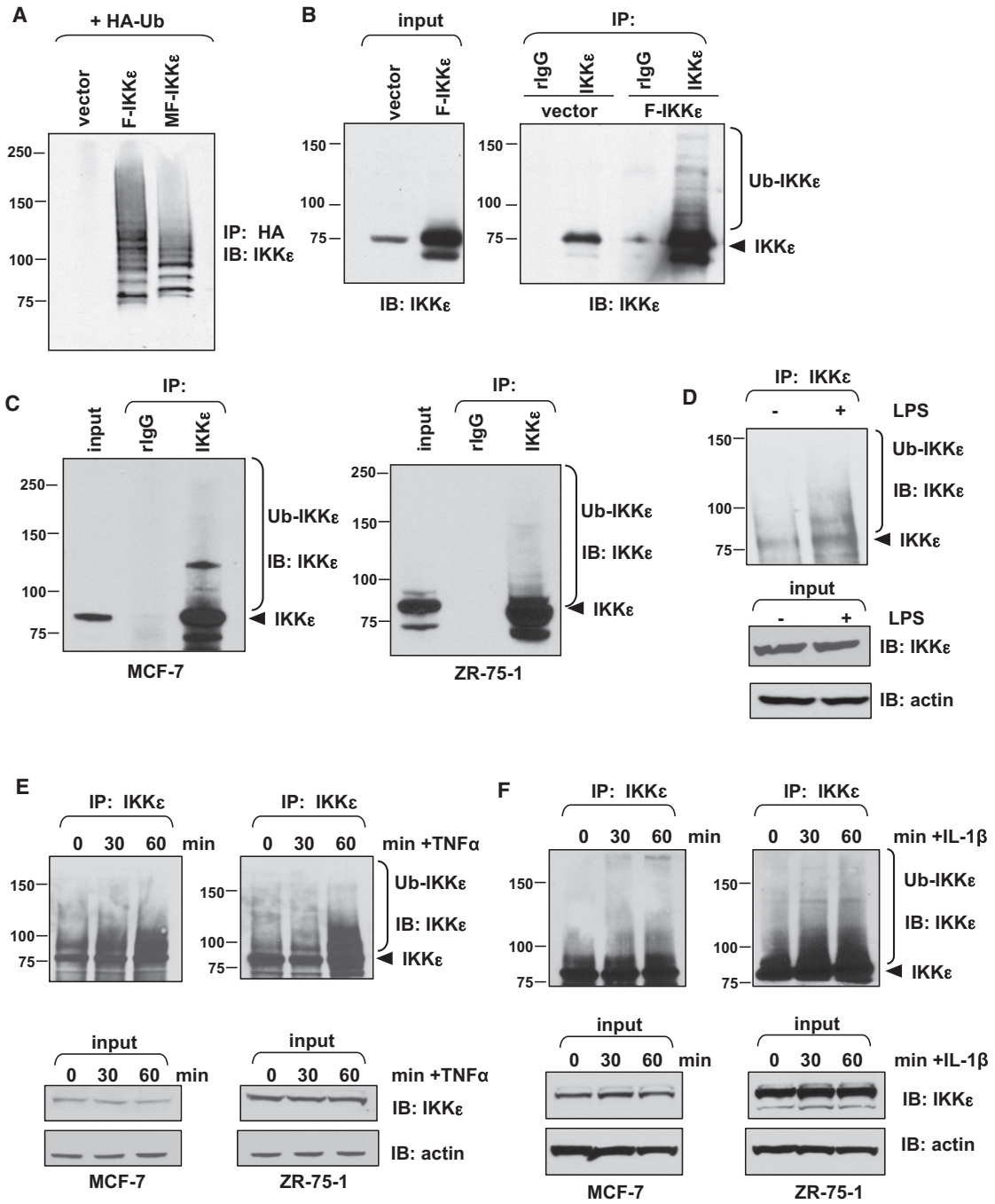

Figure 1. IKKε Is Ubiquitinated in the Context of Cell Transformation and Inflammation

(A) IKKε is ubiquitinated. HA immune complexes were isolated from HEK293T cells expressing the indicated proteins and immunoblotted with an IKKε-specific antibody.

(B) IKKε is ubiquitinated in transformed cells. IKKε immune complexes were isolated from HA1EM MF-IKKε cells using an IKKε-specific antibody and immunoblotted by the same antibody. Rabbit immunoglobulin (rIgG) was used as a control.

(C) IKKε is ubiquitinated in breast cancer cell lines. Endogenous IKKε immune complexes were isolated from MCF-7 and ZR-75-1 cells using an IKKε-specific antibody and immunoblotted by the same antibody.

(D) IKKε ubiquitination is induced by LPS treatment. RAW 264.7 gamma NO(−) macrophage cells were treated with 100 ng/ml LPS. IKKε immune complexes were isolated from cells using an IKKε-specific antibody and immunoblotted by the same antibody. Immunoblotting was performed with the indicated antibodies.

(legend continued on next page)

we failed to detect phosphorylation of CYLD in U2OS shUb-Ub(K63R) cells as assessed by a pCYLD-specific antibody.

