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A20-binding Inhibitor of Nuclear Factor-κB (NF-κB)-2 (ABIN-2) Is an Activator of Inhibitor of NF-κB (IκB) Kinase α (IKKα)-mediated NF-κB Transcriptional Activity*

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Laurent Leotoing[‡§¶1], Fanny Chereau[‡§¶1], Silvère Baron[‖2], Florent Hube[‡‡2], Hugo J. Valencia[‡§¶], Didier Bordereaux[‡§¶], Jeroen A. Demmers[§§], John Strouboulis[¶¶3], and Véronique Baud[‡§¶4]**

*From [‡]INSERM, U1016, Institut Cochin, 75014 Paris, France, [§]CNRS, UMR 8104, Paris, France, [¶]Université Paris Descartes, Sorbonne Paris Cité, Paris, France, [‖]Clermont Université, Université Blaise Pascal, Génétique Reproduction et Développement, BP 10448, F-63000 Clermont-Ferrand, France, **CNRS, UMR 6247, GReD, F-63177 Aubiere, France, [‡‡]Université Paris Diderot Paris 7, CNRS, UMR 7216, Epigenetics and Cell Fate, Paris, France, and the [§§]Department of Biochemistry and Proteomics Center and [¶¶]Department of Cell Biology, Erasmus Medical Center, Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands*

NF-κB transcription factors are pivotal players in controlling inflammatory and immune responses, as well as cell proliferation and apoptosis. Aberrant regulation of NF-κB and the signaling pathways that regulate its activity have been involved in various pathologies, particularly cancers, as well as inflammatory and autoimmune diseases. NF-κB activation is tightly regulated by the IκB kinase (IKK) complex, which is composed of two catalytic subunits IKKα and IKKβ, and a regulatory subunit IKKγ/NEMO. Although IKKα and IKKβ share structural similarities, IKKα has been shown to have distinct biological functions. However, the molecular mechanisms that modulate IKKα activity have not yet been fully elucidated. To understand better the regulation of IKKα activity, we purified IKKα-associated proteins and identified ABIN-2. Here, we demonstrate that IKKα and IKKβ both interact with ABIN-2 and impair its constitutive degradation by the proteasome. Nonetheless, ABIN-2 enhances IKKα- but not IKKβ-mediated NF-κB activation by specifically inducing IKKα autophosphorylation and kinase activity. Furthermore, we found that ABIN-2 serine 146 is critical for the ABIN-2-dependent IKKα transcriptional up-regulation of specific NF-κB target genes. These results imply that ABIN-2 acts as a positive regulator of NF-κB-dependent transcription by activating IKKα.

The NF-κB family of transcription factors regulates the expression of a wide range of genes implicated in immune and inflammatory responses, cell proliferation, apoptosis, and oncogenesis (1–9). Although activation of NF-κB occurs in response to a remarkable diversity of stimuli including cytokines (*e.g.* TNFα, IL1β, lymphotoxin β), bacterial lipopolysaccharide (LPS), stress, and viral proteins, most of the respective signaling pathways converge at the level of the IKK[5] complex (10–12). Once activated, IKK phosphorylates the IκB inhibitory proteins on two specific serine residues (*e.g.* serines 32 and 36 of IκBα), leading to their ubiquitination by the multisubunit $SCF^{\beta\text{-}TrCP}$ E3 ligase and subsequent proteosomal degradation, which in turn allows NF-κB dimers to translocate to the nucleus and activate the transcription of their specific target genes (13–16).

IKK is composed of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit IKKγ/NEMO (10, 17, 18). Although IKKα and IKKβ share a high degree of sequence similarity, they have largely distinct functions, due to their different substrate specificities and modes of regulation. IKKβ (and IKKγ) is essential for rapid and transient NF-κB induction by proinflammatory signaling cascades, such as those triggered by TNFα or LPS, via the "classical NF-κB pathway" that mainly relies on IκBα degradation (19–21). In contrast, IKKα is required for the late and sustained activation of NF-κB in response to a specific subset of TNF family members (*e.g.* lymphotoxin β, B cell activating factor (BAFF), CD40 ligand) via the "alternative NF-κB pathway" that relies on the inducible proteolysis of p100 (22, 23). Nonetheless, there is evidence indicating that IKKα contributes to cytokine-induced IκBα phosphorylation and subsequent degradation, thus participating in the classical NF-κB activation pathway as well. For instance, IKKα is essential for efficient TNFα-induced IκBα phosphorylation in HeLa cells (24), and it has been reported that IKKα is also required for receptor activator of nuclear factor κ-B ligand (RANKL) mediated classical NF-κB activation in mammary epithelial cells (25). Furthermore, IKKα exerts nuclear NF-κB transcriptional activating functions through the control of histone phosphorylation (26, 27) and can also act in specific cell types as a negative mediator of NF-κB activation by attenuating IKKβ-driven NF-κB activation or modulating RelA Ser-536 phosphorylation

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[1] Both authors contributed equally to this work.

[2] Both authors contributed equally to this work.

[3] Present address: Institute of Molecular Oncology, Biomedical Sciences Research Center "Alexander Flemming," 34 Fleming St., Vari, Greece.

[4] To whom correspondence should be addressed: Département d'Immunologie-Hématologie, Institut Cochin, INSERM U1016, Maternité Port-Royal, 123 Boulevard de Port-Royal, 75014 Paris, France. Tel.: 33153104370; Fax: 33143251167; E-mail: veronique.baud@inserm.fr.

[5] The abbreviations used are: IKK, IκB kinase; ABIN-2, A20-binding inhibitor of NF-κB; KD, kinase-inactive; MEF, mouse embryonic fibroblast; Ub, ubiquitin; RANTES, regulated on activation T cell expressed and secreted.

(28, 29). IKKα is also involved in epidermal differentiation, but independently of its kinase activity (30–32).

IKKα and IKKβ activation is dependent on phosphorylation of specific serine residues located within the activation loop (T loop), either by upstream kinases or by trans-autophosphorylation (33–36). In addition, conformational changes by protein interactions may also be a mechanism to stimulate IKK activity. For example, Cdc37 and Hsp90 form a chaperone complex with IKKα/β that is required for IKK activation in response to TNFα and DNA damage (37, 38), and NIK and IKKβ binding protein (NIBP) is an enhancer of TNFα-induced NF-κB activity by interacting with IKKβ and increasing IKK kinase activity (39).

Because IKKα is involved in many ways in the regulation of NF-κB activity, the possibility that IKKα-interacting proteins might specifically regulate its activity is a crucial issue with profound implications toward specific NF-κB response. We have purified IKKα-associated proteins in mouse embryonic fibroblasts (MEFs), and mass spectrometry analysis of the copurified proteins revealed the presence of A20-binding inhibitor of NF-κB (ABIN-2) (40, 41). In the study presented here, we show that IKKα and IKKβ both interact with ABIN-2 and impair its constitutive degradation by the proteasome. Nonetheless, ABIN-2 increases IKKα but not IKKβ-mediated NF-κB activation by specifically increasing IKKα autophosphorylation and kinase activity. In addition, we found that ABIN-2 serine 146 is critical for ABIN-2-dependent IKKα transcriptional up-regulation of specific NF-κB target genes.

MATERIALS AND METHODS

Antibodies and Reagent—The antibodies were purchased from Abcam (E tag), Roche Diagnostic (HA), Sigma (FLAG, β-actin), Cell Signaling (IKKβ, phospho-IκBα serine 32–36, phospho-IKKα/β serine 176/180), BD Biosciences Pharmingen (IKKα), Santa Cruz Biotechnology (RelA, RelB, cRel, IκBα, p105/p50, p100/p52, ABIN-2), Southern Biotech (R-phycoerythrin conjugated anti-rat), and Molecular Probes (Alexa Fluor 488-conjugated anti-mouse). MG132 and murine recombinant TNFα were purchased from Calbiochem and Sigma, respectively.

Plasmid Constructs—Expression vectors for E tag-ABIN-1 and -ABIN-2 were obtained from R. Beyaert (Ghent University, Zwijnaarde, Belgium). FLAG-ABIN-2 and GST-ABIN-2 from S. C. Ley (National Institute for Medical Research, MillHill, UK). GST-IκBα(1–54), wild-type HA-IKKα and HA-IKKβ, and kinase-inactive K44M mutants (KD) of HA-IKKα and HA-IKKβ were from M. Karin (University of California, San Diego, CA). FLAG-IKKα and FLAG-IKKβ were from M. Hu (University of Texas, Houston, TX). HA-Ub WT and mutants were from C. Pique (Institut Cochin, Paris, France), and 3×NF-κB-luciferase reporter was from D. Baltimore (California Institute of Technology, Pasadena, CA). pTrip-ABIN-2 was generated by subcloning human full-length ABIN-2 cDNA into pTRIP-ΔU3-EF1α-IRES GFP lentiviral vector (42). GST-ABIN-2 deletion mutants were obtained by subcloning ABIN-2 coding sequences from amino acids 1–97 (Δ1), 94–184 (Δ2), 180–279 (Δ3), 275–345 (Δ4), and 348–429 (Δ5) following standard recombinant DNA procedures; and details are available upon request. GST-ABIN-2 point mutants were generated by substituting serine 2, 7, 62, 113, 129, 140, and 146 to alanine using site-directed mutagenesis (Stratagene) and confirmed by sequencing.

