Nuclear IKKβ is an adaptor protein for IκBα ubiquitination and degradation in UV-induced NF-κB activation

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SUMMARY

Proinflammatory cytokines activate NF-κB using the IκB kinase (IKK) complex that phosphorylates inhibitory proteins (IκBs) at N-terminal sites resulting in their ubiquitination and degradation in the cytoplasm. Although ultraviolet (UV) irradiation does not lead to IKK activity, it activates NF-κB by an unknown mechanism through IκBα degradation without N-terminal phosphorylation. Here, we describe an adaptor function of nuclear IKKβ in UV-induced IκBα degradation. UV irradiation induces the nuclear translocation of IκBα and association with IKKβ, which constitutively interacts with β-TrCP through heterogeneous ribonucleoprotein-U (hnRNP-U) leading to IκBα ubiquitination and degradation. Furthermore, casein kinase 2 (CK2) and p38 associate with IKKβ and promote IκBα degradation by phosphorylation at C-terminal sites. Thus, nuclear IKKβ acts as an adaptor protein for IκBα degradation in UV-induced NF-κB activation. NF-κB activated by the nuclear IKKβ adaptor protein suppresses anti-apoptotic gene expression and promotes UV-induced cell death.

INTRODUCTION

NF-κB is a critical mediator of the cellular response to inflammatory cytokines, developmental signals, pathogens, and cellular stresses. NF-κB activity is regulated through its interaction with inhibitory proteins (IκBs), the most prominent and well-studied of which is IκBα, which prevents the DNA binding of NF-κB (Hayden and Ghosh, 2008; Perkins, 2007). The NF-κB/IκBα complex is localized exclusively in the cytosol because of a nuclear export sequence (NES) encoded in the IκBα subunit and the masking of a nuclear localization signal (NLS) in the NF-κB subunit. Proinflammatory stimuli, including tumor

necrosis factor α (TNFα), activate IKKβ to phosphorylate IκBα at two N-terminal serines, Ser32 and Ser36. This phosphorylation triggers ubiquitination at two N-terminal lysines, Lys20 and Lys21, by recruiting the SKP1-CUL1-F-box protein ubiquitin ligase through association with an F-box protein β-TrCP (Hayden and Ghosh, 2008; Karin and Ben-Neriah, 2000; Nakayama et al., 2003). This sequential cascade of reactions leads to the proteasomal degradation of IκBα and subsequent NF-κB activation. IKKβ is mainly localized in the cytoplasm, although a certain quantity of IKKβ, with unknown function, is present in the nucleus (Anest et al., 2003; Birbach et al., 2002).

Mammalian cells respond to ultraviolet (UV) light by inducing or suppressing the expression of specific genes involved in DNA damage repair, cell cycle arrest and apoptosis, which is mediated by the activation of transcription factors such as AP-1, NF-κB, and p53 (Herrlich et al., 2008). Whereas the activation of p53 is a direct consequence of nuclear signals generated by damaged DNA, the molecular mechanisms by which UV light activates AP-1 and NF-κB involve cytoplasmic signals generated independently of DNA damage. In contrast to the strong, rapid, and transient NF-κB activation induced by inflammatory stimuli, UV-induced NF-κB activation appears to be weak, slow, and prolonged. However, the mechanism by which UV induces the proteolysis of IκBs remains unclear, as the conclusions drawn from studies of UV-induced NF-κB activation are confusing and conflicting.

Initially, it was proposed that IKK activity is not required for IκBα degradation, as UV irradiation does not activate IKK (Huang et al., 2002; Li and Karin, 1998), and an IκBα mutant (IκBαAA), in which the IKKβ phosphorylation sites were replaced with alanines, is degraded upon UV irradiation (Bender et al., 1998; Li and Karin, 1998). Instead, several studies have suggested that UV irradiation causes IκBα degradation through phosphorylation

in the C-terminal PEST domain by casein kinase 2 (CK2) via an interaction with p38 MAP kinase (Bender et al., 1998; Kato et al., 2003). There is strong evidence, however, that IKK is required for UV-induced IκB proteolysis because UV-induced NF-κB activity is not detected in IKK-knockout cells, particularly in IKKβ-deficient (*Ikkβ*$^{-/-}$) cells (Huang et al., 2002; O'Dea et al., 2008). More recently, a second mechanism was proposed wherein UV-induced translational inhibition through phosphorylation of the eukaryotic initiation factor-2α (eIF2α) is responsible for NF-κB activation (Jiang and Wek, 2005; Wu et al., 2004). As the half-life of IκBs is shorter than that of NF-κB, the inhibition of protein synthesis results in NF-κB induction. Furthermore, a recent mathematical approach using computational modeling has suggested that constitutive phosphorylation of IκBα by basal IKK activity, which affects the turnover rate of IκBα, and IκBα synthesis inhibition are crucial for UV-induced NF-κB activation (O'Dea et al., 2008).

Here, we performed a detailed investigation of the role of IKKβ and confirmed that IKKβ is required for UV-induced IκBα proteolysis and NF-κB activation, consistent with previous findings (Huang et al., 2002; O'Dea et al., 2008). An intriguing and surprising result is that kinase activity is not required for the UV response. IKKβ acts as an adaptor protein for IκBα degradation that is mediated through N-terminal ubiquitination by β-TrCP and C-terminal phosphorylation by CK2-p38.

RESULTS

IKKβ is an adaptor protein interacting with β-TrCP and IκBα

To elucidate functions of IKKβ in UV-induced NF-κB activation, IκBα degradation was analyzed in wild-type (*wt*) and *Ikkβ*$^{-/-}$ fibroblasts. Cycloheximide (CHX)

treatment induced a gradual decrease in IκBα, which reflects its constitutive degradation, and UV irradiation markedly promoted its degradation in *wt* fibroblasts (Figure 1A). By contrast, constitutive degradation and UV-induced degradation were suppressed in *Ikkβ*$^{-/-}$ fibroblasts (Figure 1A and S1A). An electrophoretic mobility shift assay (EMSA) also revealed that NF-κB activation was attenuated in *Ikkβ*$^{-/-}$ fibroblasts due to the suppression of IκBα degradation (Figure S1B).

An intriguing aspect is that UV irradiation dose not induce either IKKβ kinase activity or IκBα phosphorylation (data not shown), consistent with previous findings (Huang et al., 2002; O'Dea et al., 2008). These results raise a fundamental question as to the function of IKKβ, that is, is kinase activity required for UV-induced IκBα degradation? To address this, we introduced either wild-type IKKβ (IKKβWT) or the kinase-negative IKKβ mutant (IKKβKN) into *Ikkβ*$^{-/-}$ fibroblasts and analyzed IκBα degradation after UV irradiation. Surprisingly, a kinase-negative IKKβ mutant (IKKβKN) induced IκBα degradation in a time-dependent manner with similar kinetics to wild-type IKKβ (IKKβWT), suggesting that IKKβ induces IκBα degradation independently of its kinase activity during the UV response (Figure 1B). Furthermore, IκBαAA was degraded with similar kinetics to wild-type IκBα (IκBαWT) following UV irradiation, whereas the degradation of an IκBα mutant lacking the β-TrCP ubiquitination sites (IκBαKKm) was slower than that of IκBαWT and IκBαAA (Figure 1C). Although UV induced IκBα ubiquitination in *wt* fibroblasts, ubiquitination was markedly abolished in *Ikkβ*$^{-/-}$ fibroblasts (Figure 1D). Therefore, IKKβ is essential for IκBα degradation via ubiquitination, but not phosphorylation, during UV-induced NF-κB activation. The absence of a requirement for kinase activity is also supported by the observations that an IKKβ inhibitor failed to prevent UV-induced IκBα degradation (Figure

S1C) and that IκBαAA was degraded in response to UV irradiation in many cell types, whereas it was stable in *Ikkβ*$^{-/-}$ fibroblasts (Figure S1D and E).

IκBα degradation is delayed in *β-TrCP1*$^{-/-}$ fibroblasts, suggesting that ubiquitination by β-TrCP is a prerequisite for UV-induced IκBα degradation (Figure 1E). We transfected IκBαAA and an F-Box deletion mutant of β-TrCP (β-TrCPΔF), which demonstrates improved substrate-binding (Spencer et al., 1999), and analyzed the association of these two components in *wt* and *Ikkβ*$^{-/-}$ fibroblasts. Although UV irradiation induced the association of β-TrCPΔF with IκBαAA in *wt* fibroblasts, β-TrCPΔF did not bind to IκBαAA in *Ikkβ*$^{-/-}$ fibroblasts (Figure 1F). IKKβKN constitutively associated with β-TrCPΔF, and UV irradiation induced the association of IκBαAA (Figure 1G). The immunoprecipitation assay of the endogenous proteins also indicates that a portion of IKKβ constitutively associated with β-TrCP, and that UV irradiation induced the association of IKKβ with IκBα (Figure 1H). Therefore, UV irradiation results in the formation of a β-TrCP-IKKβ-IκBα complex wherein IKKβ mediates IκBα ubiquitination as an adaptor protein bringing β-TrCP to IκBα independently of its kinase activity.