To determine the effect of the K30 and K401 mutants on IKKε kinase activity, we assessed the ability of WT and mutant IKKε to phosphorylate CYLD in vivo. We cotransfected HEK293T cells with Myc-tagged CYLD and WT, K30R, or K401R IKKε. After isolating Myc immune complexes, we determined CYLD phosphorylation by IKKε pSubstrate immunoblot (Hutti et al., 2009). These observations confirmed that WT but not K30R or K401R IKKε phosphorylates CYLD (Figure 4B). We found both K30R and K401R IKKε mutants in CYLD immune complexes, indicating that these mutants still retained the ability to bind CYLD.

We recently solved the structure of the close IKKε homolog TBK1 and found that TBK1 forms a homodimer (Tu et al., 2013). When we expressed WT or mutant IKKε in HEK293T cells, we found that WT and mutant IKKε all formed homodimers (Figure S3A). In addition, we found that the K30R/K401R TBK1 mutant that cannot be ubiquitinated showed significantly decreased kinase activity as compared to WT TBK1 (Tu et al., 2013). These observations provide further evidence that these ubiquitination-deficient mutants do not disrupt the structure of the IKKε protein.

The activation of NF-κB signaling is essential for IKKε-mediated transformation (Boehm et al., 2007). To assess the effects of IKKε ubiquitination on NF-κB activation, we used a NF-κB luciferase reporter assay (Figure 4C) and we found that WT but neither IKKε mutant induced this NF-κB reporter. IKKε and TBK1 also interact with the canonical NF-κB proteins, IKKβ and IKKγ (NEMO), through the adaptor TANK (Chariot et al., 2002). This interaction allows IKKε and TBK1 to activate the canonical NF-κB pathway and TLR signaling. We found that WT IKKε robustly recruited IKKβ and IKKγ, while the IKKε mutants were defective in their ability to recruit these proteins (Figure 4D). This decreased binding resulted in a consequent decrease in TLR signaling as assessed by MyD88 recruitment (Figure S3B). These observations demonstrate that IKKε ubiquitination is required for NF-κB pathway activation.

IKKε Ubiquitination at K30 and K401 and IKKε-Mediated Transformation

To interrogate the role of ubiquitination in IKKε-mediated cell transformation, we assessed if mutant IKKε was able to transform cells (Figure 4E; Figures S3C and S3D). In HA1EM cells, expression of WT IKKε induces robust anchorage-independent colony growth. In contrast, the K30 and K401 IKKε mutants were markedly defective in anchorage-independent colony growth. This transformation phenotype was identical in both the lysine-to-arginine and lysine-to-alanine mutants.

To confirm these in vitro findings, we then assessed if expression of WT or mutant IKKε conferred tumorigenicity. We found that WT IKKε induced tumor formation. In contrast, the K30 and K401 IKKε mutants exhibited markedly impaired tumorigenicity (Figure 4F). These observations indicate that the K63-linkage-specific ubiquitination of IKKε at K30 and K401 are essential for IKKε-mediated cell transformation.

The cIAP1/cIAP2/TRAF2 E3 Ubiquitin Ligase Complex Ubiquitinates IKKε

Prior work has shown that IKKε forms a complex that includes TBK1, TRAF2, cIAP-1, and TANK (Pomerantz and Baltimore, 1999; Vince et al., 2009). In particular, the cIAP1/cIAP2/TRAF2 complex forms an active E3 ubiquitin ligase complex that K63-linkage ubiquitinates RIP1 during activation of the canonical NF-κB pathway (Bertrand et al., 2008; Shih et al., 2011; Vince et al., 2009; Zarnegar et al., 2008). Thus, we tested if the cIAP1/cIAP2/TRAF2 complex is an E3 ubiquitin ligase for IKKε.

To confirm that IKKε interacts with TRAF2 and cIAP1, we isolated IKKε immune complexes in MCF-7 cells and confirmed that IKKε binds to cIAP1 and TRAF2 (Figure 5A). We then performed an in vitro ubiquitination assay to identify which member(s) of the cIAP1/cIAP2/TRAF2 complex are responsible for IKKε ubiquitination. We found that expression of immunopurified TRAF2 induced a low level of IKKε ubiquitination and that either recombinant cIAP1 or cIAP2 alone induced strong ubiquitination of purified IKKε (Figure 5B). To confirm this observation, we introduced IKKε with either WT or E3 ligase-deficient mutant cIAP1, WT or E3 ligase-deficient mutant cIAP2, and TRAF2 into HEK293T cells. We found that WT cIAP1 alone and in complex with WT cIAP2 and TRAF2 sufficed to induce IKKε ubiquitination (Figure 5C). When expressed in these cells, TRAF2 also induced IKKε ubiquitination. However, mutant cIAP1 and cIAP2 disrupted the ability of TRAF2 to ubiquitinate IKKε. Together, these observations support a model in which the cIAP1/cIAP2/TRAF2 E3 ligase complex is responsible for IKKε ubiquitination.