Cell Culture and Transient Transfections—HEK293, 293T, HeLa and MEF cells were cultured in DMEM (Invitrogen) supplemented with 10% heat-inactivated FBS and 100 units/ml penicillin-streptomycin. For transfections, 10^6 HEK293 cells in 35-mm dishes were transfected with 0.8–1 μg of DNA using Lipofectamine (Invitrogen) in Opti-MEM (Invitrogen) as described (43). Cells were harvested 24 h after transfection.

Lentiviral Production and Transduction—Production of infectious recombinant lentiviruses was performed by transient transfection of 293T cells as described in Ref. 42. For infections, 10^5 cells in 35-mm dishes were transduced with 5000 ng/ml p24 (HIV-1 capsid protein). 48 h later, cells were washed, and fresh medium was added. The culture was then continued as described above.

In Vivo Biotinylation Approach—The approach was adapted from the method described by de Boer *et al.* based on efficient biotinylation *in vivo* and single-step purification of tagged transcription factors in mammalian cells (44). Briefly, we first generated a lentiviral vector named pTRIP-BirA by subcloning the bacterial protein-biotin ligase BirA cDNA into the lentiviral vector pTRIP-ΔU3-EF1α-IRES GFP (42) and then subcloned the cDNA encoding full-length IKKα tagged at the N terminus by a peptide of 23 amino acids that can be biotinylated by BirA *in vivo* (IKKα-BP). IKKα-deficient MEFs were then stably transduced with either pTRIP-BirA empty vector or pTRIP-BirA expressing IKKα-BP. Following large scale cell culture, cytoplasmic and nuclear extracts were incubated with streptavidin beads. Eluted proteins were separated by SDS-PAGE and then stained with Colloidal blue. Each entire lane was divided into 2–3-mm gel slices (at least 20 gel plugs/lane) that were each treated by an in-gel-digested method using modified trypsin and further analyzed by nanoflow liquid chromatography-tandem coupled to a Q-TOF system. Data base searches were performed using MASCOT and Profound.

Indirect Immunofluorescence Microscopy—Cells seeded on coverslips were transfected using Lipofectamine. 24 h later, cells were fixed in 4% (w/v) paraformaldehyde in PBS for 25 min, washed twice in PBS, and permeabilized for 15 min in 0.25% Triton X-100 in PBS at room temperature. After blocking with 10% FBS in PBS for 10 min at room temperature, cells were then incubated for 1 h at room temperature with the primary antibody in 3% FBS in PBS, washed twice in PBS, and further incubated with the fluorophore-conjugated secondary antibody for 1 h. After two washes in PBS, the coverslips were mounted with Vectashield® mounting medium containing 1.5 μg/ml DAPI (Vector Laboratories). Fluorescence imaging was performed using a Leica DMI6000 inverted microscope and a MicroMAX-1300Y/HS (Princeton Instruments) camera using the Metamorph v.7 software (Molecular Devices).

Coimmunoprecipitation and Immunoblotting—Coimmunoprecipitation and immunoblotting were performed as reported in Ref. 45.

EMSAs—EMSAs were realized as described in Ref. 45. For supershift assays, nuclear extracts were incubated with specific

antibodies for 30 min on ice before incubation with the labeled probe.

Kinase Assays—For IKK immunocomplex kinase assays, transfected cells were collected in whole cell lysis buffer (300 mM NaCl, 25 mM Hepes, pH 7.7, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5% Triton X-100 supplemented with 10 mM *p*-nitrophenyl phosphate disodium salt, 20 mM β-glycerol phosphate, 100 μM Na_3VO_4, 1 mM PMSF, 1 × Complete protease inhibitor mixture (Roche Diagnostics)). HA-IKKα or HA-IKKβ was immunoprecipitated from 100 μg of cell lysates with HA antibody for 2 h or overnight at 4 °C, after which protein A/G-agarose beads were added and incubation continued for 90 min at 4 °C. The immunoprecipitates were collected, washed three times in lysis buffer and once in kinase buffer (20 mM Hepes, pH 7.6, 10 mM $MgCl_2$), and then incubated at 30 °C for 25 min in 30 μl of kinase reaction mixture containing 1 μCi of [γ-^{32}P]ATP, 1.5 μg of either bacterial expressed GST-IκBα(1–54) or GST-ABIN-2 full-length and mutant forms as substrate. The reaction was stopped by addition of an equal volume of Laemmli buffer and heat denaturation for 5 min at 90 °C. Proteins were separated on 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes, stained with Ponceau S, subjected to autoradiography to visualize phosphorylated GST fusion proteins, and finally analyzed by immunoblotting for normalization on amount of immunoprecipitated IKKs.

Ubiquitination Assays—Cells were lysed for 20 min on ice in ubiquitination assay buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% deoxycholic acid, 1% Nonidet P-40, 50 μM $MgCl_2$, 0.1% SDS, supplemented with 10 mM *N*-ethylmaleimide, 50 μM MG132, 10 mM *p*-nitrophenyl phosphate disodium salt, 20 mM β-glycerol phosphate, 100 μM Na_3VO_4, 1 mM PMSF, and 1 × Complete protease inhibitor mixture), after which the SDS concentration was adjusted to 1%, and whole cell extracts boiled for 10 min, diluted 10-fold with ubiquitination assay buffer without SDS, and subjected to immunoprecipitation and immunoblotting.

Calf Intestinal Phosphatase Assays—Whole cells extracts prepared in ubiquitination assay buffer as described above were incubated for 30 min at 37 °C with 1 unit of calf intestinal phosphatase (New England Biolabs) either untreated or heat-inactivated for 10 min at 95 °C and analyzed by immunoblotting.

Luciferase Reporter Assays—Firefly and *Renilla* luciferase activities were determined using the Dual Luciferase Assay System (Promega) according to the manufacturer's instructions. Experiments were conducted three times in triplicate.

RT-qPCR—Total RNA extraction and RT were performed as described previously (45). Real-time PCR analysis was carried out with LightCycler FastStart DNA Master plus SYBR Green I on a Light Cycler 1.5 (Roche Applied Science). All values were normalized to the level of hypoxanthine-guanine phosphoribosyltransferase (HPRT) mRNA. Primer sequences are available upon request.

RESULTS

ABIN-2 Interacts with IKKα and IKKβ—Because IKKα plays pivotal and complex roles in the regulation of NF-κB activation, we undertook to identify new IKKα-interacting partners that could participate in the activation of NF-κB transcriptional activity. We applied a biochemical approach based on specific biotinylation *in vivo* using IKKα as a bait (see "Materials and Methods"), taking advantage of the very high affinity of avidin/streptavidin for biotinylated templates and efficient single-step purification and identified ABIN-2 as a new IKKα-interacting partner candidate.

To examine further the significance of this ABIN-2-IKKα interaction, we first examined the subcellular localization of ABIN-2 when coexpressed with IKKα/β in HeLa cells. As shown in Fig. 1*A*, when expressed alone, ABIN-2 formed punctate structures of various sizes in the cytoplasm. However, when coexpressed with IKKα or IKKβ, ABIN-2 relocalizes uniformly all over the cytoplasm, indicating that IKKα/β have an effect on the subcellular localization of ABIN-2. We next performed coimmunoprecipitation experiments using whole cell extracts from HEK293 cells cotransfected with E tag-ABIN-2 along with either FLAG-IKKα or FLAG-IKKβ. As shown in Fig. 1*B*, reciprocal experiments with anti-FLAG- and anti-E tag specific antibodies showed that ABIN-2 coimmunoprecipitates with both IKKα and IKKβ at similar levels. Finally, we confirmed the association between ABIN-2 and IKKα/β on endogenous proteins in HEK293 and HeLa cells (Fig. 1*C*).

IKKα and IKKβ Impair ABIN-2 Constitutive Degradation by the Proteasome through Deubiquitination of Lys-48-linked Polyubiquitin Chains—In the course of our experiments, we noted that when expressed alone in HEK293 cells, ABIN-2 protein was hardly detectable (Fig. 1*B*), although the total mRNA level of ABIN-2 mRNA presented a 500-fold increase over basal level of untransfected cells (Fig. 2*B*). Remarkably, ABIN-2 protein expression is also extremely low in wild-type MEFs stably transduced with a lentivirus expressing E tag-ABIN-2 (Fig. 2*A*, *left*, *lane 1*). These observations suggest that ABIN-2 might be constitutively targeted for degradation. Treatment of E tag-ABIN-2 expressing MEFs (Fig. 2*A*, *left*), as well as HEK293 cells (Fig. 2*A*, *right*), with the proteasome inhibitor MG132 led to a strong increase in ABIN-2 protein levels, indicating that ABIN-2 is constitutively degraded in a proteasome-dependent manner. Coexpression of IKKα/β with ABIN-2 in HEK293 cells resulted in a strong increase of ABIN-2 protein expression levels in an IKK kinase activity-dependent manner (Fig. 2*C*), whereas it had only a slight effect on ABIN-2 mRNA level (Fig. 2*B*). Taken together, these observations led us to speculate that IKKα/β might induce a reduction in the extent of conjugation of ABIN-2 to the Lys-48-linked polyubiquitin chains which provide a tag for recognition by the 26 S proteasome (46). To test this hypothesis, we first examined whether ABIN-2 can be ubiquitinated via a Lys-48 linkage by cotransfection of HEK293 cells of E tag-ABIN-2 with an HA-tagged ubiquitin Lys-48 mutant containing only one lysine at position 48 (Lys-48-Ub). After immunoprecipitation of ABIN-2 (E tag antibody), Lys-48-linked polyubiquitination was analyzed by immunoblotting with an HA antibody. As shown in Fig. 2*D*, *left*, ABIN-2 is efficiently ubiquitinated with ubiquitin conjugated through Lys-48. Most importantly, coexpression of either HA-IKKα or HA-IKKβ led to a marked reduction in Lys-48-linked ubiquitination of ABIN-2. Significantly, coexpression of IKKα/β with wild-type ubiquitin also decreased ABIN-2 polyubiquitination

