

RESEARCH ARTICLE

# Structural instability of IκB kinase β promotes autophagic degradation through enhancement of Keap1 binding

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**Citation:** Kanamoto M, Tsuchiya Y, Nakao Y, Suzuki T, Motohashi H, Yamamoto M, et al. (2018) Structural instability of IκB kinase β promotes autophagic degradation through enhancement of Keap1 binding. *PLoS ONE* 13(11): e0203978. <https://doi.org/10.1371/journal.pone.0203978>

**Editor:** Wenhui Hu, Lewis Katz School of Medicine at Temple University, UNITED STATES

**Received:** August 28, 2018

**Accepted:** November 10, 2018

**Published:** November 30, 2018

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**Data Availability Statement:** All relevant data are within the manuscript and figures.

**Funding:** H.K. was supported, in whole or in part, by KAKEN Grants-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (#15H01406, # 15H01382, and #23370062), <https://kaken.nii.ac.jp/en/search/>.

**Competing interests:** The authors have declared that no competing interests exist.

## Abstract

IKKβ, an essential kinase of NF-κB signaling, is composed of an N-terminal kinase domain (KD) and a C-terminal scaffolding domain, containing a ubiquitin-like domain (ULD). The Hsp90 chaperon has special responsibility for folding of protein kinases including IKKβ. Here, we found that Hsp90 inhibition induced IKKβ degradation, which is partially mediated by Keap1. Geldanamycin (GA), a Hsp90 inhibitor, enhances association of IKKβ with Keap1 through the binding site in KD, and translocates IKKβ to detergent-insoluble fractions leading to its autophagic degradation. An electrophile tBHQ suppressed Keap1-mediated proteasomal Nrf2 degradation but not autophagic IKKβ degradation. Substitution mutation of Leu353 to Ala in the ULD destabilizes IKKβ, enhances its association with Keap1, translocates it to detergent-insoluble fractions, and causes its autophagic degradation. These results suggest that Keap1 is involved in the degradation of structural destabilized IKKβ and negative regulation of NF-κB under proteotoxic stress.

## Introduction

NF-κB is a critical transcription factor, regulating oxidative stress response, inflammation and carcinogenesis [1, 2]. In resting cells, NF-κB is inactivated by association with inhibitory proteins such as IκBα that prevent the DNA binding and nuclear transport. A vast array of stimuli including inflammatory cytokines and cellular stress activate NF-κB through the IκB kinase (IKK) complex, which consists of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit NEMO/IKKγ [3–6]. While IKKβ mediates activation of the canonical NF-κB pathway in response to pro-inflammatory stimuli, IKKα has an indispensable role in non-canonical NF-κB signaling by phosphorylating NF-κB2 [7]. IKKβ is composed of kinase domain (KD), ubiquitin-like domain (ULD), scaffold/dimerization domain (SDD), and NEMO-binding domain (NBD). Crystal structure analysis showed that IKKβ is activated by trans auto-

phosphorylation of Ser171 and Ser181 in the activation loop of IKK $\beta$  through higher order oligomerization [8, 9]. IKK $\beta$  phosphorylates I $\kappa$ B $\alpha$  at N-terminal serines, Ser32 and Ser36, which leads to its ubiquitination and proteasomal degradation. These reactions result in nuclear translocation of NF- $\kappa$ B and binding of its cognate  $\kappa$ B sites in the promoters of target genes. The NF- $\kappa$ B signaling pathways are associated with many human diseases, including inflammatory disease and cancer, which suggests that IKK $\beta$  can be a potential therapeutic target [10].

Maintenance of proteome integrity and protein homeostasis depend on a complex network of molecular chaperones, including conserved heat shock proteins (HSPs) [11, 12]. Environmental insults perturb cellular proteome homeostasis, triggering the proteotoxic stress response, or heat shock response, that includes the induction of expression genes encoding HSPs. HSPs are classified into several groups based on their molecular weight. Hsp90, Hsp70, and Hsp60, refer to families of HSPs on the order of 90, 70, and 60 kilodaltons in size, respectively. Hsp90, one of the most conserved HSPs, is essential not only in the protective heat shock response but also due to its chaperoning function in folding client proteins to their active conformations [13]. The Hsp90 chaperon is responsible for folding of protein kinases, and Hsp90 inhibitors such as geldanamycin (GA), stimulate kinase degradation [14]. It has been reported that Hsp90 is essential for IKK $\beta$  activity [15, 16], and Hsp90 inhibition results in IKK $\beta$  degradation [15–17]. Degradation of IKK $\beta$  is mediated by two major pathways that degrade cellular proteins in cells: the ubiquitin–proteasome system (UPS) and autophagy [17–19].