IKKβ is composed of an N-terminal kinase domain and a C-terminal regulatory region (Figure 1I). The immunoprecipitation assay revealed that β-TrCP bound to the N-terminal kinase domain constitutively (Figure 1J). By contrast, IκBα associated with the C-terminal region in response to UV irradiation (Figure 1K). Reconstitution of *Ikkβ*$^{-/-}$ fibroblasts with full-length IKKβ restored IκBα degradation; however, the N-terminal and C-terminal fragments failed to promote its degradation (Figure S1F).

IκBα is composed of three structural domains: the N-terminal region containing phosphorylation sites and β-TrCP ubiquitination sites, the middle domain containing six

ankyrin repeats, and the C-terminal region containing the PEST sequence and several CK2 phosphorylation sites (Figure 2A). UV irradiation induced the association of IKKβ and β-TrCP with IκBα through the middle domain containing ankyrin repeats (Figure 2B and C). Although binding through the ankyrin repeat domain is a prerequisite for UV-induced IκBα degradation, the binding is insufficient to induce its degradation, as an IκBα mutant lacking the N- and C-terminal regions was not degraded after UV irradiation (Bender et al., 1998). Overexpression of the ankyrin repeats domain prevented the association of IκBα with β-TrCP by competing for the binding and attenuated UV-induced IκBα degradation (Figure 2D and E).

Nuclear translocation of IκBα

NF-κB/IκBα subunits are not statically localized in the cytoplasm, but rather shuttle between the cytoplasm and the nucleus (Anest et al., 2003; Birbach et al., 2002; Ghosh and Karin, 2002). Although the nuclear export inhibitor leptomycin B (LMB) markedly inhibited UV-induced IκBα degradation and NF-κB activation with a similar potency to the proteasome inhibitor MG132 (Figure 3A and B), it did not inhibit IκBα ubiquitination (Figure 3C). Immunofluorescent staining of cells revealed that IκBα and RelA translocated from the cytoplasm to the nucleus within 2 h of UV irradiation, with IκBα being degraded 8 h after exposure (Figure 3D, S2A, and S2B). IκBα and RelA localized in the nucleus 8 h after UV irradiation in the presence of LMB, whereas they were distributed in both the nucleus and the cytoplasm in cells exposed to MG132 (Figure 3D, S2A, and S2B). In *Ikkβ*$^{-/-}$ fibroblasts, IκBα was not degraded and remained in the nucleus (Figure 3E).

UV irradiation failed to degrade NLS-IκBα and NES-IκBα which localized

exclusively in the nucleus and cytoplasm, suggesting that nucleocytoplasmic shuttling is crucial for IκBα degradation (Figure 3F and S2C). NLS-IκBα was effectively ubiquitinated after UV irradiation, whereas NES-IκBα was ubiquitinated to a lesser extent (Figure 3G). UV irradiation resulted in the translocation of IκBα from the cytoplasm to the nucleus, and induced its association with IKKβ and β-TrCP after 2 h (Figure 3H). These results suggest that IκBα translocates into the nucleus in response to UV irradiation where it associates with β-TrCP through IKKβ and is subjected to ubiquitination; it then returns to the cytoplasm and undergoes proteasomal degradation. Consistent with this model, NLS-IKKβ, which localized in the nucleus, did not induce IκBα degradation when cells were stimulated with TNFα, but degraded IκBα more effectively than NES-IKKβ after UV irradiation (Figure 3I and S2D). IκBα binds to NES-IKKβ and NLS-IKKβ with a similar affinity, whereas β-TrCP associates preferentially with NLS-IKKβ, suggesting that the prominent effect of nuclear IKKβ on IκBα degradation is due to its association with β-TrCP (Figure 3J).

A recent study revealed that UV irradiation triggers Ca^{2+} mobilization in cells (Lao and Chang, 2008). The Ca^{2+} chelator BAPTA-AM markedly suppressed nuclear accumulation of IκBα (Figure 3K and S2E) and its degradation (Figure 3L). BAPTA-AM inhibited the UV-induced association of IκBα with IKKβ without interfering with the binding of IKKβ and β-TrCP (Figure S2F and G). Treatment of cells with a Ca^{2+} ionophore did not induce the nuclear translocation of IκBα, indicating that Ca^{2+} mobilization is required but not sufficient for the UV response (Figure 3K and S2E).

β-TrCP-hnRNP-U-IKKβ complex

β-TrCP recognizes its substrate through a DSGXXS degron (Frescas and Pagano, 2008). As IKKβ lacks this motif, we searched for a protein capable of mediating interactions with β-TrCP and found that hnRNP-U, previously reported as a pseudosubstrate of β-TrCP1 (Davis et al., 2002), associated with IKKβ (Figure 4A). An immunoprecipitation assay of the endogenous proteins demonstrated that hnRNP-U constitutively associated with β-TrCP and IKKβ, and UV irradiation recruited IκBα to this complex (Figure 4B). The UV-induced association of IκBα depends on IKKβ since IκBαAA did not bind to hnRNP-U in *Ikkβ*$^{-/-}$ fibroblasts (Figure 4C). The binding site of hnRNP-U on IKKβ is located at the N-terminal kinase domain, which is consistent with the β-TrCP binding site (compare Figure 4D and 1J). NLS-IKKβ preferentially associated with hnRNP-U, consistent with their dual nuclear localization (Figure 4E).

hnRNP-U is composed of an N-terminal DNA and β-TrCP binding region (Davis et al., 2002), a middle RNA polymerase II binding region (Kukalev et al., 2005), and a C-terminal RNA binding region (Figure 4F). β-TrCP associated with the N-terminal region, whereas IKKβ and IκBα bound to the middle region (Figure 4G). Overexpression of either the N-terminal or middle region prevented the association of β-TrCP and IκBα by competing for the binding (Figure 4H), and then impeded UV-induced IκBα degradation (Figure 4I). These results suggest that β-TrCP constitutively associates with IKKβ through hnRNP-U and that IκBα is recruited to the β-TrCP-hnRNP-U-IKKβ complex in which IκBα is subjected to ubiquitination. This scenario was supported by observations that knockdown of hnRNP-U decreased the association of β-TrCP with IKKβ and attenuated UV-induced IκBα degradation (Figure 4J and K).

p38 and CK2 associate with IKKβ and promote IκBα degradation

We next investigated the involvement of CK2 and p38 in the UV response. UV-induced IκBα degradation was delayed in *p38*$^{-/-}$ fibroblasts (Figure 5A). An immunoprecipitation assay of endogenous proteins revealed that UV induced the association of p38 and CK2β (Figure 5B). The IκBα mutant IκBα7MA lacking both the N-terminal and C-terminal phosphorylation sites was degraded more slowly than IκBαAA after UV irradiation (Figure 5C). Thus, C-terminal phosphorylation by the p38-CK2 complex, although not essential, accelerates IκBα degradation. The marked suppression of IκBα degradation in *Ikkβ*$^{-/-}$ fibroblasts (Figure 1A) suggests a possible mechanism that IκBα proteolysis mediated by the p38-CK2 complex is also dependent on IKKβ. Indeed, although the CK2-p38 complex is formed in *wt* and *Ikkβ*$^{-/-}$ fibroblasts, the association of IκBα with p38 and CK2 was not induced in *Ikkβ*$^{-/-}$ fibroblasts (Figure 5D and S3A). Transfection of IKKβKN into *Ikkβ*$^{-/-}$ fibroblasts restored the interaction of IκBα with p38 and CK2, suggesting that IKKβ again acts as an adaptor protein (Figure 5E). IKKβ mediates the interaction between the p38-CK2 complex and β-TrCP (Figure S3B) and may form a complex consisting of β-TrCP, hnRNP-U, IKKβ, p38, CK2, and IκBα. An immunoprecipitation assay showed that p38 and CK2 bound to the N-terminal kinase domain of IKKβ (Figure 5F and G).

p38 and CK2 interactions were dependent on p38 phosphorylation of two essential tyrosine and serine residues in the T-loop, since a p38 mutant in which these phosphorylation sites were substituted with alanine (p38AA) did not associate with CK2, IKKβ, and IκBα (Figure S3C). p38AA failed to promote IκBα degradation (Figure S3D). Nonetheless, UV irradiation induced the association of IκBα with β-TrCP in *p38*$^{-/-}$ fibroblasts, suggesting that p38 promoted IκBα degradation independently of the association of β-TrCP with IκBα

(Figure S3E). BAPTA-AM did not interfere with the binding of p38 and CK2 to IKKβ (Figure S3F).

Nuclear IKKβ promotes cell death in the UV response

NF-κB activation predominantly induces survival functions, although it also promotes UV-induced cell death (Campbell et al., 2004; Kasibhatla et al., 1998; Liu et al., 2006). Consistent with these reports, loss of IKKβ promoted TNFα-induced cell death but suppressed UV-induced death (Figure 6A). IKKβKN and NLS-IKKβ did not suppress TNFα-induced death but instead promoted UV-induced death, suggesting that NF-κB activation by nuclear IKKβ enhances cell death during the UV response (Figure 6B). UV irradiation reportedly induces the association of RelA with histone deacetylase and then suppresses anti-apoptotic genes such as Bcl-xL and X-IAP (Campbell et al., 2004). A real time PCR assay revealed that IKKβKN and NLS-IKKβ, as well as IKKβWT, repressed Bcl-xL and X-IAP expression when cells were irradiated with UV (Figure 6C).