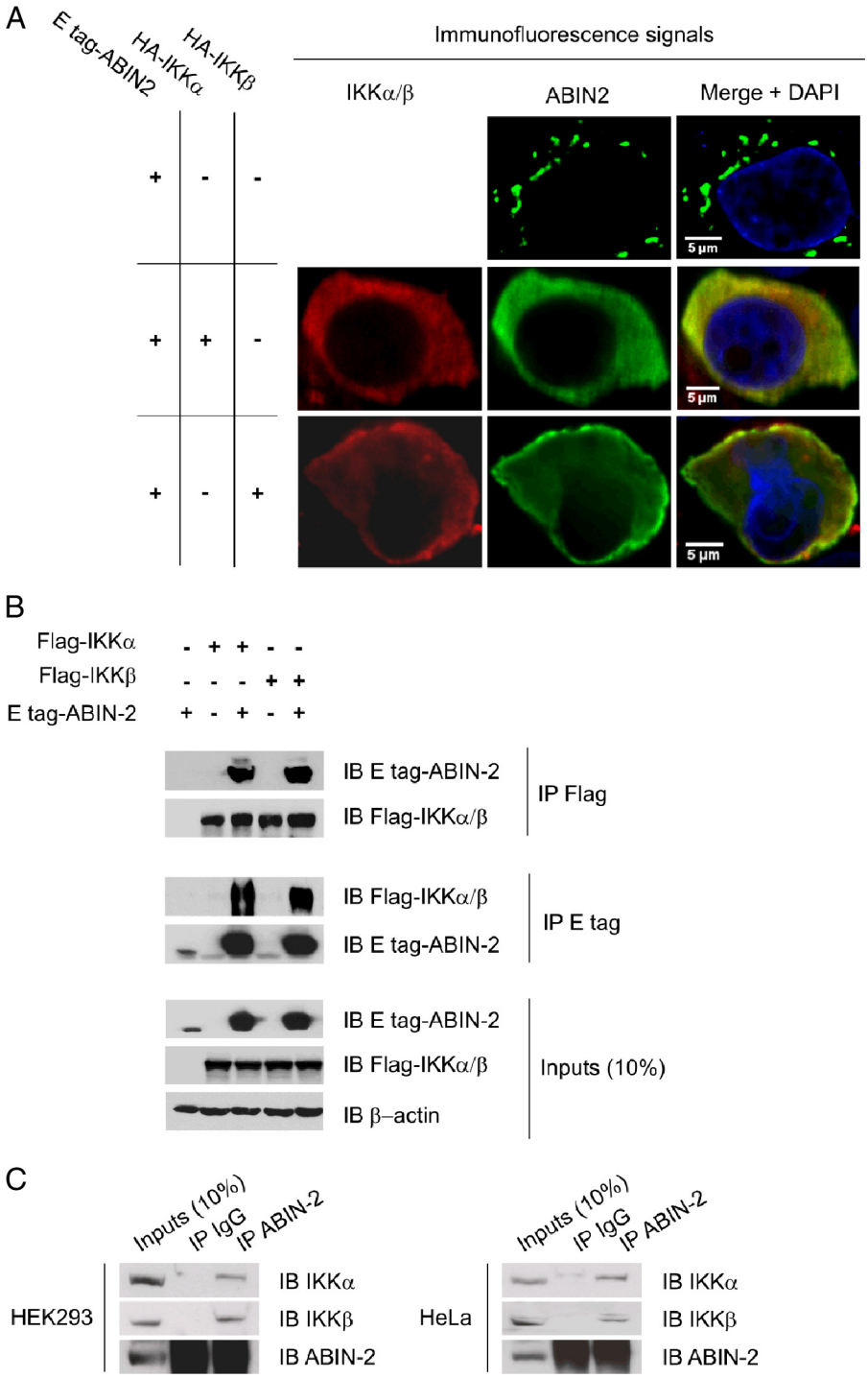

FIGURE 1. **ABIN-2 interacts with IKKα and IKKβ.** *A*, subcellular relocalization of ABIN-2 upon IKKα/β ectopic expression. HeLa cells were transfected either with E tag-ABIN-2, HA-IKKα, or HA-IKKβ alone, or with E tag-ABIN-2 along with either HA-IKKα or HA-IKKβ. Localization of transiently expressed proteins was evaluated by indirect immunofluorescence microscopy using anti-E tag or anti-HA antibody, followed by immunofluorescent staining with Alexa Fluor 488-conjugated anti-mouse or phycoerythrin-conjugated anti-rat, respectively. Nuclei were stained with DAPI. The *merged images* are shown on the *right*. *B*, interaction of ABIN-2 with IKKα and IKKβ within transfected HEK293 cells. HEK293 cells were transiently transfected with equivalent amounts of the indicated expression plasmids and whole cell extracts were subjected to immunoprecipitation (*IP*) with anti-FLAG (*upper panel*) or anti-E tag (*central panel*) and analyzed by immunoblotting (*IB*) for the indicated proteins. *C*, ABIN-2 constitutively interacts with IKKα and IKKβ *in vivo*. Whole cell extracts from HEK293 (*left*) and HeLa (*right*) cells were subjected to immunoprecipitation with anti-ABIN-2 or nonimmune control antibody and analyzed by immunoblotting for the indicated proteins. Of note, immunoprecipitated endogenous ABIN-2 comigrates with the IgG heavy chains.

(Fig. 2*D*, *center*), whereas it does not affect Lys-63-linked ubiquitination (Fig. 2*D*, *right*). Together, these results indicate that ABIN-2 is constitutively degraded by the proteasome and that IKKα/β can increase ABIN-2 stability, possibly via the induction of deubiquitination of Lys-48-linked polyubiquitin chains of ABIN-2.