To confirm that cIAP1, cIAP2, and TRAF2 are required for IKKε ubiquitination, we suppressed the expression of these proteins in MCF-7 cells and assessed IKKε ubiquitination. We suppressed TRAF2 with two independent *TRAF2*-specific shRNAs (shTRAF2 #1 and shTRAF2 #2). We then isolated IKKε immune complexes and found that cells in which TRAF2 was suppressed exhibited a decrease in polyubiquitinated IKKε proportional to the amount of TRAF2 suppression (Figure 5D). We next suppressed cIAP1 and cIAP2 expression alone or in combination with two independent *cIAP1*-specific shRNAs (shcIAP1 #1, shcIAP1 #2) and *cIAP2*-specific shRNAs (shcIAP2 #1, shcIAP2 #2), respectively. When we isolated IKKε immune complexes, we found that cells in which cIAP1 or cIAP2 was suppressed independently or in combination exhibited a decrease in polyubiquitinated IKKε (Figure 5E). These observations show that all three components of the cIAP1/cIAP2/TRAF2 E3 ligase complex are essential for IKKε ubiquitination.

(E) IKKε ubiquitination is induced by TNF-α treatment. MCF-7 and ZR-75-1 were treated with 20 ng/ml TNF-α as indicated. IKKε immune complexes were isolated from cells using an IKKε-specific antibody and immunoblotted by the same antibody.

(F) IKKε ubiquitination is induced by IL-1β treatment. MCF-7 and ZR-75-1 were treated with 20 ng/ml IL-1β as indicated. IKKε immune complexes were isolated from cells using an IKKε-specific antibody and immunoblotted by the same antibody. A total of 5% of the whole cell lysate (WCL) was used as an input control for all panels.

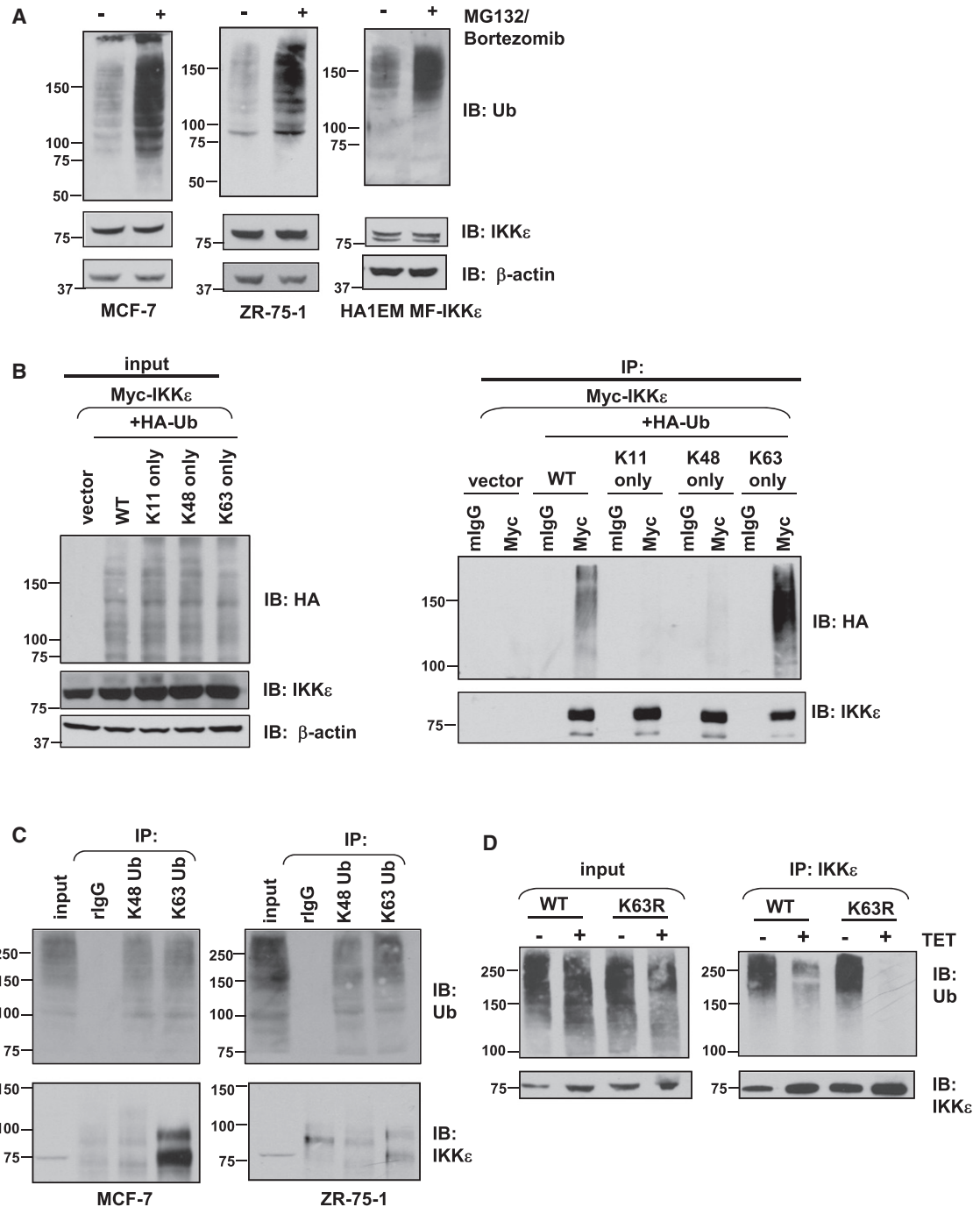

Figure 2. IKKε Is Modified by K63-Linked Ubiquitination

(A) Proteasome inhibitor treatment does not affect IKKε protein levels. MCF-7 and ZR-75-1 and HA1EM MF-IKKε cells were treated with 10 μM MG-132 and 1 μM bortezomib. Immunoblotting was performed with the indicated antibodies.