Based on these experimental results, we propose a model of the adaptor function of nuclear IKKβ in UV-induced IκBα degradation. It has been suggested that NF-κB activity is induced by UV-induced translational inhibition by phosphorylation of eIF2 in conjunction with constitutive and UV-induced IκBα degradation (Figure 7A). Following UV irradiation, IκBα translocates into the nucleus with RelA, associates with the pre-existing β-TrCP-hnRNP-U-IKKβ complex and is subjected to ubiquitination (Figure 7B). UV also induces the association of IκBα with the CK2-p38 complex through IKKβ, and then CK2 phosphorylates the C-terminal region. Finally, IκBα is degraded in the cytoplasm, and then NF-κB is translocated to the nucleus and suppresses anti-apoptotic gene expression. Among

the many proteins involved in these reactions, IKKβ is a key factor since it plays an essential role in IκBα degradation.

DISCUSSION

The absolute requirement for IKKβ in UV-induced IκBα degradation appears contradictory to the findings that UV irradiation induced neither IKKβ activity nor IκBα phosphorylation (Huang et al., 2002; Li and Karin, 1998), an IκBαAA mutant was degraded by UV irradiation (Bender et al., 1998; Li and Karin, 1998), a dominant negative IKKβ mutant did not prevent IκBα degradation (Li and Karin, 1998), and β-TrCP associated with IκBα in the absence of IKKβ kinase activation (Huang et al., 2002). However, the above results, which initially appear contradictory, all strongly provide support for a function of IKKβ as an adaptor protein.

Several proteins in the NF-κB signaling pathway have dual functions as kinases and adaptors or regulators. IKKα, an essential kinase in the alternative NF-κB activation pathway, functions as a transcriptional regulator in keratinocyte and epidermal differentiation (Descargues et al., 2008). Receptor-interacting protein kinase 1 (RIP1) functions as a kinase in TNFα-induced programmed necrosis (Cho et al., 2009; He et al., 2009); however, its kinase activity is not required for NF-κB activation (Festjens et al., 2007; Meylan and Tschopp, 2005). Interleukin-1 (IL-1) receptor-activated kinase 1 and 4 also have two functions, as adaptor proteins transducing signals and as kinases (Janssens and Beyaert, 2003). Likewise, IKKβ has two functions, as an adaptor protein in the UV response and as a kinase in cytokine signaling.

An adaptor function of IKKβ in IκBα ubiquitination depends on the interaction

with hnRNP-U which associates with β-TrCP. hnRNP-U was originally described as a component of the nuclear matrix and was thought to mediate RNA processing. Furthermore, hnRNP-U regulates transcription by association with RNA polymerase II through binding at its N-terminal domain (Obrdlik et al., 2008). Thus, hnRNP-U is a multifunctional protein that mediates gene expression through interactions with several proteins including IKKβ and β-TrCP.

CK2 is a nuclear-matrix-associated ubiquitous serine/threonine kinase and has been regarded as a constitutive, non-regulated protein kinase. Recent studies, however, revealed that CK2 is activated by UV radiation and osmotic stress in a p38-dependent manner and regulates the stress response (Kato et al., 2003; Sayed et al., 2000; Scaglioni et al., 2006). As shown in the present study, the CK2-p38 complex promoted IκBα degradation through phosphorylation at the C-terminal region during the UV response mediated by IKKβ. It should be noted, however, that IκBα was gradually degraded in UV-irradiated *p38*$^{-/-}$ fibroblasts, in contrast to the complete suppression of IκBα degradation in *Ikkβ*$^{-/-}$ fibroblasts (compare Figures 1A and 5A). Therefore, IKKβ is prerequisite and critical, whereas CK2 and p38 are not absolute requirements.

Nuclear IKKβ might be important for synergistic crosstalk between oxidative stress and inflammatory signals. Stimulation of cells with IL-1 or lipopolysaccharide results in NF-κB activation via IκBα degradation, and then NF-κB is rapidly down-regulated by the negative feedback loop in which NF-κB induces IκBα gene expression. Alternatively, concomitant treatment of cells with UV irradiation and inflammatory reagents enhances NF-κB activation via accelerated IκBα degradation (Bender et al., 1998; O'Dea et al., 2008). It is likely that, under UV stress, resynthesized IκBα associates with nuclear IKKβ and is

then degraded. Importantly, IκBα translocates into the nucleus and associates with IKKβ when cells are subjected not only to UV stress but also to other types of oxidative stress (data not shown). Inflammatory signals, such as TNFα, potentially generate reactive oxygen species in cells and increase oxidative stress (Kamata et al., 2005; Sakon et al., 2003). It is plausible that nuclear IKKβ and cytoplasmic IKKβ have a synergistic effect under oxidative stress in inflammatory diseases. We suggest that nuclear IKKβ is a target for therapeutic intervention in inflammatory disease.

EXPERIMENTAL PROCEDURES

Plasmids and cell culture

β-TrCP1, p38α, IκBα, and CK2β cDNA were amplified from a human cDNA library by polymerase chain reaction (PCR). The plasmid encoding HA-ubiquitin was provided by Dr. Zhijian Chen, and the plasmid encoding hnRNP-U was provided by Dr. Yinon Ben-Neriah. IκBαAA and IκBα7MA were described previously (Kato et al., 2003). Substitution mutants and deletion mutants of IKKβ, IκBα, p38, β-TrCP, and hnRNP-U were generated by PCR. The IKKβKN mutant in which the conserved lysine44 was changed to alanine was provided by Dr. David V. Goeddel. β-TrCPΔF was generated by deletion of the F-box amino acid sequence (188-229) from β-TrCP cDNA. p38AA was generated by exchange of Thr180 and Tyr182 to alanines.

The cDNA was inserted into pRK-HA, pRK-Flag, or pRSGFP expression vectors. NES- or NLS-fused IKKβ and IκBα genes were prepared by insertion of oligonucleotides encoding NES (GSLALKLAGLDIS) or NLS (GSKKKRKVRSR) to the N-terminal site of each gene. Plasmids were transfected into fibroblasts cultured in Opti-MEM (Invitrogen)

using Lipofectamine Plus (Invitrogen) following the manufacturer's instructions. Immortalized fibroblasts derived from *Ikkβ*$^{-/-}$, *RelA*$^{-/-}$, *IκBα*$^{-/-}$, and *β-TrCP1*$^{-/-}$ mouse embryos were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin. For UV irradiation as well as for mock treatment, the growth medium was aspirated and the cell layer was covered with a small amount of phosphate buffered saline (PBS) and subjected to UV irradiation with CL1000 Ultraviolet Crosslinker (UVP). After completion of treatment, growth medium was replenished.

RNAi

Double stranded stealth siRNAs for mouse hnRNP-U were prepared by Invitrogen as follows: 5'-UAUUAUAUCCGCCACGAUUCCCAGG-3', 5'-UGUUUGAGUAACUACCACGGCCAGG-3', 5'-AAAUAUCCACGGCCAUGAUCUUCUC-3'. A pool of three stealth siRNAs or control siRNAs (Invitrogen) were transfected using Oligofectamine (Invitrogen). At 48 h after transfection, protein interactions were analyzed by immunoprecipitation. Alternatively, cells were incubated in the presence of CHX following UV irradiation to analyze IκBα degradation.

Adenovirus transduction

Recombinant adenoviruses for GFP, GFP-IKKβ, GFP-IKKβKN, GFP-IKKβ-NLS and GFP-IKKβ-NES were prepared using the Adenovirus Expression Vector kit (Dual Version) Ver.2 (Takara), and amplified using HEK293T cells. NES- or NLS-IKKβ were

prepared by insertion of oligonucleotides encoding NES or NLS between the coding sequences of GFP and IKKβ.