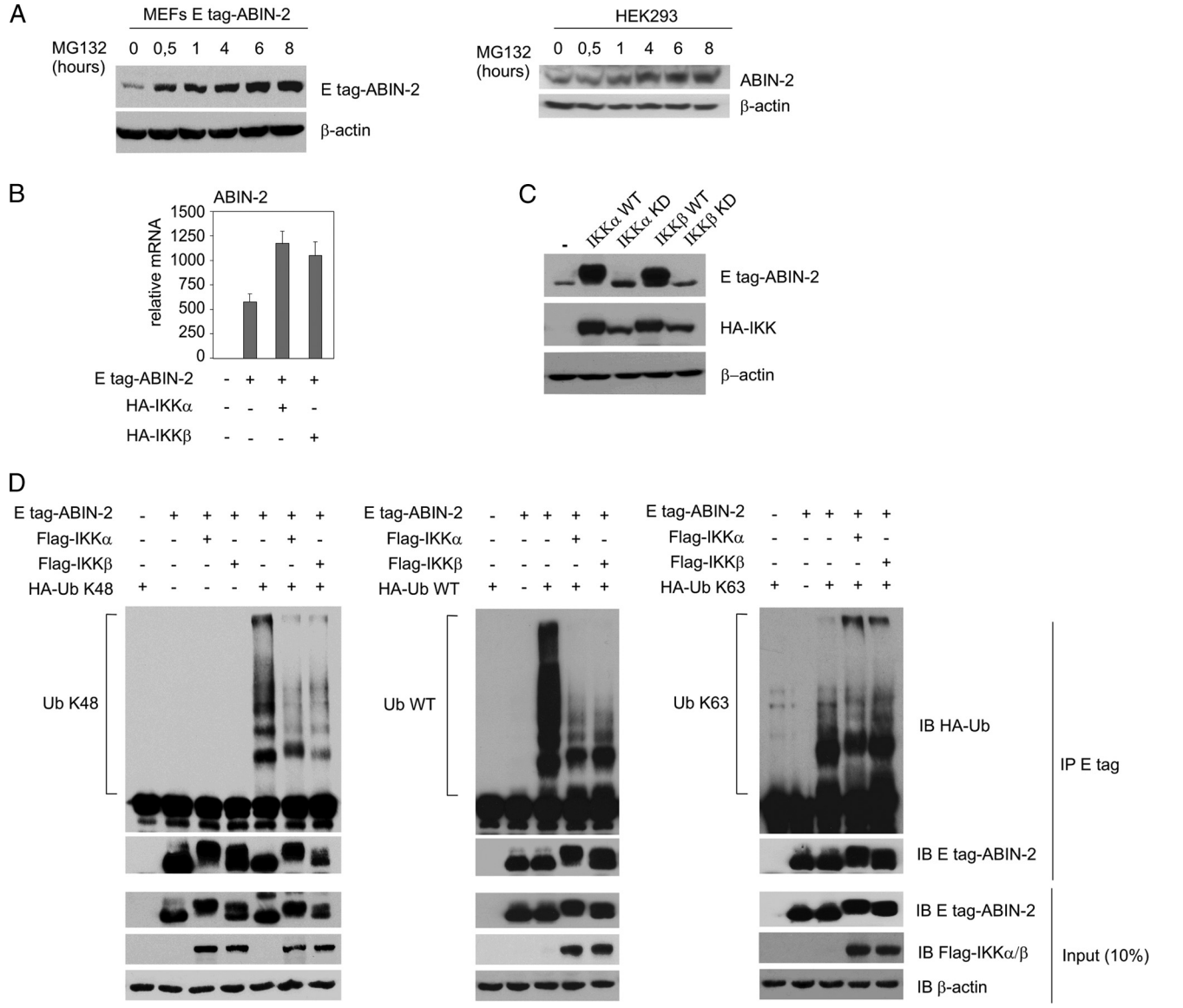

FIGURE 2. **IKKα and IKKβ impair constitutive degradation of ABIN-2 by the proteasome through deubiquitination of Lys-48-linked polyubiquitin chains.** *A*, ABIN-2 is constitutively degraded by the ubiquitin-proteasome pathway. ABIN-2-deficient MEFs stably transduced with a lentivirus encoding E tag-ABIN-2 (*left*) or HEK293 cells (*right*) were treated with MG132 (10 μM) for the indicated periods of time, and whole cell extracts were analyzed by immunoblotting for the indicated proteins. *B*, ABIN-2 mRNA level upon ABIN-2 overexpression. Total RNA was isolated from HEK293 cells transfected with either empty vector or E tag-ABIN-2 either alone or along with HA-IKKα or HA-IKKβ expression plasmids. ABIN-2 mRNA expression was quantified by real-time PCR analysis. Data are normalized to hypoxanthine-guanine phosphoribosyltransferase mRNA expression and shown as mean ± S.D. (*error bars*; $n = 3–4$). *C*, IKKα/β kinase activity is required for ABIN-2 stabilization. Whole cell extracts from HEK293 cells transfected with E tag-ABIN-2 along with either wild-type or KD mutants of IKKα/β were analyzed by immunoblotting for the indicated proteins. *D*, Lys-48-linked poly(Ub) chains of ABIN-2 deubiquinated by IKKα and IKKβ. HEK293 cells were transiently transfected with E tag-ABIN-2 and either HA-WT Ub or HA-Ub Lys-48 or HA-Ub Lys-63 along with an empty vector or FLAG-IKKα/β. Whole cell extracts prepared as described under the section on ubiquitination assays ("Materials and Methods") were subjected to immunoprecipitation (*IP*) with anti-E tag and analyzed by immunoblotting for the indicated proteins.

ABIN-2 Enhances IKKα-mediated NF-κB Activation—Given the ability of ABIN-2 to interact with and to be stabilized by IKKα and IKKβ, we wanted to see whether ABIN-2 could affect IKKα/β-mediated NF-κB activation. To our surprise, the expression of ABIN-2 led to a strong increase in IKKα-mediated NF-κB DNA binding activity in an ABIN-2 dose-dependent manner (Fig. 3*A*). In contrast, although the basal level of NF-κB DNA binding complexes was slightly higher when IKKβ was expressed alone compared with that seen with IKKα alone (*lane 3 versus lane 8*), ABIN-2 expression had little effect on IKKβ-mediated NF-κB DNA binding activity (Fig. 3*A*). We next examined the subunit composition of the NF-κB DNA-binding complexes. Supershift analysis of nuclear extracts from HEK293 cells transfected with HA-IKKα and E tag-ABIN-2 (extracts from Fig. 3*A*, *lane 7*) revealed that κB-binding complexes were mainly composed of RelA-p50 and cRel-p50 dimers (Fig. 3*B*). Not surprisingly, expression of ABIN-2 had no effect on catalytically inactive K44M IKKα and IKKβ mutants (KD) (Fig. 3*C*). Most importantly, coexpression of IKKα with ABIN-1, another ABIN family member (41), had no effect on the overall NF-κB DNA binding activity, indicating that ABIN-2 exerts specific function in enhancing IKKα-mediated NF-κB activation (Fig. 3*D*). To address whether ABIN-2-activating function could also be seen on IKKα-dependent NF-κB

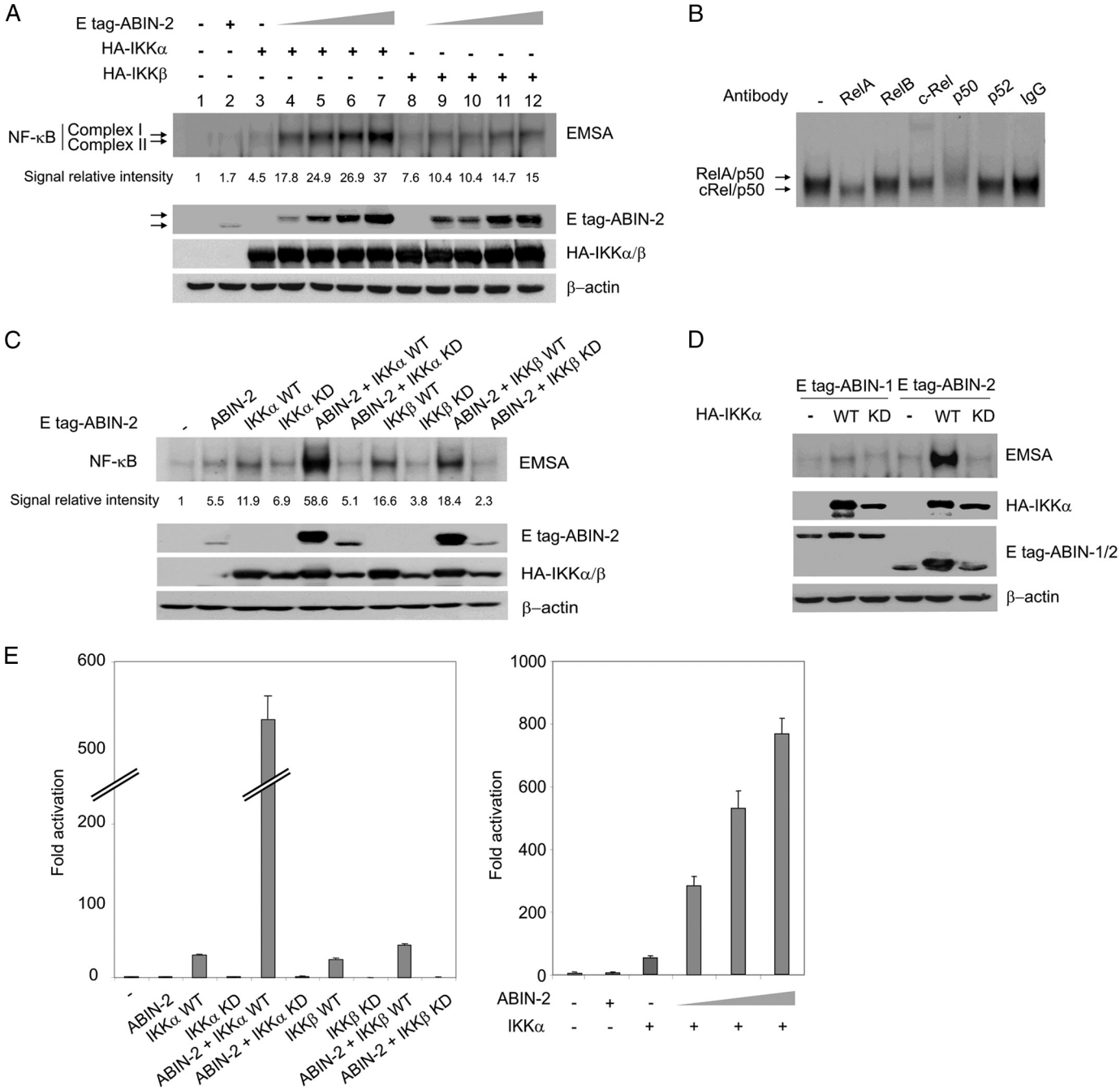

FIGURE 3. **ABIN-2 enhances IKKα-mediated NF-κB activation.** *A*, ABIN-2 enhances IKKα- but not IKKβ-mediated NF-κB DNA binding activity. Nuclear extracts from HEK293 cells transfected with either HA-IKKα or HA-IKKβ alone, or along with increasing amounts of E tag-ABIN-2 were analyzed by EMSA using a ^{32}P-labeled HIV-LTR tandem κB oligonucleotide as a probe (*upper panel*). Protein expression levels were analyzed by immunoblotting for the indicated proteins (*lower panels*). *Arrows* indicate various migrating forms of ABIN-2. *B*, for supershift analysis, nuclear extracts from HEK293 cells cotransfected with HA-IKKα and E tag-ABIN-2 (conditions of *panel A*, *lane 7*) were incubated with the indicated antibodies before incubation with the labeled probe. Complex I, RelA/p50; complex II, cRel/p50. *C*, IKKα kinase activity is required for the ABIN-2-dependent IKKα-mediated NF-κB DNA binding activity. Nuclear extracts from HEK293 cells transfected with E tag-ABIN-2 along with either WT IKKα/β or KD mutants were analyzed for NF-κB activity by EMSA. Protein expression levels were analyzed by immunoblotting for the indicated proteins. *D*, ABIN-2 but not ABIN-1 synergizes with IKKα to induce NF-κB DNA binding activity. Nuclear extracts from HEK293 cells transfected with either E tag-ABIN-1 or E tag-ABIN-2 alone or with either wild-type or KD mutant of HA-IKKα were analyzed for NF-κB activity by EMSA. Protein expression levels were analyzed by immunoblotting for the indicated proteins. *E*, ABIN-2 enhances IKKα-mediated NF-κB transcriptional activity. HEK293 cells were transfected with 3×NF-κB-luciferase reporter along with the indicated expression plasmids, and luciferase activity was determined. *F*, ABIN-2 increases IKKα activity *in vitro*. Whole cell extracts from HEK293 cells transiently transfected with HA-IKKα alone or with E tag-ABIN-2 were subjected to immunoprecipitation with anti-HA, and kinase assays were performed using either GST alone or GST-IκBα(1–54) as substrates. Immunoprecipitated HA-IKKα protein was detected by immunoblotting with anti-HA. Ponceau S staining ensures equal amounts of substrates. *Arrows* indicate phosphorylated substrate and IKKα autophosphorylation. *G*, ABIN-2 increases IKKα activity *in vivo*. Whole cell extracts from HEK293 cells transiently transfected with equivalent amounts of the indicated expression plasmids were analyzed by immunoblotting for the indicated proteins. *H*, ABIN-2 controls NF-κB DNA binding activity upon prolonged stimulation by TNFα in primary MEFs. Nuclear extracts from WT and ABIN-2-deficient MEFs treated with TNFα for the indicated periods of time were analyzed for NF-κB activity by EMSA. *I*, ABIN-2 controls IKKα activity upon prolonged stimulation by TNFα in primary MEFs. *Upper*, whole cell extracts from WT and ABIN-2-deficient MEFs treated with TNFα for the indicated periods of time were analyzed for IKKα kinase activity and expression as described above. *Lower*, whole cell extracts from WT and ABIN-2-deficient MEFs were analyzed by immunoblotting for the indicated proteins. * indicates nonspecific band.