(B) K63-linked ubiquitination of IKKε. HA-tagged WT, K11-only, K48-only, or K63-only ubiquitin mutants were cotransfected into HEK293T cells. Myc immune complexes (IKKε) were isolated followed by immunoblotting with the indicated antibodies. Murine immunoglobulin (mIgG) was used as a control.

(C) K63-linked ubiquitination of IKKε in breast cancer cell lines. Endogenous K48-linked polyubiquitin and K63-linked polyubiquitin immune complexes were isolated followed by immunoblotting with the indicated antibodies in MCF-7 and ZR-75-1 cells. rIgG was used as a control.

(D) U2OS-shUb-Ub(WT) or U2OS-shUb-Ub(K63R) cells were treated with tetracycline (TET) (1 μg/ml). IKKε immune complexes were isolated followed by immunoblot analysis with the indicated antibodies. A total of 5% of the WCL was loaded for comparison (input).

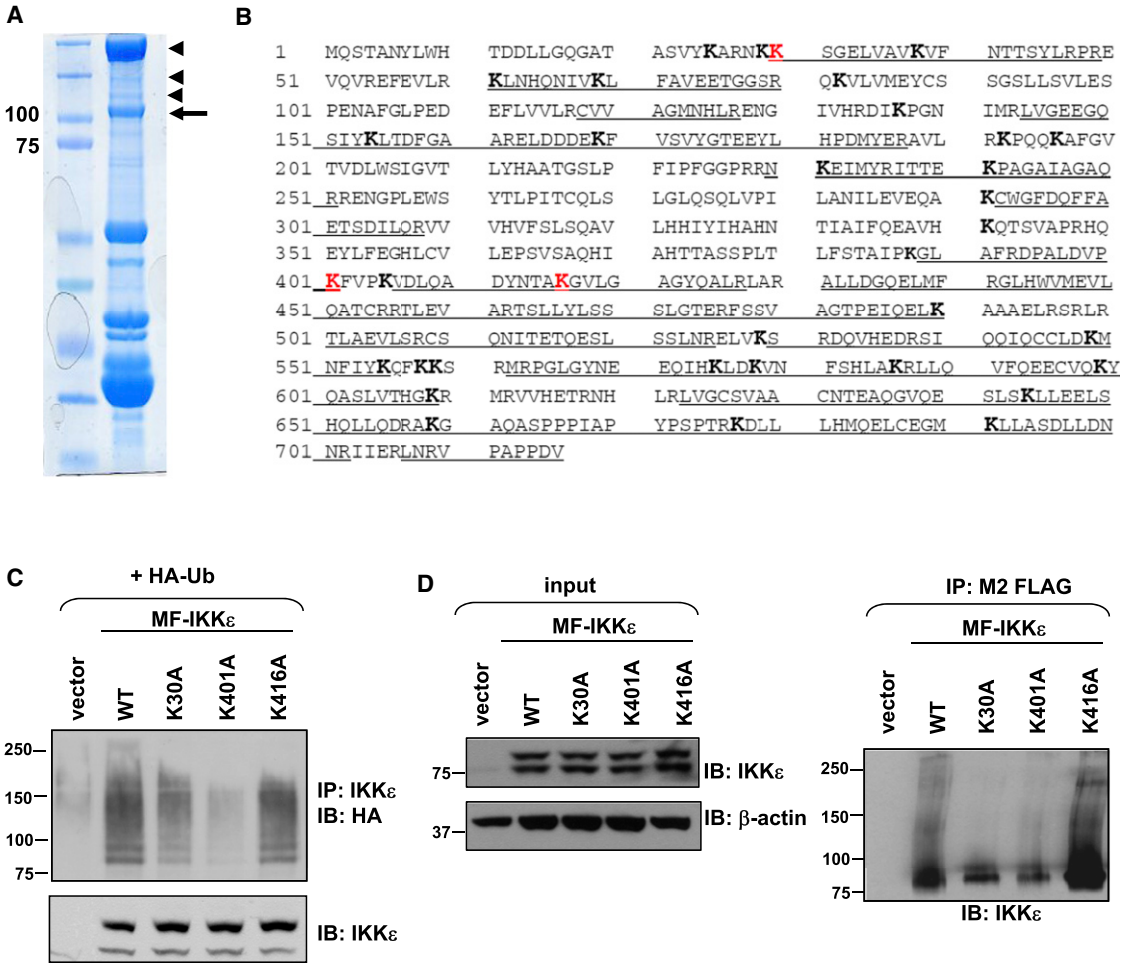

Figure 3. IKKε Is Ubiquitinated on K30, K401, and K416

(A) Ubiquitinated IKKε analysis by mass spectrometry. GST-IKKε was cotransfected into HEK293T cells with HA-Ub. GST immune complexes were isolated and subjected to SDS-PAGE and Colloidal Blue staining. The band corresponding to IKKε (arrow) and three additional bands (arrowheads) were excised from the gel and digested with trypsin and chymotrypsin. Ubiquitination sites were mapped by microcapillary liquid chromatography MS/MS.