Immunoprecipitation and immunoblotting

Cells were washed with PBS and solubilized with buffer A consisting of 20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 10 mM EGTA, 10 mM $MgCl_2$, 60 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM 4-amidino phenyl methyl sulfonyl fluoride, 50 KIU/ml aprotinin, 20 μg/ml pepstatin, 20 μg/ml leupeptin, 2 mM dithiothreitol and 1% Triton X-100. After centrifugation at 16,000 x *g* for 20 min at 4^{o}C, the supernatants were used as cell lysates. For the ubiquitination assay, cells were solubilized with buffer A containing 20 mM N-ethylmaleimide. Nuclear and cytoplasmic fractions were prepared using the Nuclear/Cytosol Fraction kit (BioVision). For the immunoprecipitation assay of transfected cells, cell lysates were incubated with an antibody together with Protein A and Protein G Sepharose (GE Healthcare) or with anti-Flag (M2) Sepharose (Sigma) at 4^{o}C, and subjected to immunoblotting using HRP-conjugated anti-mouse or anti-rabbit IgG antibodies (GE Healthcare). For the immunoprecipitation assay of endogenous proteins, cell lysates were incubated with an antibody together with TrueBlot^{TM} anti-mouse or anti-rabbit IP beads (eBioscience) and subjected to immunoblotting using TrueBlot^{TM} HRP-conjugated anti-mouse or anti-rabbit IgG antibodies (eBioscience). Gel separated proteins were transferred to polyvinylidene difluoride membranes (Millipore) with an electroblotting apparatus (Mighty Small Transphor; Amersham) and subjected to immunoblotting using the SuperSignal West Pico Chemiluminescence System (Pierce). Anti-IκBα (C-21), anti-p38 (C-20), anti-β-TrCP (H-85), anti-IKKα/β (H-470), anti-CK2β (FL-215), anti-RelA (C-20),

anti-tubulin (H-300), anti-HA (Y-11) rabbit antibodies, and anti-IκBα (H-4) and anti-hnRNP-U (3G6) mouse antibodies were obtained from Santa Cruz Biotechnology. Anti-IKKβ rabbit antibody was obtained from Cell Signaling. Anti-IKKα (14A431) mouse antibody was obtained form Imgenex. Anti-IKKβ mouse antibody was obtained from Upstate Biotechnology. Anti-Flag (M2) mouse antibody was obtained from Sigma. Anti-HA mouse antibody was obtained from Roche. Anti-CK2β mouse antibody was obtained from BD Bioscience. Anti-GFP antibody was prepared from a rabbit immunized with bacterially expressed GFP protein.

EMSA

Nuclear cell extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce). Biotin-labeled oligomers containing the NF-κB-binding site sequence (GGATCCTCAACAGAGGGGACTTTCCGAGGCCA) and the NF-Y-binding site sequence (TTTTCTGATTGGTTAAAA) were prepared using the Biotin3 End DNA Labeling kit (Pierce) following the manufacturer's instructions. Probes were incubated with nuclear extracts for 20 min at room temperature. Following electrophoresis through 6% non-denaturing polyacrylamide gels, detection was performed using the LightShift Chemiluminescent EMSA kit (Pierce) according to the manufacturer's instructions.

Immunofluorescent microscopy

Cells were cultured on sterile glass coverslips in six-well plates and fixed with PBS containing 3.7% paraformaldehyde for 15 min at room temperature. Cells were permeabilized with PBS containing 0.2% Triton X-100. After incubating with the antibodies, the cells were

washed with PBS and incubated with secondary fluorescein isothiocyanate-conjugated anti-rabbit IgG antibody or Cy3-conjugated anti-mouse antibody (Jackson ImmunoResearch). Cells were mounted in GEL/MOUNT (Biomeda), and immunostaining images were analyzed using a Carl Zeiss inverted laser scanning confocal microscope LSM 510.

Cell death

Cell death was analyzed by dye exclusion assay. Cells were stained with 1 μg/ml propidium iodide and Hoechst 33342, and cell death was analyzed using a Carl Zeiss inverted microscope Axio Obsserber Z1.

RT-PCR analysis

Total RNA was extracted from cells using Trizol (Invitrogen) according to the manufacturer's instructions. RNA (5 μg) was converted to cDNA with the SuperScript™ III First-Strand Synthesis System (Invitrogen). The following oligonucleotides were used for each gene: Bcl-xL forward, 5'-CAAGGAGATGCAGGTATTG-3'; Bcl-xL reverse, 5'-CCTCCTTGCCTTTCCGG-3'; X-IAP forward, 5'-CAAGGAGATGCAGGTATTG-3'; X-IAP reverse, 5'-CCTCCTTGCCTTTCCGG-3'; CPH forward, 5'-ATGGTCAACCCCACCGTGT-3'; CPH reverse, 5'-TTCTTGCTGTCTTTGGAACTTTGTC-3'. Gene expression was analyzed by quantitative real-time PCR using CYBR Premix EX Taq (Takara). PCR amplifications were performed in Opticon Monitor 3 (BioRad) using 39 cycles of denaturation at 95^{o}C for 5 s, and annealing and elongation at 60^{o}C for 30 s.

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FIGURE LEGENDS

Figure 1. IKKβ acts as an adaptor protein for recruiting β-TrCP to IκBα in UV-induced NF-κB activation

(A) UV irradiation induces IκBα degradation in an IKKβ-dependent manner. Fibroblasts were irradiated with UVC (100 J/m^2) and incubated in the presence of 10 μg/ml CHX to monitor IκBα degradation. IκBα in cell lysates was analyzed by immunoblotting

(B) Kinase-negative IKKβ mutant (IKKβKN) induces IκBα degradation. *Ikkβ*$^{-/-}$ fibroblasts were transfected with IKKβWT or IKKβKN, and incubated in the presence of CHX following UV irradiation.

(C) Phosphorylation of IκBα at the N-terminal sites is not necessary for UV-induced degradation. *IκBα*$^{-/-}$ fibroblasts were transfected with HA-tagged IκBα mutants and incubated in the presence of CHX following UV irradiation.

(D) UV induces IκBα ubiquitination in an IKKβ-dependent manner. Following transfection with HA-ubiquitin, fibroblasts were irradiated with UV and incubated in the presence of 50 μM MG132 to prevent IκBα degradation. Lysates were immunoprecipitated with anti-IκBα antibody and subjected to immunoblotting.

(E) β-TrCP1 is involved in IκBα degradation. *Wt* and *β-TrCP1*$^{-/-}$ fibroblasts were irradiated with UV and incubated for the indicated periods in the presence of CHX.

(F) UV-irradiation induces the association of IκBα with β-TrCP in an IKKβ-dependent manner. Following transfection with the indicated plasmids, *wt* and *Ikkβ*$^{-/-}$ fibroblasts were irradiated with UV and incubated for 2 h in the presence of MG132. Lysates were immunoprecipitated with anti-FLAG antibody and subjected to immunoblotting with anti-HA antibody.

(G) Kinase activity is not required for association of IκBα with β-TrCP. *Ikkβ*$^{-/-}$ fibroblasts were transfected with the indicated plasmids, and the association of IκBα and β-TrCP was analyzed by the immunoprecipitation assay.

(H) Association of endogenous IKKβ with IκBα and β-TrCP. *Wt* fibroblasts were irradiated with UV and incubated for 2 h in the presence of MG132. Association of endogenous proteins was analyzed by the immunoprecipitation assay.

(I) Schematic representation of IKKβ mutants used in this study. IKKβ is composed of an N-terminal kinase domain and a C-terminal regulatory region consisting of a ubiquitin-like (UBL) domain, a leucine zipper (LZ) domain, a helix-loop-helix (HLH) domain, and an NF-κB essential modulator (NEMO)-binding (NBD) domain.

(J and **K)** Constitutive association of IKKβ with β-TrCP through the N-terminal region (J) and UV-induced association with IκBα through the C-terminal region (K). Following transfection with the indicated plasmids into *Ikkβ*$^{-/-}$ fibroblasts, cells were irradiated with UV and incubated for 2 h in the presence of MG132.

Figure 2. IκBα associates with IKKβ through the ankyrin repeat domain and is degraded by UV irradiation

(A) Schematic representation of IκBα mutants used in this study.

(B) UV induces the association of IκBα with IKKβ through the ankyrin repeat domain. Following transfection of the indicated plasmids, *Ikkβ*$^{-/-}$ fibroblasts were incubated in the presence of MG132 for 2 h after UV irradiation.

(C) UV induces the association of IκBα with β-TrCP through the ankyrin repeat domain.

Following transfection of the indicated plasmids, *wt* fibroblasts were incubated in the presence of MG132 for 2 h after UV irradiation.

(D) Overexpression of the ankyrin repeat domain prevents the association of IκBα with β-TrCP. Following transfection of the indicated plasmids, *wt* fibroblasts were incubated in the presence of MG132 for 2 h after UV irradiation.

(E) The ankyrin repeat domain acts as a dominant negative mutant for UV-induced IκBα degradation. *Wt* fibroblasts were transfected with the indicated plasmids, and then were irradiated with UB. Cells were incubated in the presence of CHX.

Figure 3. UV irradiation induces nuclear translocation of IκBα and association with the IKKβ complex

(A) LMB prevents UV-induced IκBα degradation. Fibroblasts were incubated with CHX in the presence or absence of 15 ng/ml LMB or MG132.

(B) LMB prevents UV-induced NF-κB activation. NF-κB activity was analyzed by EMSA after 8 h UV irradiation.

(C) LMB does not inhibit IκBα ubiquitination. Following transfection with HA-ubiquitin, fibroblasts were irradiated with UV and incubated for 2 h in the presence of MG132.

(D) UV induces the nuclear translocation of IκBα and RelA. After UV irradiation, *wt* fibroblasts were incubated in the presence or absence of LMB or MG132.

(E) Nuclear accumulation of undegraded IκBα in *Ikkβ*$^{-/-}$ fibroblasts. After UV irradiation, *Ikkβ*$^{-/-}$ fibroblasts were incubated for the indicated periods.

(F) Nucleocytoplasmic shuttling is crucial for IκBα degradation. Fibroblasts were transfected with HA-IκBα, HA-NLS-IκBα, and HA-NES-IκBα, and were incubated with CHX after UV

irradiation.