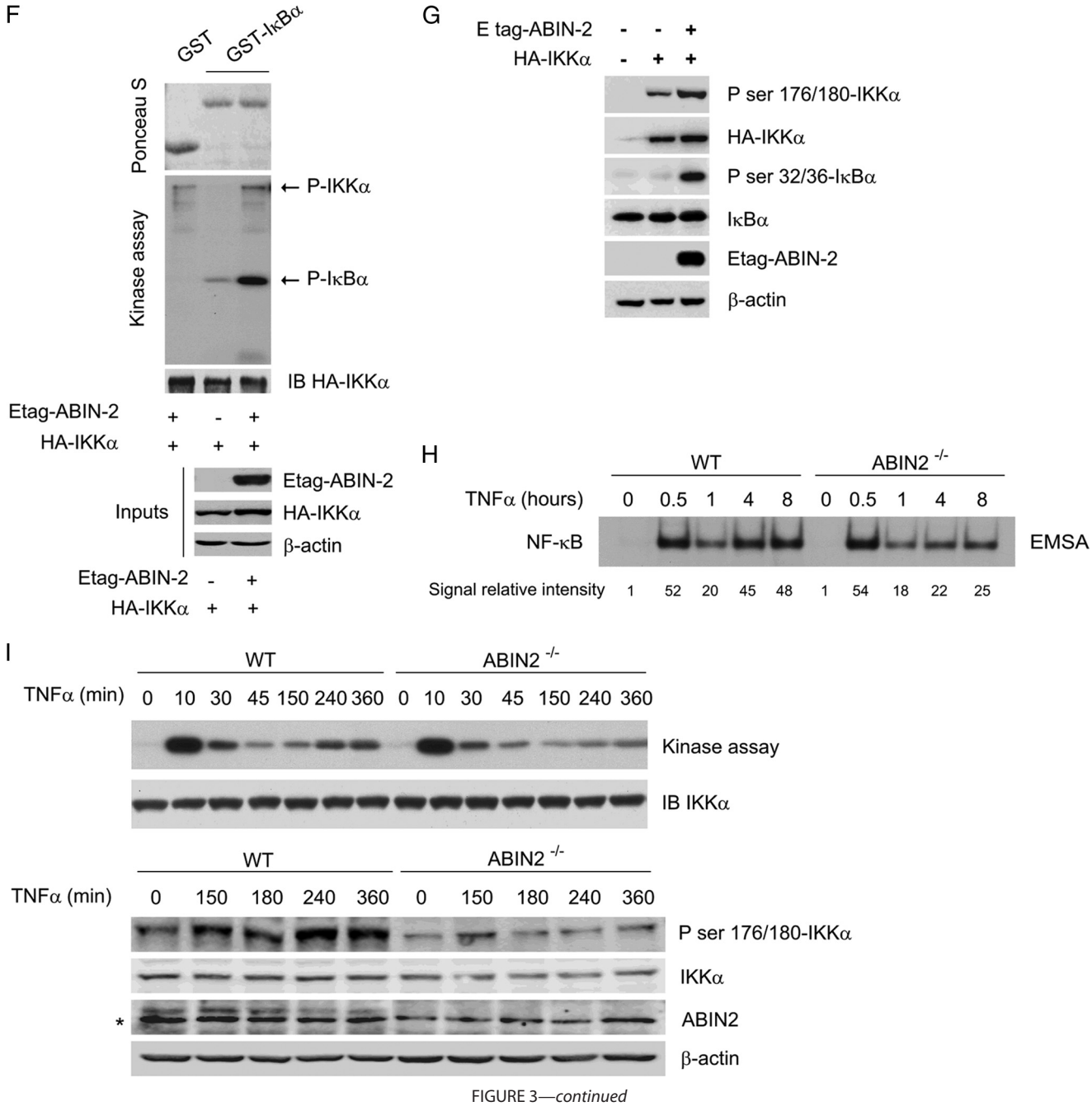

FIGURE 3—*continued*

transcriptional activity, we compared the ability of ABIN-2 to potentiate IKKα and IKKβ activation of an NF-κB-dependent reporter gene in transiently transfected HEK293 cells. Whereas IKKα and IKKβ alone led to similar activation of the NF-κB reporter, overexpression of ABIN-2 greatly enhanced IKKα-induced NF-κB activation in a dose-dependent manner but had little or no effect on IKKβ-mediated activation (Fig. 3*E*).

Because ABIN-2 interacts with IKKα and specifically activates IKKα-dependent NF-κB activation, we wondered whether ABIN-2 might increase IKKα activity. We first measured IKKα activity in an *in vitro* kinase assay. Whereas in the absence of ABIN-2, only a weak IKKα kinase activity toward IκBα could be detected (Fig. 3*F*), this activity was enhanced markedly when cells were cotransfected with E tag-ABIN-2 (Fig. 3*F*, *lane 2 versus lane 3*). Interestingly, ABIN-2 also greatly enhanced IKKα autophosphorylation. We next examined IKKα by immunoblotting (Fig. 3*G*) and similarly observed that ABIN-2 also markedly increased IκBα phosphorylation on serine 32 and serine 36 *in vivo* as well as IKKα phosphorylation.

Further *in vivo* evidence for a role for ABIN-2 in regulating IKKα activity was obtained by comparing IKK/NF-κB activity in WT and ABIN-2-deficient primary MEFs upon prolonged stimulation with TNFα. As shown in Fig. 3*H*, TNFα treatment of WT MEFs resulted in the characteristic biphasic time course of NF-κB DNA binding activity. In contrast, the late phase of NF-κB activity that occurs at 4 h and persisted for at least 8 h in WT MEFs was strongly decreased in ABIN-2-deficient MEFs.

Because temporal control of IKK activity has been reported previously to be crucial for dynamic of NF-κB activation (47), we thus compared the IKKα activation profile in WT and ABIN-2-deficient MEFs at late time points of TNFα treatment. Examining IKKα kinase activity in WT MEFs revealed that IKKα was highly active at early time points, dropped to low levels after about 45 min, and was reactivated at 4 h of TNFα stimulation and persisted over 6 h in WT MEFs (Fig. 3*I*, *upper*). In contrast, the late phase of TNFα-induced IKKα activity was almost completely abolished in absence of ABIN-2. Similarly, IKKα phosphorylation induced by prolonged stimulation by TNFα was markedly reduced in ABIN-2-deficient MEFs compared with that seen in WT MEFs (Fig. 3*I*, *lower*). Together, these results indicate that ABIN-2 induces IKKα autophosphorylation and kinase activity *in vivo*, thus resulting in increased IKKα-mediated NF-κB transcriptional activity.

IKKα Induces the Phosphorylation of ABIN-2—During the course of ABIN-2 immunoblotting assays, we noticed that more slowly migrating ABIN-2 species could be detected when ABIN-2 was coexpressed with HA-IKKα or HA-IKKβ (Fig. 1*B*, *Inputs*, and Fig. 2*C*). Importantly, highly resolutive SDS-polyacrylamide gels indicated that coexpression of ABIN-2 with IKKα resulted in the appearance of an even more shifted band compared with the one observed upon coexpression of ABIN-2 with IKKβ (Fig. 2*C*, *lane 2 versus lane 4*). Because coexpression of ABIN-2 with IKKα led to a strong increase in IKKα kinase activity and subsequent IKKα-mediated NF-κB activation, these results raise the intriguing possibility that IKKα may also directly phosphorylate ABIN-2. Thus, we decided to investigate whether ABIN-2 might be phosphorylated by IKKα and whether such a phosphorylation might be, at least in part, responsible for the enhancement of IKKα-dependent NF-κB target gene expression.