Keap1 is a ubiquitin ligase which interacts with a transcription factor Nrf2, a master regulator of the antioxidant response [20, 21]. Under quiescent conditions, Nrf2 is anchored in the cytoplasm by binding to Keap1, which in turn facilitates its proteolysis by UPS [22]. Oxidative stress and electrophiles disrupt the Keap1–Nrf2 complex, dissociating Nrf2 from Keap1 and translocating it into the nucleus where it can act as a transcription factor for series of antioxidant genes to defend against oxidative stress. Keap1 also binds to IKK $\beta$  and causes its degradation through UPS [18] or autophagy [23]. Recent studies have revealed a functional connection between IKK $\beta$ /NF- $\kappa$ B and Keap1/Nrf2 signaling pathways, which modulates inflammatory and carcinogenic processes.

Inhibition of HSPs or heat shock leads to proteotoxic stress that causes cellular protein damage or conformational changes such as protein misfolding, resulting in protein degradation. IKK $\beta$  degradation caused by Hsp90 inhibition may reflect an important adaptive cellular response which links proteotoxic stress response to inflammatory stimuli. However, the mechanism of IKK $\beta$  degradation by Hsp90 inhibition is yet to be studied. In this study, we investigated the mechanism by which Hsp90 inhibition causes IKK $\beta$  degradation and found that structural destabilization of IKK $\beta$  enhances its binding to Keap1 and promotes its autophagic degradation by different mechanism from that of Nrf2.

## Materials and method

### Reagents and antibody

Anti-tubulin (H-300) and anti-HA (Y-11) rabbit antibodies were obtained from Santa Cruz Biotechnology. Anti-HA (11867423) rat monoclonal antibody and anti-Keap1 (10503-2-AP) rabbit antibody were purchased from Roche and Proteintech, respectively. Anti-IKK $\beta$  (2684), anti-phospho-IKK $\beta$  (2697) and anti-phospho-I $\kappa$ B $\alpha$  (2859) rabbit antibodies were procured from Cell Signaling. Anti-Flag (M2) mouse antibody was obtained from Sigma. MG132, Chloroquine, GA, 3-methyladenine(3-MA), and cycloheximide (CHX) were procured from Sigma-Aldrich. Tertiary Butylhydroquinone (tBHQ) was purchased from Tokyokasei.

## Cell culture, plasmid transfection, and luciferase assay

Human embryonic kidney (HEK293) and mouse embryonic fibroblast (MEF) cells 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. HEK293 cells were obtained from RIKEN cell bank. WT and *Keap1*<sup>-/-</sup> MEFs were prepared in the laboratory of Masayuki Yamamoto. Keap1 and Nrf2 were amplified from a human cDNA library by polymerase chain reaction (PCR), and cDNAs were cloned in expression vectors encoding HA and Flag sequences. Expression plasmids encoding IKKβ, IKKα, NEMO/IKKγ and IκBα were described previously [24]. Substitution mutants were generated by PCR. Plasmids were transfected into fibroblasts, which were cultured in Opti-MEM (Invitrogen) using Lipofectamine Plus (Invitrogen), following the manufacturer's instructions. For luciferase assay, cells were transfected with reporters encoding NF-κB binding sites (pNF-κB luciferase plasmids), or Nrf2 binding sites (pNQO1 luciferase plasmids), together with pRK-TK Renilla-luciferase control plasmids. Luciferase activity was measured using Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's instructions.