(G) Nuclear translocation is crucial for IκBα ubiquitination. Following transfection with HA-IκBα, HA-NLS-IκBα, and HA-NES-IκBα together with Flag-ubiquitin, fibroblasts were irradiated with UV and incubated for 2 h in the presence or absence of MG132.

(H) IκBα associates with IKKβ and β-TrCP in the nucleus. Following UV irradiation, *wt* fibroblasts were incubated for 2 h with MG132 and then were fractionated into cytosolic and nuclear subfractions. Nuclear translocation of IκBα and association with IKKβ were analyzed by the immunoprecipitation assay.

(I) Nuclear IKKβ mediates UV-induced IκBα degradation. *Ikkβ*$^{-/-}$ fibroblasts were transfected with GFP-IKKβ, GFP-NES-IKKβ, and GFP-NLS-IKKβ and were incubated with CHX after UV irradiation or TNFα stimulation.

(J) IKKβ associates with β-TrCP in the nucleus. *Ikkβ*$^{-/-}$ fibroblasts were transfected with the indicated plasmids and incubated for 2 h in the presence of MG132 following UV irradiation.

(K) Ca^{2+} is required for the nuclear translocation of IκBα and RelA. *Wt* fibroblasts were incubated for 2 h after UV irradiation in the presence or absence of 20 μM BAPTA-AM or with 1 μM ionomycin.

(L) Ca^{2+} is required for IκBα degradation. *Wt* fibroblasts were incubated for the indicated periods after UV irradiation with CHX in the presence or absence of 20 μM BAPTA-AM.

Figure 4. hnRNP-U mediates the association of β-TrCP with IKKβ

(A) hnRNP-U constitutively binds to IKKβ. *Ikkβ*$^{-/-}$ fibroblasts were transfected with plasmids encoding hnRNP-U and IKKβ, and irradiated with UV. After 2 h incubation, the association of these two components was analyzed by immunoprecipitation assay.

(B) UV induces the association of endogenous IκBα, hnRNP-U and IKKβ. After UV irradiation, *wt* fibroblasts were incubated for 2 h in the presence of MG132, and the association of proteins was analyzed by immunoprecipitation assay.

(C) UV induces the association of IκBα with hnRNP-U in an IKKβ-dependent manner. *Wt* and *Ikkβ*$^{-/-}$ fibroblasts were transfected with the indicated plasmids and irradiated with UV. After 2 h incubation in the presence of MG132, the association of proteins was analyzed by immunoprecipitation assay.

(D) hnRNP-U binds to the N-terminal region of IKKβ. Following transfection of the indicated plasmids, the association of proteins was analyzed in *Ikkβ*$^{-/-}$ fibroblasts.

(E) Nuclear IKKβ preferentially binds to hnRNP-U. *Ikkβ*$^{-/-}$ fibroblasts were transfected with the indicated plasmids, and the association of IKKβ and hnRNP-U was analyzed by immunoprecipitation assay.

(F) Schematic representation of hnRNP-U mutants used in this study.

(G) IKKβ and IκBα bind to the middle region of hnRNP-U, and β-TrCP binds to the N-terminal region. *Ikkβ*$^{-/-}$ fibroblasts were transfected with the indicated plasmids. After UV irradiation, cells were incubated for 2 h in the presence of MG132, and the association of proteins was analyzed by the immunoprecipitation assay.

(H) Overexpression of the N-terminal and middle regions of hnRNP-U competes with the association of β-TrCP and IKKβ. *Ikkβ*$^{-/-}$ fibroblasts were transfected with the indicated plasmids, and the association of proteins was analyzed by immunoprecipitation assay.

(I) The N-terminal and middle regions of hnRNP-U prevent UV-induced IκBα degradation. *Wt* fibroblasts were transfected with the indicated plasmids. After UV irradiation, cells were incubated for the indicated periods in the presence of CHX to monitor IκBα degradation.

(J) Knockdown of hnRNP-U interferes with the association of IKKβ with β-TrCP but not with IκBα. HeLa cells were transfected with siRNA, and irradiated with UV. After 2 h incubation in the presence of MG132, the association of proteins was analyzed by immunoprecipitation assay.

(K) Knockdown of hnRNP-U attenuates UV-induced IκBα degradation. HeLa cells were transfected with siRNA, and irradiated with UV. After UV irradiation, cells were incubated for the indicated periods in the presence of CHX to monitor IκBα degradation.

Figure 5. UV induces the association of the p38-CK2 complex with IKKβ and promotes IκBα degradation

(A) UV-induced IκBα degradation is attenuated in *p38*$^{-/-}$ fibroblasts. *Wt* and p38$^{-/-}$ fibroblasts were incubated for the indicated periods after UV irradiation in the presence of CHX.

(B) UV induces the association of endogenous IκBα with CK2 and p38. *Wt* fibroblasts were incubated for 2 h after UV irradiation in the presence of MG132. Association of proteins was analyzed by immunoprecipitation assay.

(C) The C-terminal CK2 phosphorylation sites are involved in UV-induced IκBα degradation. *IκBα*$^{-/-}$ fibroblasts were transfected with IκBα mutants and incubated for the indicated periods after UV irradiation in the presence of CHX.

(D) UV induces the association of endogenous IκBα with p38 and CK2 in an IKKβ-dependent manner. *Wt* and *Ikkβ*$^{-/-}$ fibroblasts were irradiated with UV and incubated for 2 h in the presence of MG132.

(E) IKKβ mediates the association of IκBα with p38 and CK2 in a kinase activity-independent manner. *Ikkβ*$^{-/-}$ fibroblasts were transfected with the indicated plasmids.

After UV irradiation, cells were incubated for 2 h in the presence of MG132, and the association of proteins was analyzed by the immunoprecipitation assay.

(F and **G)** UV induces the association of IKKβ with p38 (F) and CK2β (G) through the N-terminal region. *Ikkβ*$^{-/-}$ fibroblasts were transfected with the indicated plasmids and incubated in the presence of MG132 for 2 h after UV irradiation.

Figure 6. Nuclear IKKβ promotes UV-induced cell death

(A) Loss of IKKβ promotes TNFα-induced death but attenuates UV-induced death. *Wt* and *Ikkβ*$^{-/-}$ fibroblasts were treated with TNFα for 12 h or incubated for 36 h after UV irradiation, respectively. Cell death was analyzed by dye exclusion assay with 1 μg/ml propidium iodide.

(B) Nuclear IKKβ promotes UV-induced death in a kinase activity-independent manner. *Ikkβ*$^{-/-}$ fibroblasts were infected with adenoviruses expressing GFP, GFP-IKKβWT, GFP-IKKβKN or GFP-NLS-IKKβ, and were treated with TNFα for 12 h or incubated for 36 h after UV irradiation.

(C) UV irradiation suppresses anti-apoptotic gene expression through nuclear IKKβ in a kinase activity-independent manner. *Ikkβ*$^{-/-}$ fibroblasts were infected with the indicated adenoviruses and incubated for 12 h after UV irradiation. Gene expression was analyzed by RT-PCR. The results are presented as means $\pm$ standard errors and represent three independent experiments. Statistical significance was determined by the Student's *t* test.

Figure 7. Theoretical model of UV-induced NF-κB activation

(A) UV irradiation induces NF-κB activity through several mechanisms including translational inhibition and IκBα degradation.

(B) UV irradiation induces IκBα degradation through nuclear IKKβ adaptor protein and NF-κB-mediated suppression of anti-apoptotic gene expression.