(B) Amino acid sequence of IKKε. Mass spectrometry analysis covered 58.2% of IKKε (underlined) and 64.7% (22/34) of the internal lysines (bold). K30, K401, and K416 (red) were identified as ubiquitinated.

(C) IKKε K30A and K401A mutants exhibit decreased ubiquitination. IKKε ubiquitination site mutants (K30A, K401A, and K416A) were cotransfected into HEK293T cells with HA-Ub. IKKε immune complexes were isolated with an IKKε-specific antibody and analyzed by immunoblotting.

(D) IKKε K30A and K401A mutants exhibit decreased ubiquitination in transformed HA1EM cells. IKKε immune complexes were isolated from HA1EM cells expressing WT, K30A, K401A, and K416A MF-IKKε with anti-M2 FLAG Sepharose and analyzed by immunoblotting. A total of 5% of the WCL was loaded for input control.

DISCUSSION

K63-Linked Ubiquitination of IKKε Is Essential for its Activity as an Oncogene

IKKε plays a key role in initiating the interferon response to viral challenge and has been identified as an oncogene that is amplified in ~30% of breast cancers. Here, we demonstrate that IKKε is specifically modified by K63-linked ubiquitination. Using a proteomic approach, we identified IKKε residues that are ubiquitinated and determined that ubiquitination of IKKε at K30 and K401 is essential for its role both as an NF-κB activator and as an oncogene.

Modification of proteins by specific types of ubiquitination is an important mechanism to regulate protein function or stability. Using a combination of biochemical assays, linkage-specific ubiquitin mutant constructs and antibodies, and a cell-based ubiquitin replacement model, we found that IKKε is modified by K63-linked polyubiquitination. Although we were unable to purify sufficient amounts of IKKε to identify linkages by mass spectrometry and cannot exclude the possibility that IKKε is also modified by other types of ubiquitination, these complementary approaches provide evidence that K63-linked polyubiquitination regulates IKKε activity in both inflammatory and oncogenic contexts.

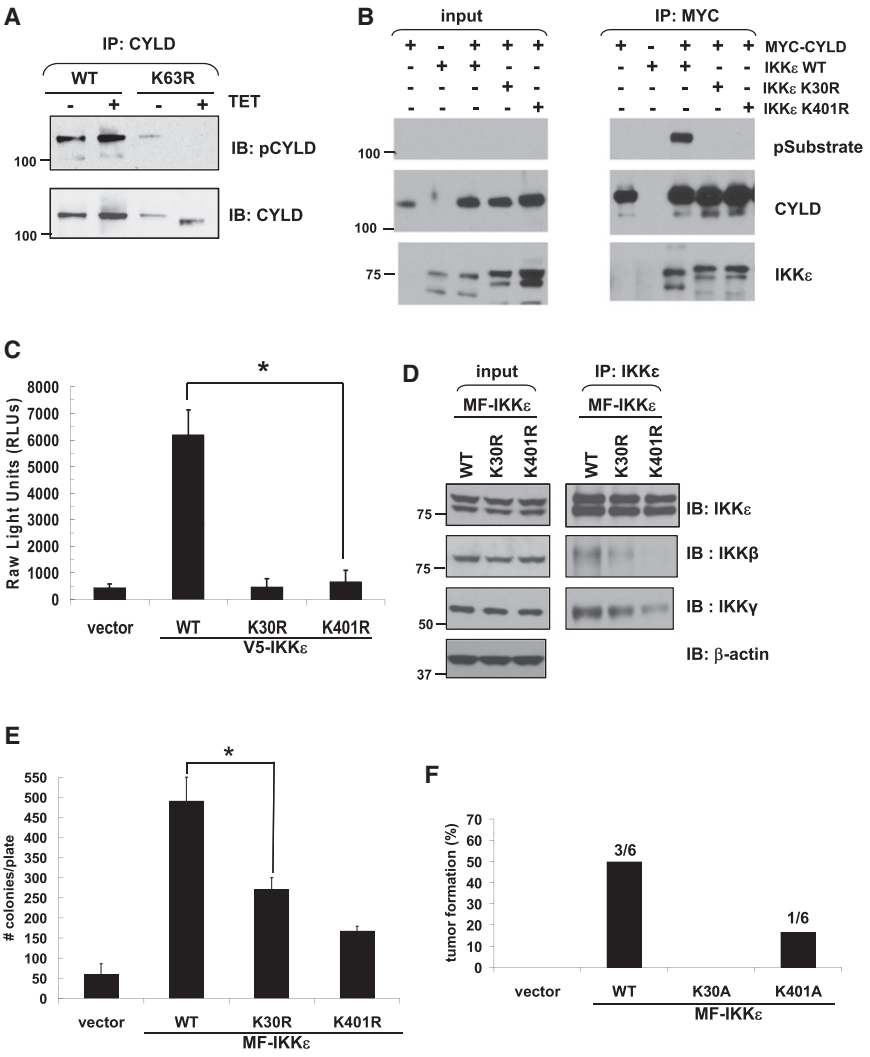

Figure 4. IKKε K63-Linked Ubiquitination at K30 and K401 Is Essential for IKKε Function

(A) IKKε kinase function is dependent on K63-linked ubiquitination. U2OS-shUb-Ub(WT) or U2OS-shUb-Ub(K63R) cells were treated with TET. CYLD immune complexes were isolated followed by immunoblotting for phospho-CYLD and total CYLD.