Interestingly, the slower migrating form of ABIN-2 was converted to its basal form upon treatment with calf intestinal phosphatase, revealing phosphorylation as the nature of ABIN-2 modification (Fig. 4*A*). In a second step, to determine whether IKKα directly phosphorylates ABIN-2, we generated a GST-ABIN-2 full-length fusion protein and performed *in vitro* IKKα immunocomplex kinase assays using IKKα that was immunoprecipitated from HEK293 cells overexpressing HA-IKKα, and the GST-ABIN-2 protein as a substrate. The GST-ABIN-2 protein was significantly phosphorylated by IKKα, the relative level of phosphorylation being slightly lower than that of IKKα autophosphorylation (Fig. 4*B*, *right*). Moreover, the ABIN-2 phosphorylation was specific, because IKKα did not phosphorylate the GST protein alone (Fig. 4*B*, *right*). To localize the region(s) of ABIN-2 that is (are) phosphorylated by IKKα, five GST-ABIN-2 deletion mutants, each containing a subdomain of the ABIN-2 coding region, as described in Fig. 4*C* (Δ1–Δ5 mutants), were used as substrates in IKKα kinase assays. As shown in Fig. 4*D*, only mutants Δ1 and Δ2 were phosphorylated efficiently by IKKα. Note that the band observed with mutant Δ3 is nonspecific, because its size does not correspond to that of the substrate detected by Ponceau S staining (Fig. 4*D*). Mutant Δ1 and mutant Δ2 contained three and four serines, respectively. Because IKKα is known to be a serine/threonine kinase (10), one or more of these serines are likely involved in ABIN-2 phosphorylation. We have mutated each of the serine residues to alanine into the Δ1 and Δ2 GST-ABIN-2 deletion mutants, and *in vitro* kinase assays revealed that a point mutation at serine 62 and serine 146 abolished phosphorylation of the Δ1 and the Δ2 ABIN-2 deletion mutant, respectively (Fig. 4*E*). Together, these results suggest that ABIN-2 serine 62 and/or serine 146 phosphorylation by IKKα might be required for enhancement of IKKα-mediated NF-κB activity.

ABIN-2 Serine 146 Is Critical to Enhance IKKα-dependent NF-κB Target Gene Expression—We therefore analyzed the functional consequence of ABIN-2 serine 62 or serine 146 mutations on endogenous NF-κB target gene expression. Using real-time PCR, we compared the expression of 15 known NF-κB target genes, including inflammatory chemokines and cytokines as well as antiapoptotic genes, in HEK293 cells transfected with HA-IKKα along with either wild-type ABIN-2 or S62A or S146A mutants. Remarkably, whereas the expression of RANTES and monocyte chemoattractant protein-1 was strongly increased in HEK293 cells transfected with IKKα and either wild-type ABIN-2 or the S62A ABIN-2 mutant (Fig. 5*A*, *upper panels*), coexpression of IKKα with the S146A mutant resulted in a marked reduction in RANTES and monocyte chemoattractant protein-1 mRNA levels. The decrease is specific, because it was not observed for TNFα and cIAP2, two other NF-κB target genes for which the expression level is also strongly induced in cells coexpressing IKKα and wild-type ABIN-2 (Fig. 5*A*, *middle panels*). Moreover, analysis of wild-type and mutant ABIN-2 expression showed that their mRNA levels are similar when coexpressed with IKKα (Fig. 5*A*, *lower left panel*). Similarly, IKKα expression is not affected by coexpression with either form of ABIN-2 (Fig. 5*A*, *lower right panel*). Taken together, our results show that ABIN-2 exerts a serine 146-dependent selective activating function serving to enhance IKKα-mediated NF-κB activity.

DISCUSSION

In this report, we reveal an unexpected and intimate relationship between IKKα and ABIN-2. We show that ABIN-2 interacts with and is stabilized by IKKα. Importantly, ABIN-2 activates IKKα kinase activity, thus enhancing IKKα-mediated NF-κB transcriptional activity. Furthermore, ABIN-2 is phosphorylated by IKKα at serine 146, a serine that appears to be critical for ABIN-2-dependent IKKα transcriptional up-regulation of specific NF-κB target genes. We propose that ABIN-2 serves as an adaptor protein to activate IKKα which in turn can also phosphorylate ABIN-2. This cross-regulation between ABIN-2 and IKKα seems to be essential for activation of selective NF-κB target gene expression (Fig. 5*B*). However, it is not yet clear whether ABIN-2 stabilization is absolutely required for an ABIN-2-dependent increase in IKKα activity.

In contrast to our findings that ABIN-2 interacts with IKKα and IKKβ both in overexpressing conditions and when the interactions of the endogenous proteins were examined, a previous report showed that ABIN-2 binds to IKKγ/NEMO, but not to IKKα and IKKβ in transfected HEK293 cells (48). The reasons for these discrepant results are not clear but could be due to differences in conditions employed for the coimmuno-

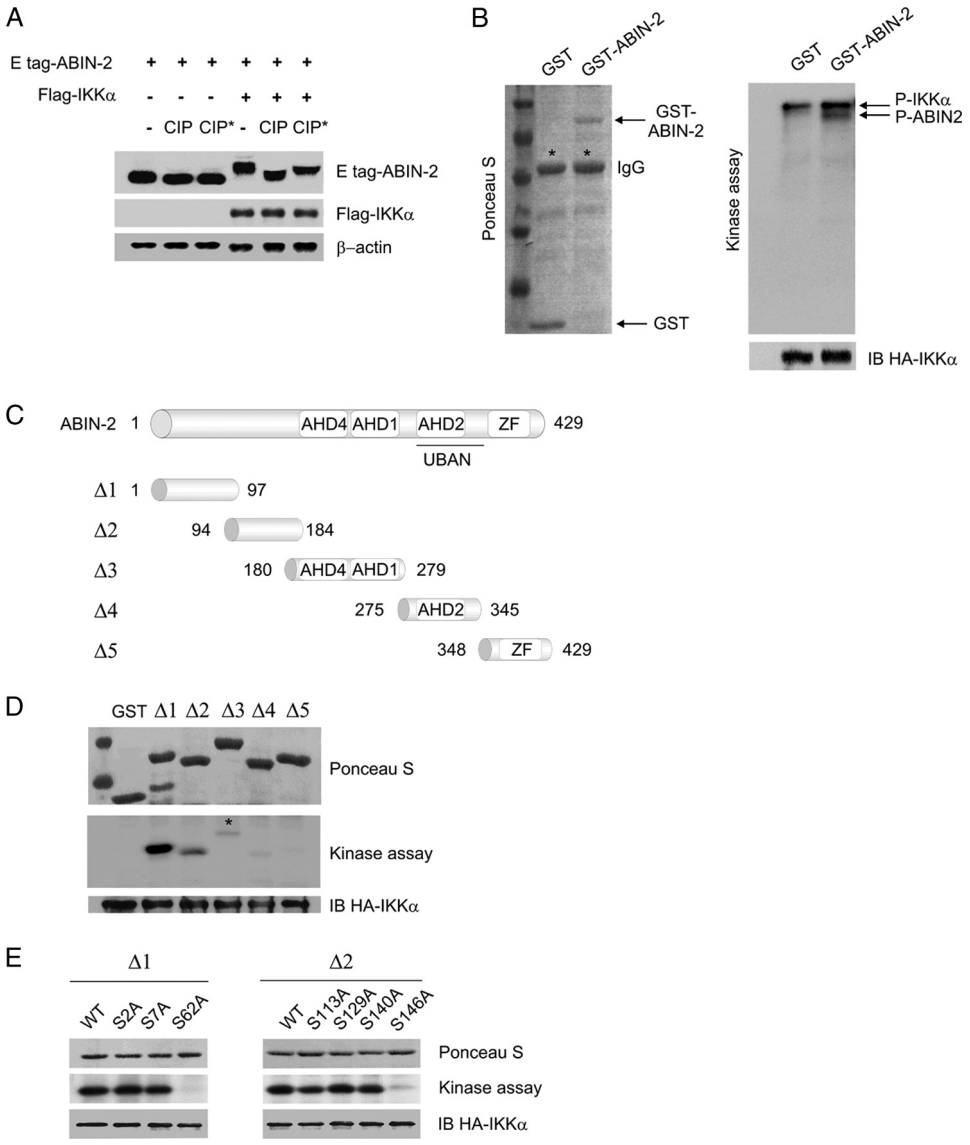

FIGURE 4. **IKKα induces the phosphorylation of ABIN-2.** *A*, differential migration of ABIN-2 upon ectopic expression of IKKα is due to phosphorylation. HEK293 cells were transfected with the indicated expression plasmids. Whole cell extracts prepared as in Fig. 2*D* were incubated with calf intestinal phosphatase, either untreated (*CIP*) or heat-inactivated (*CIP**), and analyzed by immunoblotting for the indicated proteins. *B*, IKKα phosphorylates ABIN-2 *in vitro*. Whole cell extracts from HEK293 cells transiently transfected with HA-IKKα were subjected to immunoprecipitation with anti-HA, and kinase assays were performed using either GST alone or GST-ABIN-2 full-length as substrates. Immunoprecipitated HA-IKKα protein was detected by immunoblotting with anti-HA. *Arrows* indicate phosphorylated substrate and IKKα autophosphorylation, and * indicates nonspecific bands. *C*, schematic representation of GST-ABIN-2 deletion mutants. *Numbers* refer to amino acid positions within human ABIN-2. Δ*1*, 1–97; Δ*2*, 94–184; Δ*3*, 180–279; Δ*4*, 275–345; and Δ*5*, 348–429. Structural subdomains are indicated. *AHD1/2/4*, ABIN homology domains 1, 2, and 4; *UBAN*, ubiquitin binding domain; *ZF*, zinc finger. *D*, IKKα phosphorylation of N-terminal domain of ABIN-2. Kinase assays were performed as in *B* using either GST alone or GST-ABIN-2 deletion mutants as substrates. Immunoprecipitated HA-IKKα protein was detected by immunoblotting. * indicates nonspecific band. *E*, IKKα phosphorylates ABIN-2 on serine 62 and serine 146 *in vitro*. Kinase assays were performed as in *B* using as substrates various GST-ABIN-2 point mutants in which serine (*S*) was individually replaced by alanine (*A*).

precipitation experiments. Nevertheless, because all three IKK subunits are known to form a complex *in vivo* (49), it is likely that ABIN-2 could be part of the classical tripartite IKK complex (Fig. 5*B*). Nevertheless it cannot be excluded that ABIN-2 might also bind to the alternative IKKα-containing complex independently of IKKβ and IKKγ (50).