Fig. 1

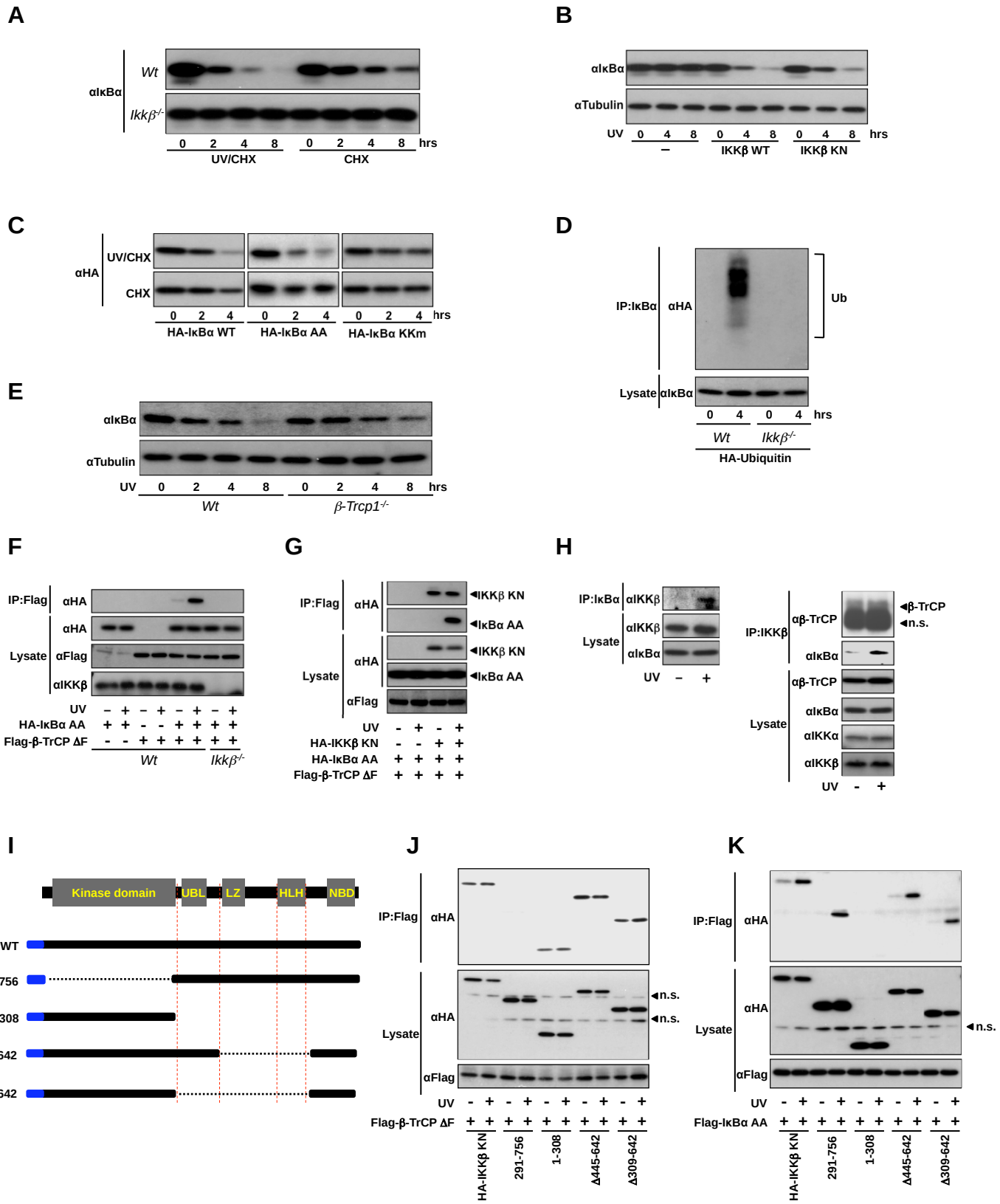
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Fig. 2

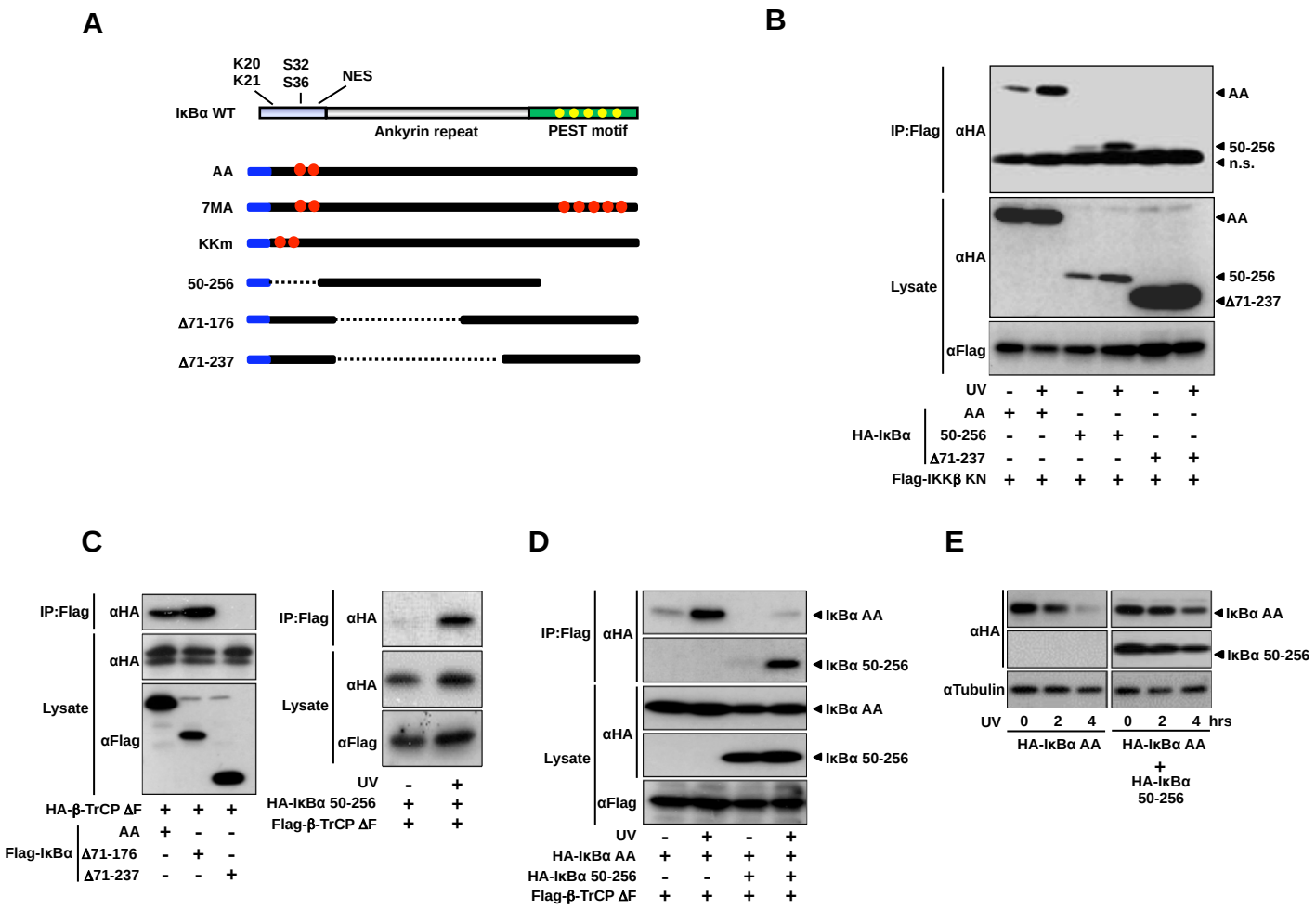
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Fig. 3