(B) Effects of IKKε ubiquitination mutants on CYLD phosphorylation. HEK293T cells were transfected as indicated. Myc-CYLD immune complexes were isolated and analyzed with the indicated antibodies.

(C) Effects of IKKε ubiquitination mutants on NF-κB activation. GloResponse NF-κB-RE-*luc2P* HEK293T cells were transfected with V5-IKKε WT, V5-IKKε K30R, and V5-IKKε K401R and analyzed by the One-Glo Luciferase assay. Results reported as RLU mean ± SD for six experiments. *p = 1.1 × 10^{-7}, calculated by a standard t test.

(D) Effects of IKKε ubiquitination on IKKβ and IKKγ recruitment. IKKε immune complexes were isolated from HA1EM cells expressing WT, K30R, or K401R IKKε and immunoblotted with the indicated antibodies. A total of 5% of the WCL was loaded for input control.

(E) Anchorage-independent growth of HA1EM MF-IKKε WT and mutant cells. Colony formation of HA1EM cells in Figure S3D expressing control vector, WT MF-IKKε, MF-IKKε K30R, or MF-IKKε K401R was analyzed after 28 days. Results reported as mean ± SD for three experiments. *p = 0.0045, calculated by standard t test.

(F) Tumorigenicity of HA1EM MF-IKKε WT and mutant cells. HA1EM cells expressing control vector, WT MF-IKKε, MF-IKKε K30A, or MF-IKKε K401A were introduced subcutaneously into immunodeficient mice (n = 6). Tumor formation is shown as a fraction.

We previously showed that IKKε induces cell transformation that is dependent upon NF-κB activation (Boehm et al., 2007). Here, we show that K63-linked IKKε ubiquitination is required for its kinase activity but that mutations that ablate ubiquitination of IKKε did not affect its interaction with other proteins such as CYLD. This latter observation makes it unlikely that these mutants disrupt the overall structure of IKKε.

In concurrent work (Tu et al., 2013), we describe the structure of TBK1, a family member that shares ~65% protein homology with IKKε, whose homodimerization is essential for activity. We show that TBK1 is ubiquitinated at the analogous residues that are ubiquitinated in IKKε. These residues are on opposing sides of one face of an IKKε/TBK1 monomer but are juxtaposed closely when TBK1 homodimerizes, suggesting that these residues may interact with an E3 ligase at this face of the dimer. Moreover, this suggests that this modification creates a new binding interface, and mutations affect recruitment of other molecules critical for kinase function. IKKε may be similarly regulated because IKKε also homodimerizes. In addition, we found that disruption of IKKε ubiquitination does not interfere with IKKε homodimerization, indicating that ubiquitination may occur after dimerization.

The cIAP1/cIAP2/TRAF2 E3 Ubiquitin Ligase Complex Modifies IKKε

We also found that the IKKε-interacting cIAP1/cIAP2/TRAF2 E3 ubiquitin ligase complex is both sufficient and essential to catalyze IKKε ubiquitination. Using both biochemical and genetic approaches, we found that cIAP1, cIAP2, and TRAF2 are all required for the ubiquitination of IKKε.

Although prior work suggests that TRAF2 may be an E3 ubiquitin ligase, the recent structure of the TRAF2 RING domain suggests that it is unlikely to have enzymatic E3 ligase activity (Yin et al., 2009). Instead, TRAF2 may act as a scaffold for the recruitment of the cIAP proteins. Consistent with this model, we found that cIAP1 and cIAP2 induce more robust IKKε ubiquitination in vitro than immunopurified TRAF2. Moreover,