## Cell fractionation

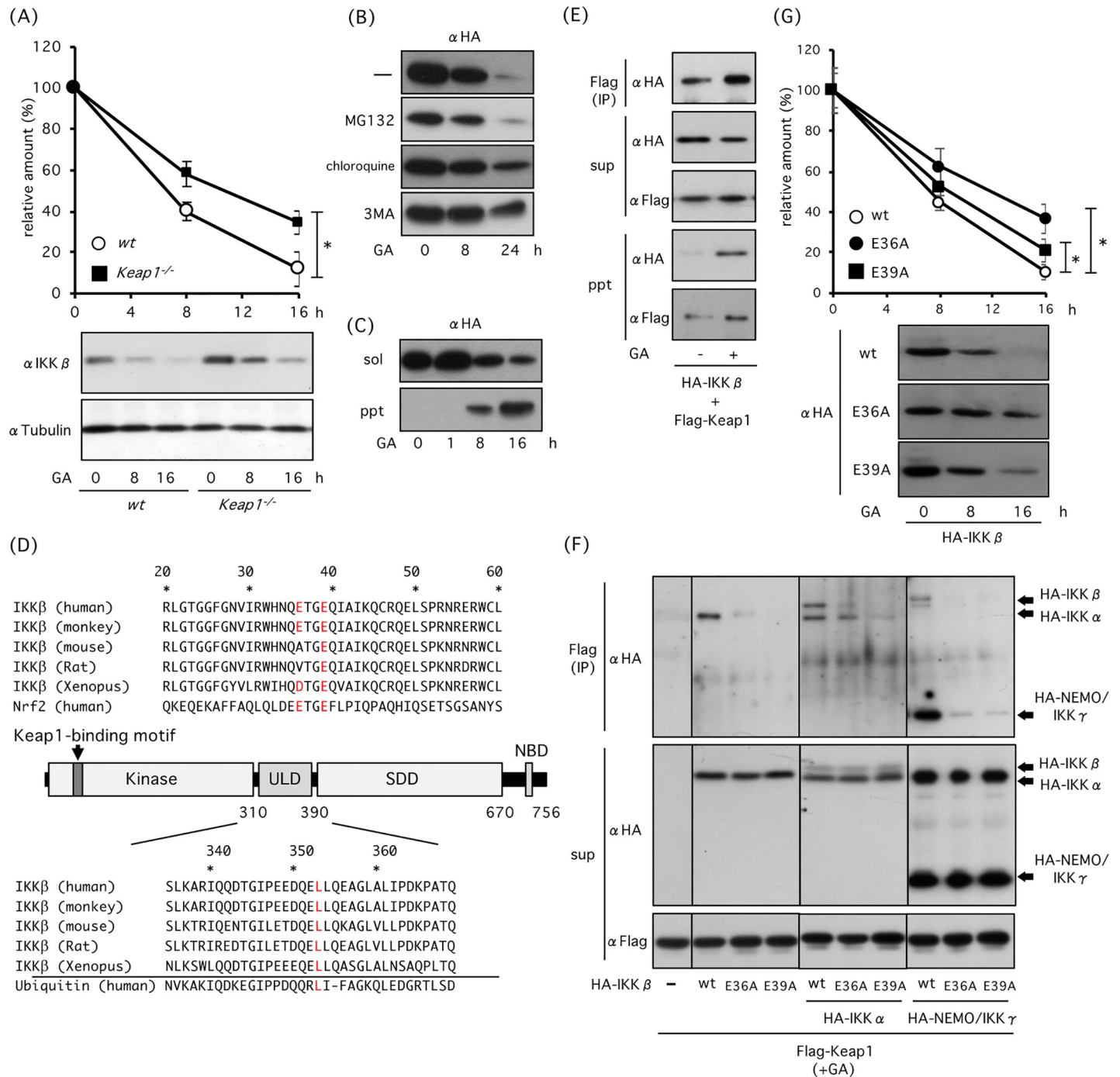
Cells were solubilized in buffer A consisting of 20 mM Tris-Cl (pH 7.5), 150 mM NaCl, 10 mM EGTA, 10 mM MgCl<sub>2</sub>, 60 mM β-glycerophosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM 4-amidino phenyl methyl sulfonyl fluoride, 50 KIU/ml aprotinin, 20 μg/ml pepstatin, 20 μg/ml leupeptin, 2 mM dithiothreitol and 1% Triton X-100. After centrifugation at 16,000 × g for 20 min at 4°C, the lysates were fractionated into supernatants and pellets. The pellets were then solubilized in buffer B containing 100 mM Tris-Cl, pH 6.8, 2% SDS, 5% glycerol, and 2.5% 2-mercaptoethanol. Cells solubilized in buffer B were used as total cell lysates.

## Immunoprecipitation and immunoblotting

For the immunoprecipitation assay of transfected cells, cell supernatants were incubated with anti-Flag (M2) Sepharose (Sigma), or with an antibody combined with Protein A and Protein G Sepharose (GE Healthcare) at 4°C. For the immunoprecipitation assay of endogenous proteins, cell lysates were incubated with an antibody together with TrueBlot anti-mouse or anti-rabbit IP beads (eBioscience) and subjected to immunoblotting using TrueBlot HRP-conjugated anti-mouse or anti-rabbit IgG antibodies (eBioscience). Proteins separated by gel electrophoresis were transferred to polyvinylidene difluoride membranes (Millipore) by an electroblotting apparatus (Mighty Small Transphor; Amersham) and subjected to immunoblotting using HRP-conjugated anti-mouse or anti-rabbit IgG antibodies (GE Healthcare). Antigen-antibody complexes were detected using SuperSignal West Pico Chemiluminescence System (Pierce).