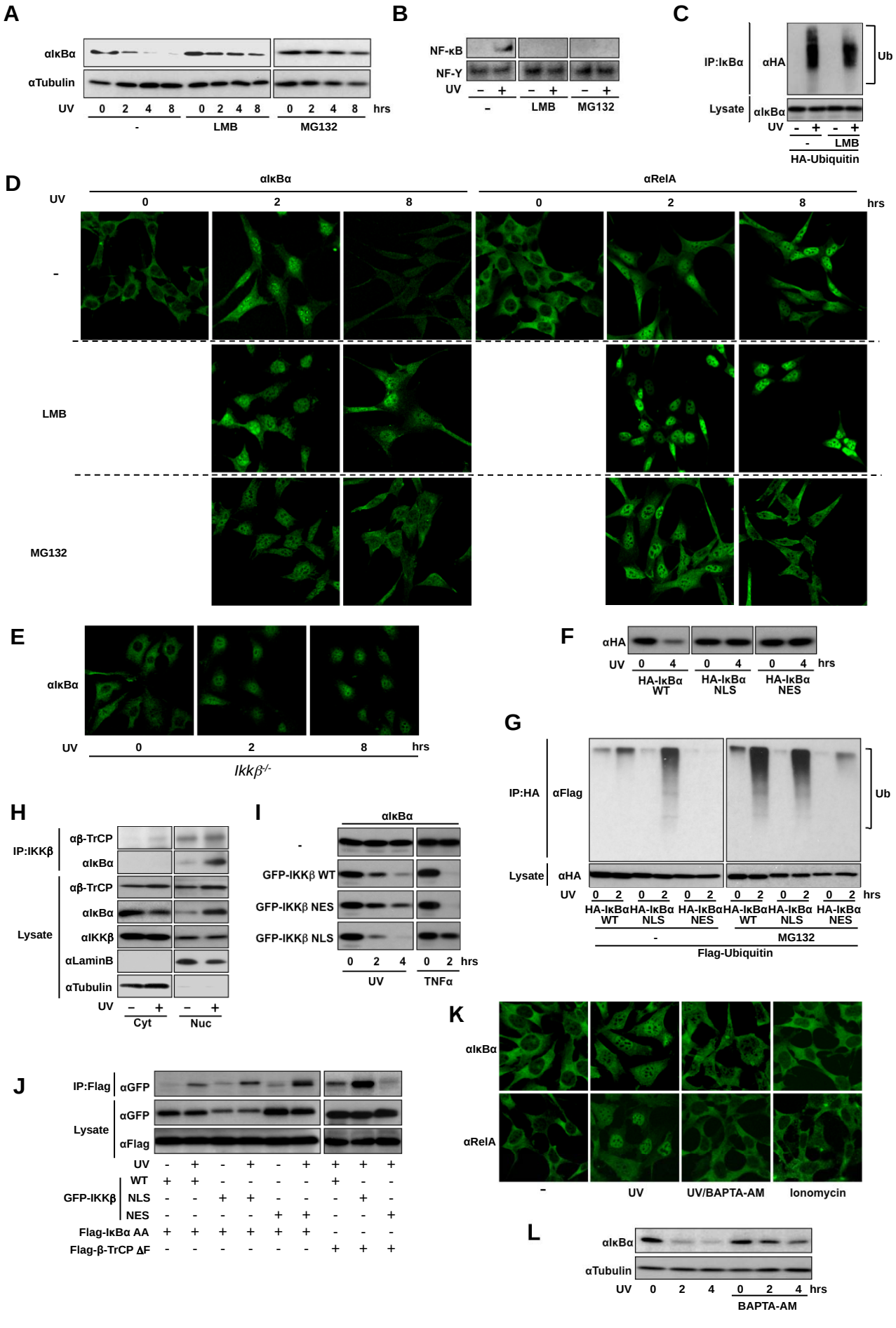
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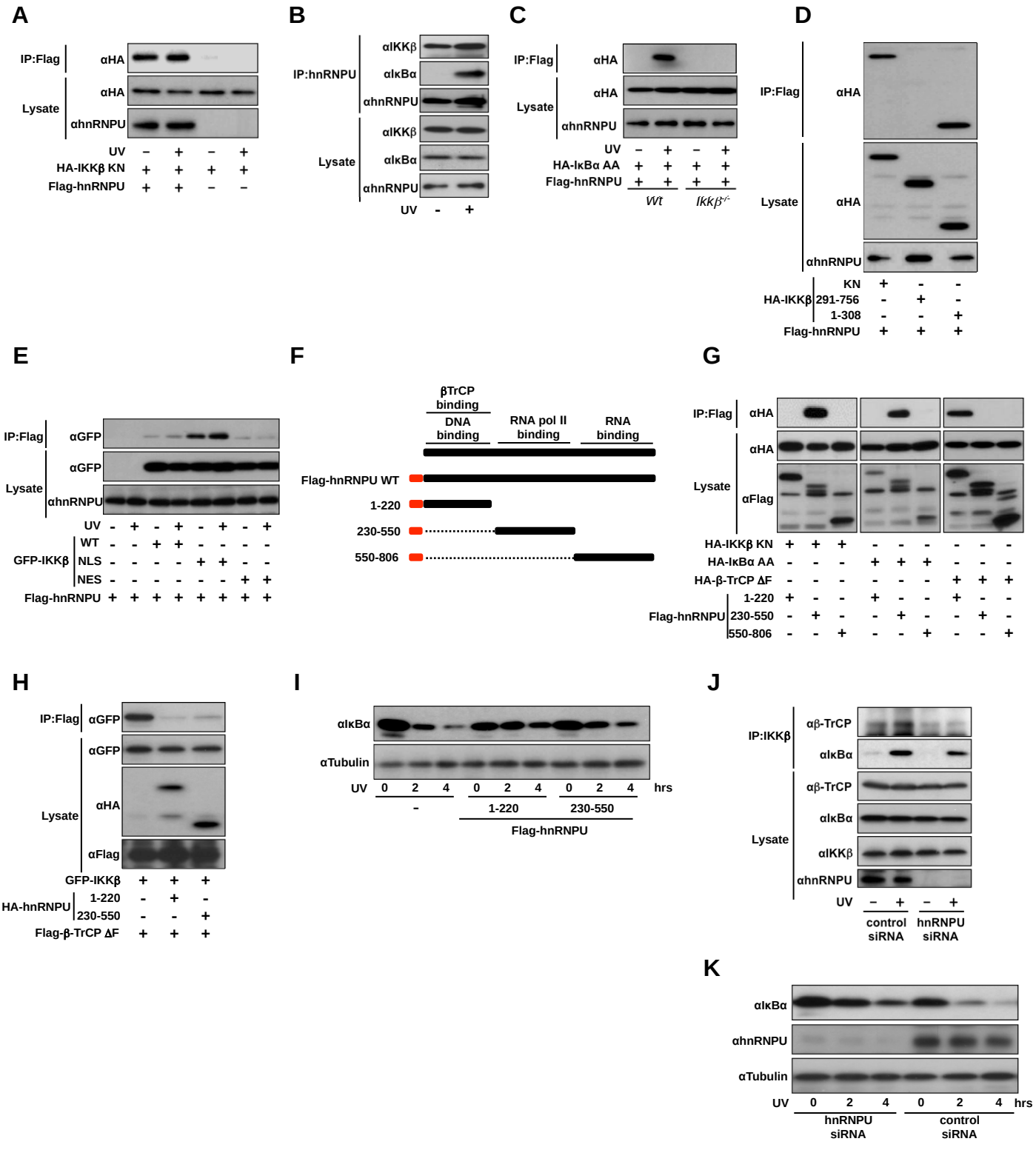
Fig. 4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K
αIκBα
αhnRNPU
αTubulin
UV
0 2 4 0 2 4 hrs
hnRNPU siRNA
control siRNA

Fig. 5

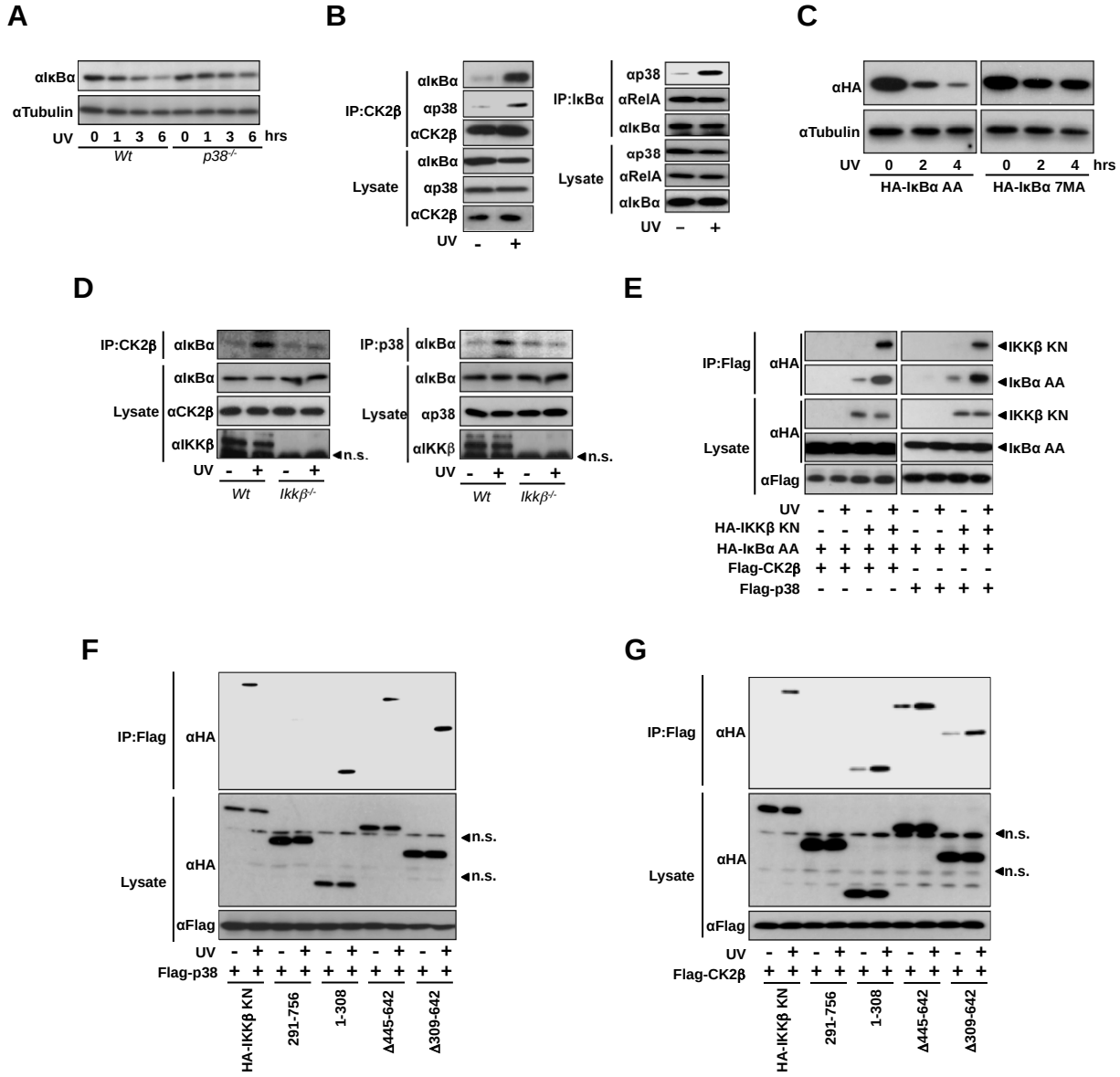

A
αIκBα
αTubulin
UV 0 1 3 6 0 1 3 6 hrs
Wt
p38-/-
B
IP:CK2β
αIκBα
αp38
αCK2β
Lysate
αIκBα
αp38
αCK2β
UV - +
IP:IκBα
αp38
αRelA
αIκBα
Lysate
αp38
αRelA
αIκBα
UV – +
C
αHA
αTubulin
UV 0 2 4 0 2 4 hrs
HA-IκBα AA
HA-IκBα 7MA
D
IP:CK2β
αIκBα
Lysate
αIκBα
αCK2β
αIKKβ
n.s.
UV - + - +
Wt
Ikkβ-/-
IP:p38
αIκBα
Lysate
αIκBα
αp38
αIKKβ
n.s.
UV - + - +
Wt
Ikkβ-/-
E
IP:Flag
αHA
IKKβ KN
IκBα AA
Lysate
αHA
IKKβ KN
IκBα AA
αFlag
UV - + - + - + - +
HA-IKKβ KN - - + + - - + +
HA-IκBα AA + + + + + + + +
Flag-CK2β + + + + - - - -
Flag-p38 - - - - + + + +
F
IP:Flag
αHA
Lysate
αHA
n.s.
n.s.
αFlag
UV - + - + - + - + - +
Flag-p38 + + + + + + + + + +
HA-IKKβ KN
291-756
1-308
Δ445-642
Δ309-642
G
IP:Flag
αHA
Lysate
αHA
n.s.
n.s.
αFlag
UV - + - + - + - + - +
Flag-CK2β + + + + + + + + + +
HA-IKKβ KN
291-756
1-308
Δ445-642
Δ309-642