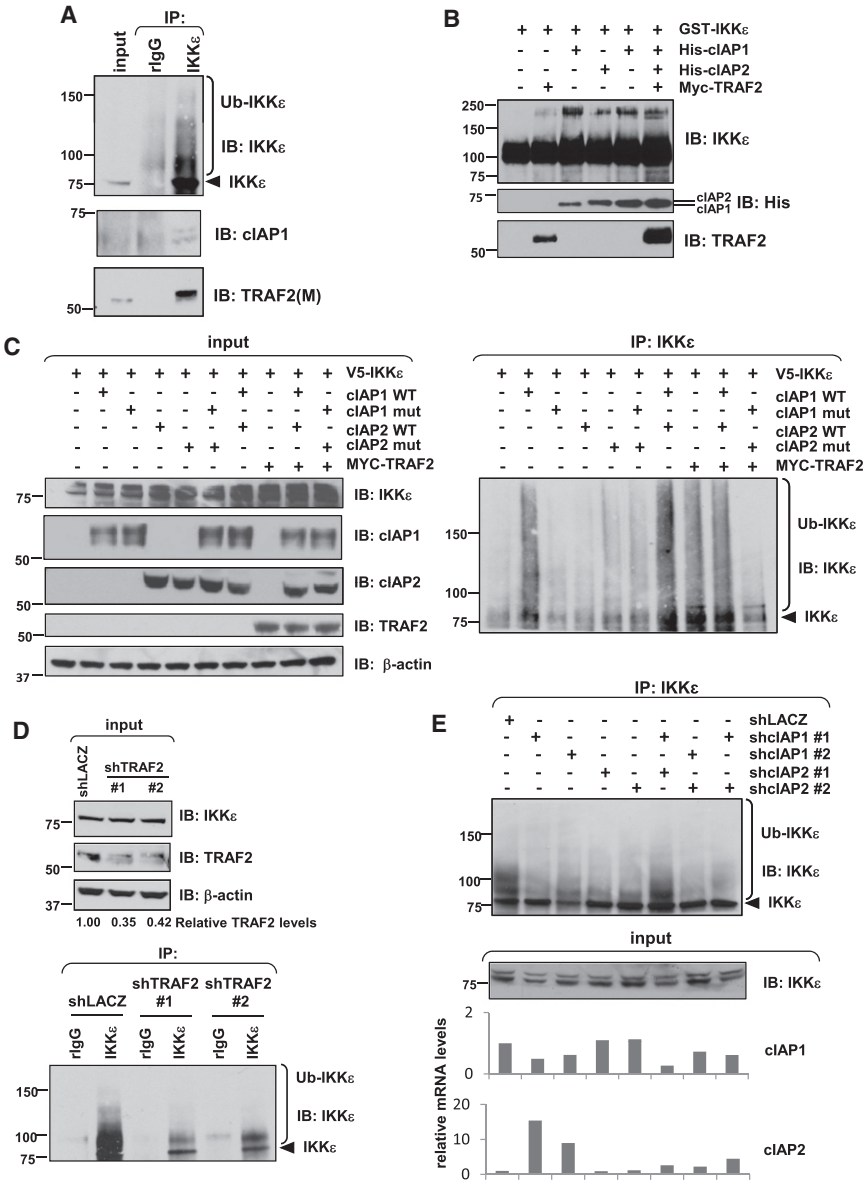

Figure 5. The cIAP1/cIAP2/TRAF2 E3 Ubiquitin Ligase Complex Ubiquitinates IKKε

(A) IKKε binds to TRAF2 and cIAP1 in MCF-7 cancer cells. Endogenous IKKε immune complexes were isolated from MCF-7 breast cancer cells using an IKKε-specific antibody and analyzed by immunoblotting as indicated. rIgG was used as a control.

(B) cIAP1/cIAP2/TRAF2 ubiquitinates IKKε in vitro. An in vitro ubiquitination assay was performed using recombinant cIAP1, recombinant cIAP2, and immunopurified TRAF2. The samples were analyzed by immunoblot.

(C) cIAP1/cIAP2/TRAF2 ubiquitinates IKKε in vivo. HEK293T cells were transfected as indicated. Lysates were immunoblotted with the indicated antibodies.

(D) Effects of *TRAF2* suppression on IKKε ubiquitination. shTRAF2#1, shTRAF2#2, or control shLACZ were expressed in MCF-7 cells. IKKε immune complexes were isolated and immunoblotted with the indicated antibodies. rIgG was used for control immunoprecipitations. Relative TRAF2 levels were calculated by densitometry analysis.

(E) Effects of *cIAP1* and *cIAP2* suppression on IKKε ubiquitination. MCF-7 cells were transduced with lentiviruses as indicated. IKKε immune complexes were isolated and immunoblotted with the indicated antibodies. Relative cIAP1 and cIAP2 levels were determined by quantitative PCR. A total of 5% of the WCL was loaded for input control and quantitative PCR analysis.

because cIAP1 and cIAP2 form a complex with TRAF2, we cannot eliminate the possibility that the immunopurified TRAF2 used herein contains low levels of cIAP1 and cIAP2 undetectable by immunoblotting. We also found that the addition of E3 ligase-deficient mutant cIAP1 and mutant cIAP2 inhibited the ability of TRAF2 to catalyze IKKε ubiquitination in vivo. These observations support the model that TRAF2 acts as a scaffold that recruits the enzymatically active cIAP1 and cIAP2 into an active E3 ubiquitin ligase complex that ubiquitinates IKKε.

Although IKKε is a serine-threonine kinase potentially amenable to inhibition by small molecule inhibitors, IKKε shares substantial homology to TBK1, which makes likely that ATP-competitive small molecule inhibitors of IKKε will also inhibit TBK1. Recent work has demonstrated that E3 ligases can also be targeted by small molecules (Lydeard and Harper, 2010), and small molecule inhibitors of the cullin-RING family of E3 ubiquitin ligases have been described (Aghajan et al., 2010). Because we found that cIAP1/cIAP2/TRAF2 E3 ligase complex-mediated K63-linked ubiquitination is essential for IKKε activity, these observations not only provide new insights into the IKKε regulation but may also identify an alternative mechanism for targeting IKKε therapeutically.