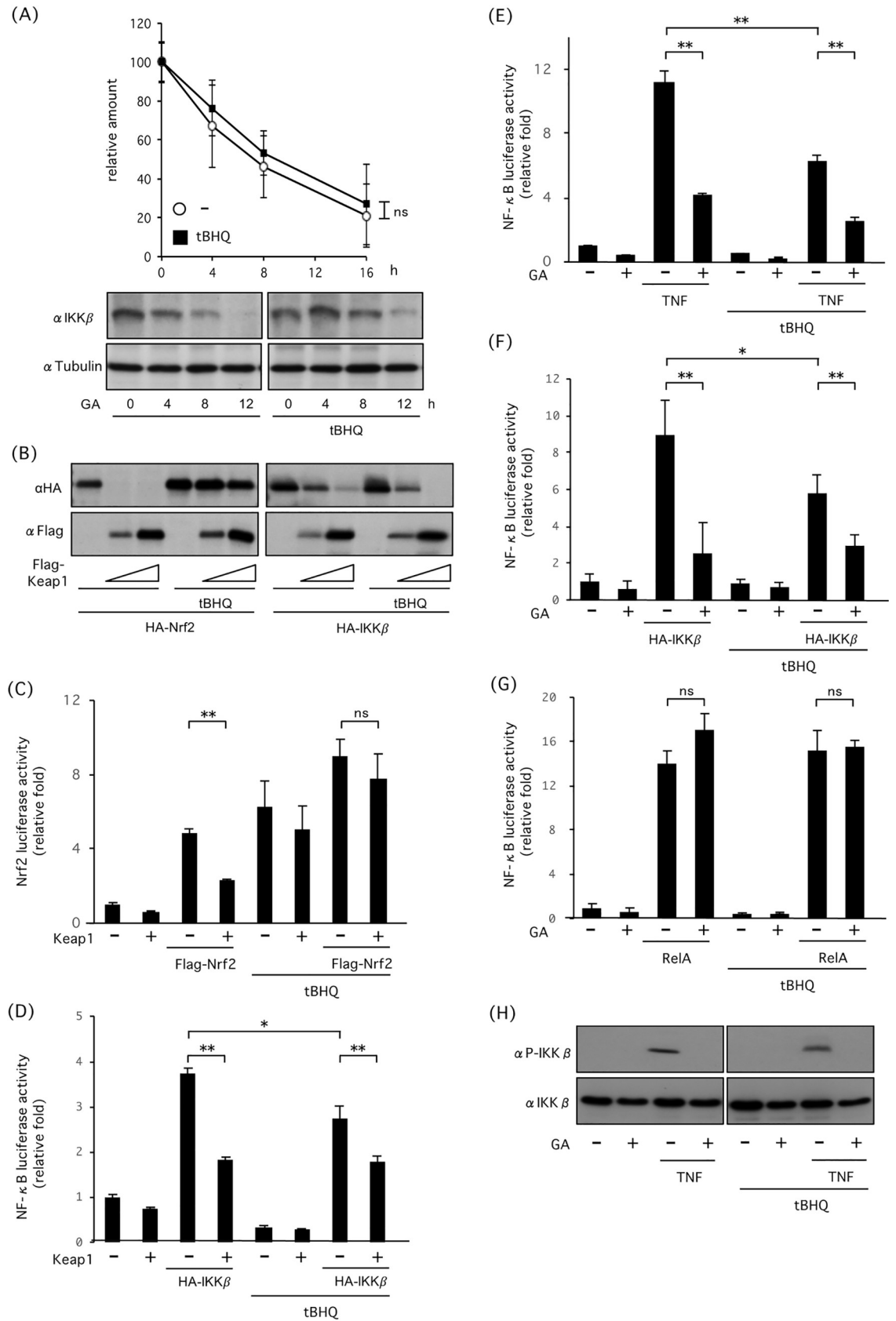
## Immunostaining of cells

HEK293 cells ( $0.5 \times 10^6$  cells) were transfected with 0.2 μg plasmids encoding HA-IKKβ, HA-IKKβ (L353A), Flag-Keap1, and GFP-LC3. Cells were grown on collagen-coated coverslips. After 18 h, cells were fixed with 4% paraformaldehyde in PBS for 30 min, and then fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 30 min at room temperature. After washing with PBS, cells were stained with anti-HA rat monoclonal antibodies or anti-Flag (M2) mouse antibodies in Can Get Signal immunostain Immunoreaction Enhancer Solution (TOYOBO) for 2 h. After washing with PBS, coverslips were incubated with Cy3-conjugated anti-mouse IgG antibodies or FITC-conjugated anti-rat IgG antibodies (Jackson



**Fig 1. Effect of Hsp90 inhibition on IKKβ degradation.** (A) Role of Keap1 in GA-induced IKKβ degradation. WT and *Keap1*<sup>-/-</sup> MEFs were incubated with 2 μM GA, and expression levels of IKKβ were investigated. (B) Role of autophagy in GA-induced IKKβ degradation. HEK293 cells were transfected with HA-IKKβ and incubated for 18 h. Then cells were treated with GA in the presence or absence of 20 μM MG132, 20 μM chloroquine, and 4 mM 3MA. (C) Role of GA in IKKβ translocation into detergent soluble or insoluble fractions in HA-IKKβ-transfected HEK293 cells. (D) Schematic representation of IKKβ. Keap1-binding motif of IKKβ is located in the KD. (E, F) Role of E36 and E39 glutamate residues of IKKβ ETGE motif in GA-enhanced IKKβ-Keap1 association. After 18 h transfection of HEK293 cells with Flag-Keap1 and HA-IKKβ (E) or with HA-IKKβ, HA-IKKα and HA-NEMO/IKKγ (F), HEK293 cells were treated with GA for 4 h and then proteins were immunoprecipitated with anti-Flag agarose. (G) Involvement of E36 and E39 in GA-induced degradation. After 18 h transfection of HEK293 cells with HA-IKKβ mutants, cells were treated with GA. Statistically significance at \* P < 0.05, \*\* P < 0.01.

<https://doi.org/10.1371/journal.pone.0203978.g001>