Fig. 6

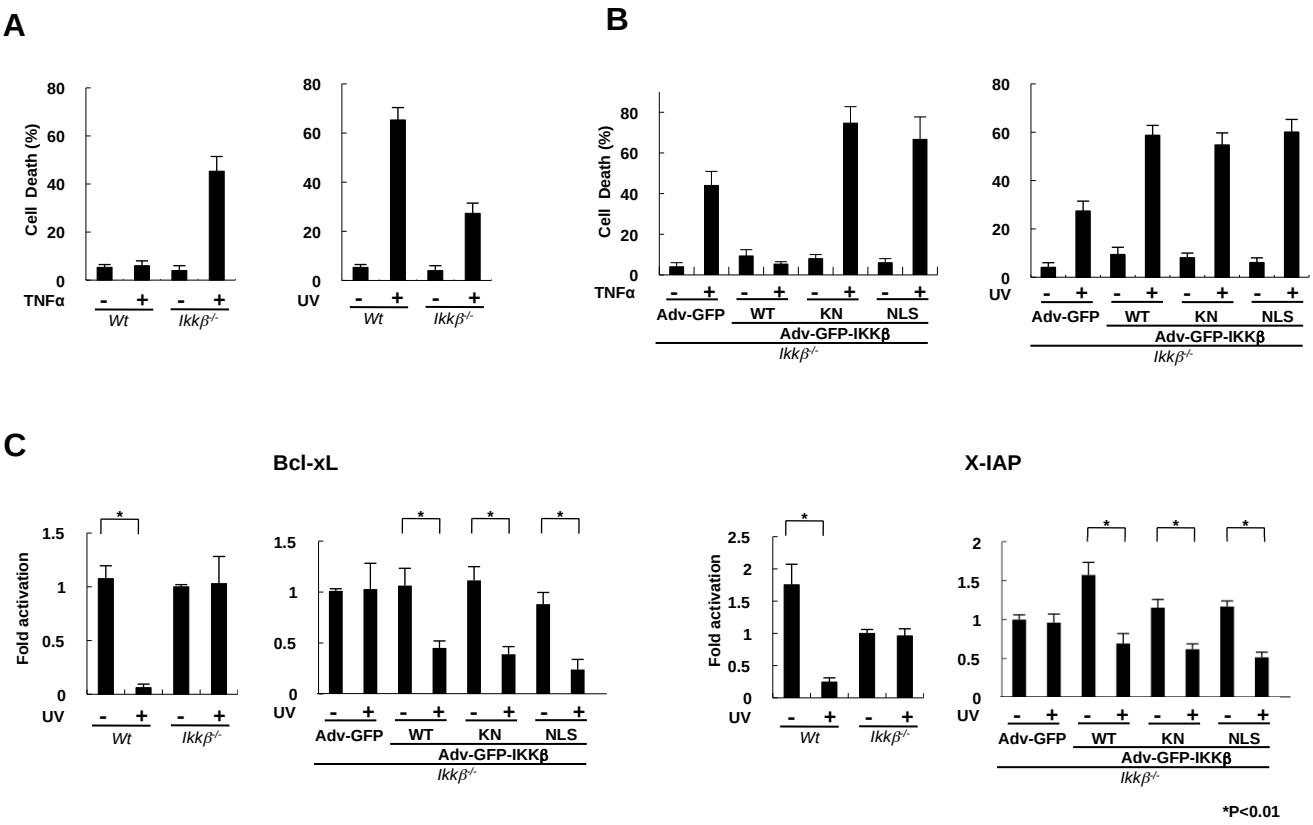
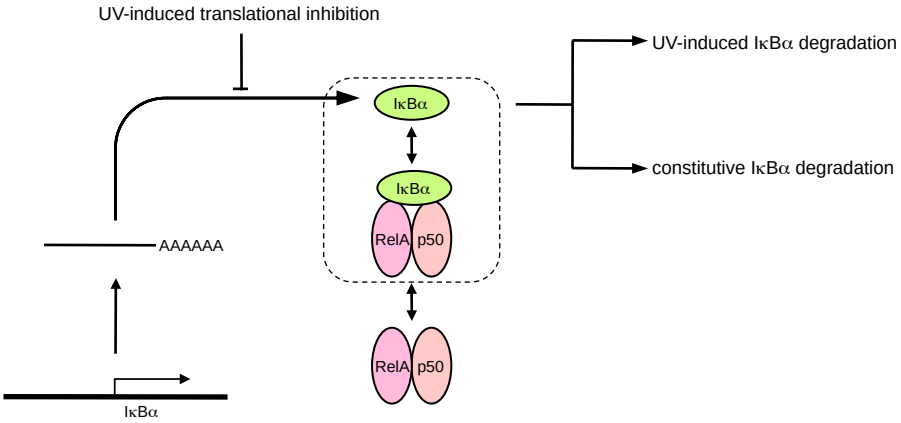
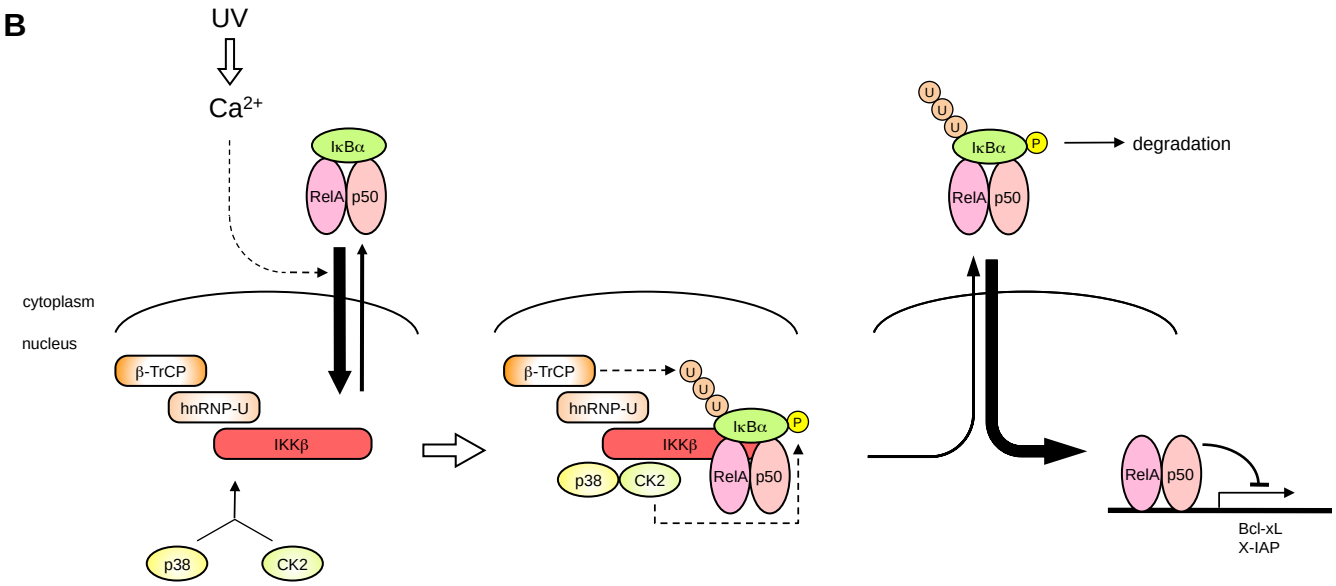

A
Cell Death (%)
80
60
40
20
0
TNFα
UV
- + - +
Wt
Ikkβ-/-
B
Cell Death (%)
TNFα
UV
Adv-GFP
WT
KN
NLS
Adv-GFP-IKKβ
Ikkβ-/-
C
Bcl-xL
X-IAP
Fold activation
1.5
1
0.5
0
2.5
2
*
*P<0.01

Fig. 7

A


UV-induced translational inhibition
UV-induced IκBα degradation
constitutive IκBα degradation
IκBα
IκBα
RelA
p50
AAAAAA
RelA
p50
IκBα

B


UV
Ca2+
IκBα
RelA
p50
cytoplasm
nucleus
β-TrCP
hnRNP-U
IKKβ
p38
CK2
U
U
U
IκBα
P
degradation
RelA
p50
Bcl-xL
X-IAP