Based on its ability to bind A20 and block inflammatory cytokine-induced NF-κB activation upon overexpression, ABIN-2 was originally described as a negative regulator of NF-κB-dependent gene expression (40). Such an inhibition was also observed in response to TPA and EGF, thus reinforcing the concept that ABIN-2 overexpression leads to inhibition of NF-κB (40, 51). Interestingly, ABIN-2 interferes with NF-κB activation induced by RIP1, TRAF2, TRAF6, and IRAK1, but not IKKβ, suggesting that the inhibitory effect of ABIN-2 occurs upstream of IKKβ (40). In the present report, we demonstrate that ABIN-2 can act as an activator of NF-κB through its interaction with IKKα and subsequent enhancement of IKKα-mediated NF-κB transcriptional activity. It thus appears that ABIN-2 may exert dual functions depending on its targets within the NF-κB activation pathways. Interestingly, IKKα was shown to associate with RelA in the nucleus and activate the

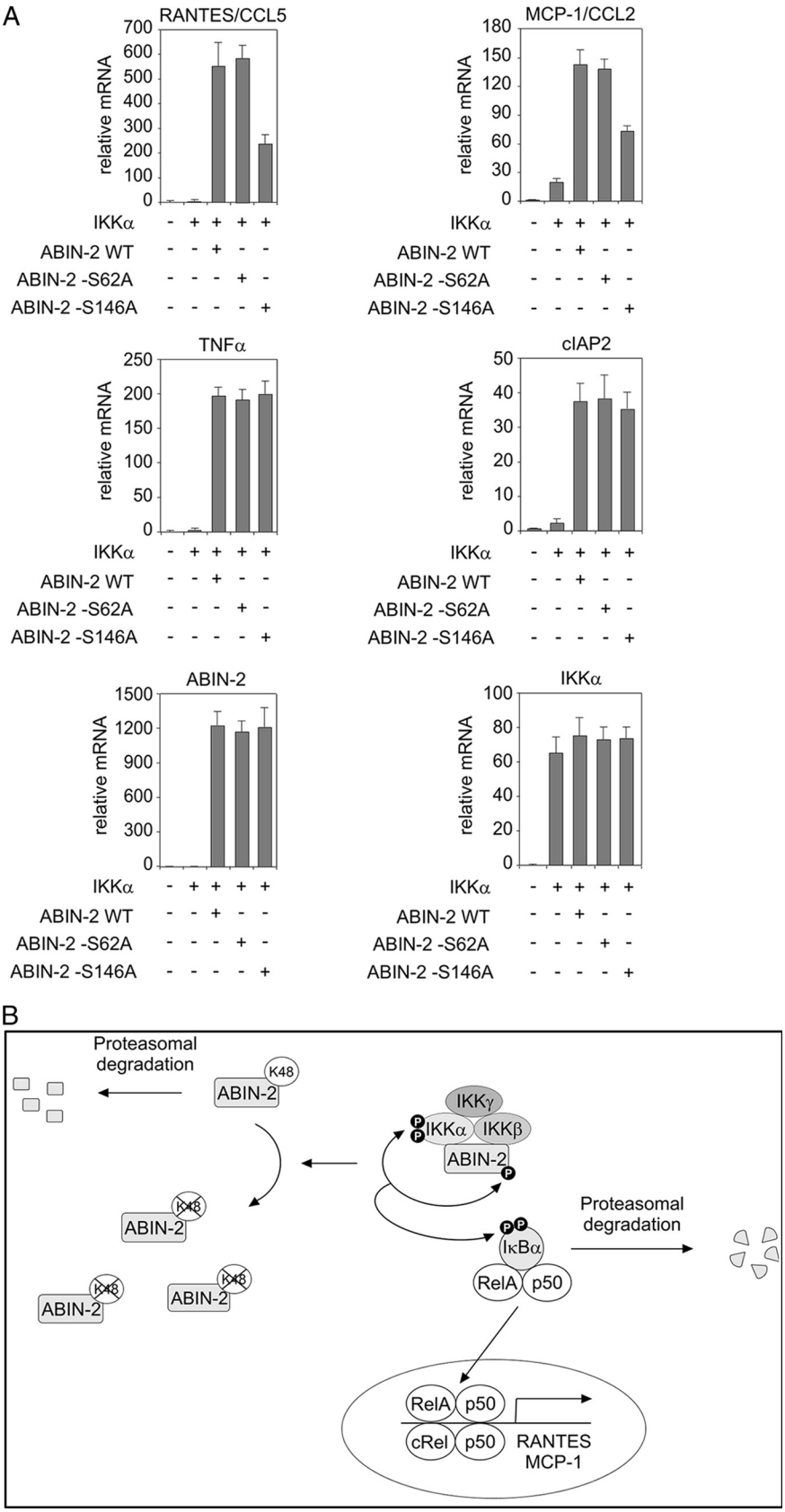

FIGURE 5. **ABIN-2 serine 146 is critical to enhance IKKα-dependent NF-κB target gene expression.** *A*, total RNA was isolated from HEK293 cells transfected with the indicated expression plasmids, and gene expression was quantified by real-time PCR analysis. Data are normalized to hypoxanthine-guanine phosphoribosyltransferase mRNA expression and shown as mean ± S.D. (*error bars*; $n = 3–4$). *B*, model of NF-κB activation by ABIN-2. Upon recruitment of ABIN-2 to the IKK complex, ABIN-2 is stabilized through deubiquitination of Lys-48-linked poly(Ub) chains; ABIN-2 is phosphorylated on serine 146; and IKKα becomes activated, thus leading to the phosphorylation, ubiquitination, and degradation of IκBα, and subsequent activation of NF-κB target gene expression.

expression of NF-κB-responsive genes through direct modulation of histone function (26, 27). Although we were unable to detect nuclear ABIN-2 by immunofluorescence microscopy experiments, there is evidence to suggest that ABIN-2 may function as a transcriptional coactivator. First, tandem affinity purification indicated that ABIN-2 forms a constitutive complex with several NF-κB subunits (RelA, p50, p52) (52); second, a fusion protein of ABIN-2 and the Gal4-DNA binding domain leads to the induction of a Gal4-dependent reporter gene (53); and third, the C-terminal domain of ABIN-2 can enter the nucleus and exert transactivating activity (53). In the near future, chromatin immunoprecipitation experiments designed to determine whether ABIN-2, potentially in conjunction with IKKα and/or RelA, is recruited to the promoter regions of NF-κB-responsive genes will provide a direct test of this hypothesis.

Analysis of ABIN-2-deficient mice revealed that ABIN-2 is required for ERK activation in response to TNFα and LPS in macrophages as well as upon CD40 ligation in B cells by stabilization of the upstream kinase TPL-2 (54). In contrast, LPS- and TNFα-induced activation of ERK was unaffected by ABIN-2 deficiency in B cells and dendritic cells, respectively (54). Therefore, it is likely that the ABIN-2-activating function in the ERK signaling pathway is both stimulus- and cell type-specific, reflecting the alternate use of TPL-2 and other MEK kinases to trigger ERK activation. No defect in NF-κB activation triggered by LPS was observed in primary B cells isolated from ABIN-2-deficient mice (54). Similarly, activation of NF-κB induced by TNFα and LPS was unaffected in ABIN-2-deficient macrophages (54). However, because overexpression experiments point to a role of ABIN-2 in the control of NF-κB transcriptional activity, it is tempting to hypothesize that ABIN-2, just as it does for the ERK pathway, plays a role in the regulation of NF-κB activation *in vivo* in a cell type- and stimulus-specific manner. In support of this idea, a recent report demonstrates the existence of recurrent ABIN-2 somatic mutations in diffuse large B cell lymphoma that altered the capacity of ABIN-2 to inhibit NF-κB (55).

In an effort to address the physiological relevance of the activation of NF-κB activity by ABIN-2, we have performed real-time PCR on several known NF-κB target genes with roles in inflammation and cell survival. We observed that coexpression of ABIN-2 and IKKα leads to a strong induction of several inflammatory cytokines and chemokines compared with what was seen upon expression of either IKKα or ABIN-2 alone, suggesting a proinflammatory role for ABIN-2, most likely due to its NF-κB-activating properties described in this report. Interestingly, serine 146, located within the N-terminal domain of ABIN-2, seems to be a critical phosphorylation site for ABIN-2-dependent IKKα transcriptional up-regulation of specific NF-κB target genes. Because ABIN-2 serine 146 is phosphorylated by IKKα *in vitro*, our data suggest an intimate interplay between ABIN-2 and IKKα that is crucial for activation of selective NF-κB target gene expression. However, further experiments are required to determine whether IKKα is responsible for ABIN-2 phosphorylation *in vivo* and whether other kinases might be involved.