EXPERIMENTAL PROCEDURES

Reagents

The antibodies used were Myc (clone 4A6) (Millipore), cIAP1, K48-linkage specific Ubiquitin, K63-linkage specific Ubiquitin, phospho-CYLD, TRAF2 (Rabbit) and TANK (Cell Signaling Technologies), V5-HRP (Invitrogen), Ubiquitin (FL-76 and PD-1), β-actin-HRP, cIAP2, CYLD, TRAF2 (mouse; Santa Cruz Biotechnology), IKKε (Sigma-Aldrich), and HA (Clone12C5; Boehringer Mannheim). The IKKε phospho-substrate antibody has been described previously (Hutti et al., 2009).

MF-IKKε K30A, MF-IKKε K401A, MF-IKKε K416A, MF-IKKε K30R, MF-IKKε K401R, and MF-IKKε K416R were created using the QuikChange site-directed mutagenesis protocol (Stratagene). V5-TRAF2, V5-TRAF2 ΔRING, V5-IKKε, V5-IKKε K30R, V5-IKKε K401R, V5-IKKε K416R, Myc-IKKε, Myc-IKKε K30R,

Myc-IKKε K401R, and Myc-IKKε K416R were generated by Gateway cloning into the pLEX-V5-Blast vector. HA-ubiquitin, HA-Ub K63-only, and HA-Ub K48-only were used as described previously (Abbott et al., 2004; Boehm et al., 2007). shRNA constructs were obtained from the RNAi Consortium. FLAG-CIAP1 (plasmid 27972), FLAG-CIAP2 (plasmid 27973), pcdna3.1 hciap1mut (plasmid 8337), pcdna3.1 hciap2mut (plasmid 8339), pCMV-HA-MyD88 (plasmid 12287), and pRK5-HA-Ubiquitin-K11 (plasmid 22901) were obtained from Addgene.

Transfection, Immunoprecipitation, and Immunoblotting

Transfection experiments were performed using Fugene (Roche). U2OS shUb-Ub(WT) and U2OS shUb-Ub(K63R) cells were cultured as described previously (Xu et al., 2009). Immunoprecipitations in which IKKε ubiquitination was assessed were performed as described previously (Xu et al., 2009) in Buffer "A" (20 mM Tris [pH 7.5], 150 mM NaCl, 10% glycerol, 1% Triton X-100). Conditions that did or did not include boiling denaturation did not affect IKKε ubiquitination. Densitometry was assessed using ImageJ software.

Mass Spectrometry Analysis

HEK293T cells were cotransfected with GST-IKKε and HA-Ub. GST immune complexes were isolated using Glutathione Sepharose (GE Healthcare) and the sample was resolved on SDS-PAGE and visualized with Colloidal Blue (Invitrogen). Four bands were excised and subjected to in-gel trypsin digestion. Peptides were separated across a 50 min gradient ranging from 7% to 30% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid in a microcapillary (125 μm × 18 cm) column packed with C_{18} reverse-phase material (Magic C18AQ, 5-μm particles, 200-Å pore size, Michrom Bioresources) and analyzed online on a hybrid linear ion trap-Orbitrap mass spectrometer (Thermo-Electron). For each cycle, one full mass spectrometry (MS) scan acquired on the Orbitrap at high mass resolution was followed by ten MS/MS spectra on the linear ion trap from the ten most abundant ions. MS/MS spectra were searched using the Sequest algorithm against the human IPI protein database. Dynamic modifications of 114.0429275 Da on lysine was allowed for ubiquitination. All peptide matches were initially filtered based on enzyme specificity, mass measurement error, Xcorr, and dCorr scores and further manually validated for peptide identification and site localization.

NF-κB Reporter Assays

GloResponse NF-κB-RE-*luc2P* HEK293T cells (Promega) were transfected with V5-IKKε WT, V5-IKKε K30R, and V5-IKKε K401R. NF-κB activity was measured 36 hr posttransfection according to the One-Glo Luciferase assay protocol (Promega). Luciferase values are reported directly in raw light units (RLU).

Transformation Assays

Growth of HA1EM cells in soft agar was determined by plating 5×10^4 cells in triplicate in 0.4% Noble agar. Colonies greater than 100 μm in diameter were counted 28 days after plating, and 2×10^6 cells were subcutaneously implanted into immunodeficient mice (Balb/c Nude, Charles River Laboratories) anesthetized with isoflurane. Six independent tumors were tested for each condition. Tumors were measured at 21 days after implantation.

In Vitro Ubiquitination Assay

Immunopurified TRAF2 was isolated by Myc immunoprecipitation from HEK293T cells that were transfected with 3xMyc-TRAF2 for 48 hr. Recombinant His-cIAP1(818-IA-050) and His-cIAP2(817-P2-050) were purchased from R&D Systems. Recombinant E1 ubiquitin activating enzyme (E-304), Ubc13 E2 enzyme (E2-664), and ubiquitin (U-100H) were purchased from Boston Biochem. Recombinant IKKε protein (PV4875) was purchased from Invitrogen. Reactions were carried out at 35°C for 2 hr in 50 nM HEPES (pH 7.8), 10 mM $MgCl_2$, and 4 mM ATP with 50 nM E1, 150 nM E2, 50 ng IKKε, 10 μg ubiquitin, 100 ng cIAP1, 100 ng cIAP2, and 20 μl immunopurified TRAF2 Protein G Sepharose. Reactions were stopped after 2 hr by adding 10 μl SDS loading dye and were subsequently analyzed by immunoblot.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.01.031.

LICENSING INFORMATION

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