In conclusion, our study has highlighted a new and unexpected NF-κB-activating function for ABIN-2 and defined a previously unrecognized mechanism of cross-regulation between ABIN-2 and IKKα that seems to be an essential regulator of NF-κB-dependent gene expression.

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REFERENCES

1. Li, Q., and Verma, I. M. (2002) *Nat. Rev. Immunol.* **2,** 725–734
2. Vallabhapurapu, S., and Karin, M. (2009) *Annu. Rev. Immunol.* **27,** 693–733
3. Pasparakis, M. (2009) *Nat. Rev. Immunol.* **9,** 778–788
4. Baker, R. G., Hayden, M. S., and Ghosh, S. (2011) *Cell Metab.* **13,** 11–22
5. Luo, J. L., Kamata, H., and Karin, M. (2005) *J. Clin. Immunol.* **25,** 541–550
6. Perkins, N. D., and Gilmore, T. D. (2006) *Cell Death Differ.* **13,** 759–772
7. Fan, Y., Dutta, J., Gupta, N., Fan, G., and Gélinas, C. (2008) *Adv. Exp. Med. Biol.* **615,** 223–250
8. Kim, H. J., Hawke, N., and Baldwin, A. S. (2006) *Cell Death Differ.* **13,** 738–747
9. Baud, V., and Karin, M. (2009) *Nat. Rev. Drug Discov.* **8,** 33–40
10. Häcker, H., and Karin, M. (2006) *Sci. STKE* 2006, re13
11. Perkins, N. D. (2007) *Nat. Rev. Mol. Cell Biol.* **8,** 49–62
12. Solt, L. A., and May, M. J. (2008) *Immunol. Res.* **42,** 3–18
13. Hoffmann, A., Natoli, G., and Ghosh, G. (2006) *Oncogene* **25,** 6706–6716
14. Sun, S. C., and Ley, S. C. (2008) *Trends Immunol.* **29,** 469–478
15. Hayden, M. S., and Ghosh, S. (2008) *Cell* **132,** 344–362
16. Baud, V., and Derudder, E. (2011) *Curr. Top. Microbiol. Immunol.* **349,** 97–114
17. Karin, M., and Ben-Neriah, Y. (2000) *Annu. Rev. Immunol.* **18,** 621–663
18. Israël, A. (2010) *Cold Spring Harb. Perspect. Biol.* **2,** a000158
19. Ghosh, S., and Karin, M. (2002) *Cell* **109,** S81–96
20. Hayden, M. S., and Ghosh, S. (2004) *Genes Dev.* **18,** 2195–2224
21. Verstrepen, L., Bekaert, T., Chau, T. L., Tavernier, J., Chariot, A., and Beyaert, R. (2008) *Cell Mol. Life Sci.* **65,** 2964–2978
22. Dejardin, E. (2006) *Biochem. Pharmacol.* **72,** 1161–1179
23. Sun, S. C. (2011) *Cell Res.* **21,** 71–85
24. Adli, M., Merkhofer, E., Cogswell, P., and Baldwin, A. S. (2010) *PLoS One* **5,** e9428
25. Cao, Y., Bonizzi, G., Seagroves, T. N., Greten, F. R., Johnson, R., Schmidt, E. V., and Karin, M. (2001) *Cell* **107,** 763–775
26. Yamamoto, Y., Verma, U. N., Prajapati, S., Kwak, Y. T., and Gaynor, R. B. (2003) *Nature* **423,** 655–659
27. Anest, V., Hanson, J. L., Cogswell, P. C., Steinbrecher, K. A., Strahl, B. D., and Baldwin, A. S. (2003) *Nature* **423,** 659–663
28. Lawrence, T., Bebien, M., Liu, G. Y., Nizet, V., and Karin, M. (2005) *Nature* **434,** 1138–1143
29. Li, Q., Lu, Q., Bottero, V., Estepa, G., Morrison, L., Mercurio, F., and Verma, I. M. (2005) *Proc. Natl. Acad. Sci. U.S.A.* **102,** 12425–12430
30. Li, Q., Lu, Q., Hwang, J. Y., Büscher, D., Lee, K. F., Izpisua-Belmonte, J. C., and Verma, I. M. (1999) *Genes Dev.* **13,** 1322–1328
31. Takeda, K., Takeuchi, O., Tsujimura, T., Itami, S., Adachi, O., Kawai, T., Sanjo, H., Yoshikawa, K., Terada, N., and Akira, S. (1999) *Science* **284,** 313–316
32. Hu, Y., Baud, V., Oga, T., Kim, K. I., Yoshida, K., and Karin, M. (2001) *Nature* **410,** 710–714
33. Mercurio, F., Zhu, H., Murray, B. W., Shevchenko, A., Bennett, B. L., Li, J., Young, D. B., Barbosa, M., Mann, M., Manning, A., and Rao, A. (1997) *Science* **278,** 860–866

34. Ling, L., Cao, Z., and Goeddel, D. V. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **95,** 3792–3797
35. Delhase, M., Hayakawa, M., Chen, Y., and Karin, M. (1999) *Science* **284,** 309–313
36. Schomer-Miller, B., Higashimoto, T., Lee, Y. K., and Zandi, E. (2006) *J. Biol. Chem.* **281,** 15268–15276
37. Chen, G., Cao, P., and Goeddel, D. V. (2002) *Mol. Cell* **9,** 401–410
38. Hinz, M., Broemer, M., Arslan, S. C., Otto, A., Mueller, E. C., Dettmer, R., and Scheidereit, C. (2007) *J. Biol. Chem.* **282,** 32311–32319
39. Hu, W. H., Pendergast, J. S., Mo, X. M., Brambilla, R., Bracchi-Ricard, V., Li, F., Walters, W. M., Blits, B., He, L., Schaal, S. M., and Bethea, J. R. (2005) *J. Biol. Chem.* **280,** 29233–29241
40. Van Huffel, S., Delaei, F., Heyninck, K., De Valck, D., and Beyaert, R. (2001) *J. Biol. Chem.* **276,** 30216–30223
41. Verstrepen, L., Carpentier, I., Verhelst, K., and Beyaert, R. (2009) *Biochem. Pharmacol.* **78,** 105–114
42. Kieusseian, A., Chagraoui, J., Kerdudo, C., Mangeot, P. E., Gage, P. J., Navarro, N., Izac, B., Uzan, G., Forget, B. G., and Dubart-Kupperschmitt, A. (2006) *Blood* **107,** 492–500
43. Baud, V., Liu, Z. G., Bennett, B., Suzuki, N., Xia, Y., and Karin, M. (1999) *Genes Dev.* **13,** 1297–1308
44. de Boer, E., Rodriguez, P., Bonte, E., Krijgsveld, J., Katsantoni, E., Heck, A., Grosveld, F., and Strouboulis, J. (2003) *Proc. Natl. Acad. Sci. U.S.A.* **100,** 7480–7485
45. Jacque, E., Tchenio, T., Piton, G., Romeo, P. H., and Baud, V. (2005) *Proc. Natl. Acad. Sci. U.S.A.* **102,** 14635–14640
46. Finley, D. (2009) *Annu. Rev. Biochem.* **78,** 477–513
47. Werner, S. L., Barken, D., and Hoffmann, A. (2005) *Science* **309,** 1857–1861
48. Liu, W. K., Yen, P. F., Chien, C. Y., Fann, M. J., Su, J. Y., and Chou, C. K. (2004) *Biochem. J.* **378,** 867–876
49. Rothwarf, D. M., Zandi, E., Natoli, G., and Karin, M. (1998) *Nature* **395,** 297–300
50. Derudder, E., Dejardin, E., Pritchard, L. L., Green, D. R., Korner, M., and Baud, V. (2003) *J. Biol. Chem.* **278,** 23278–23284
51. Huang, L., Verstrepen, L., Heyninck, K., Wullaert, A., Revets, H., De Baetselier, P., and Beyaert, R. (2008) *Oncogene* **27,** 6131–6140
52. Bouwmeester, T., Bauch, A., Ruffner, H., Angrand, P. O., Bergamini, G., Croughton, K., Cruciat, C., Eberhard, D., Gagneur, J., Ghidelli, S., Hopf, C., Huhse, B., Mangano, R., Michon, A. M., Schirle, M., Schlegl, J., Schwab, M., Stein, M. A., Bauer, A., Casari, G., Drewes, G., Gavin, A. C., Jackson, D. B., Joberty, G., Neubauer, G., Rick, J., Kuster, B., and Superti-Furga, G. (2004) *Nat. Cell Biol.* **6,** 97–105
53. Chien, C. Y., Liu, W. K., Chou, C. K., and Su, J. Y. (2003) *FEBS Lett.* **543,** 55–60
54. Papoutsopoulou, S., Symons, A., Tharmalingham, T., Belich, M. P., Kaiser, F., Kioussis, D., O'Garra, A., Tybulewicz, V., and Ley, S. C. (2006) *Nat. Immunol.* **7,** 606–615
55. Dong, G., Chanudet, E., Zeng, N., Appert, A., Chen, Y. W., Au, W. Y., Hamoudi, R., Watkins, A. J., Ye, H., Liu, H., Gao, Z., Chuang, S. S., Srivastava, G., and Du, M. Q. (2011) *Clin. Cancer Res* **17,** 1440–1451