**Fig 2. Inhibitory effect of tBHQ on Keap1-mediated IKK $\beta$  degradation.** (A) Inhibitory effect of tBHQ on GA-mediated IKK $\beta$  degradation. Cells were treated with GA in the presence or absence of 50  $\mu$ M tBHQ. (B) Inhibitory effect of tBHQ on Keap1-mediated Nrf2 or/and IKK $\beta$  degradation. HEK293 cells were transfected with HA-IKK $\beta$ , HA-Nrf2 and Flag-Keap1, and incubated with or without tBHQ for 18 h. (C) Nrf2 luciferase activity of HEK293 cells transfected with Nrf2-reporter together with Flag-Keap1 and HA-Nrf2 in the presence or absence of tBHQ. (D) NF- $\kappa$ B luciferase activity of HEK293 cells transfected with NF- $\kappa$ B-reporter together with Flag-Keap1 and HA-IKK $\beta$  in the presence or absence of tBHQ. Both luciferase activities were measured 18 h post-transfection. (E) NF- $\kappa$ B luciferase activity of HEK293 cells transfected with NF- $\kappa$ B reporter, and incubated with or without GA, tBHQ, and 50 ng/ml TNF $\alpha$  (as an inflammation stimulator) for 18 h. (F, G) NF- $\kappa$ B luciferase activity of HEK293 cells transfected with NF- $\kappa$ B reporter together with or without HA-IKK $\beta$ (F) or Flag-RelA (G), incubated in presence or absence of GA and tBHQ for 18 h. (H) Inhibitory effect of tBHQ on GA-mediated IKK $\beta$  inactivation and degradation. HEK293 cells were treated with GA for 6 h and then stimulated with TNF $\alpha$  for 20 min. \*  $P < 0.05$ , \*\*  $P < 0.01$ .

<https://doi.org/10.1371/journal.pone.0203978.g002>

ImmunoResearch Laboratories) for 2 h and were observed under BZ-9000 Fluorescence Microscope (KEYENCE).

### Statistics

Data were expressed as means  $\pm$  standard deviation (S.D.) from at least three independent experiments and analyzed by two-tailed t-tests. Differences were considered statistically significant if at \*  $P < 0.05$ , \*\*  $P < 0.01$ .

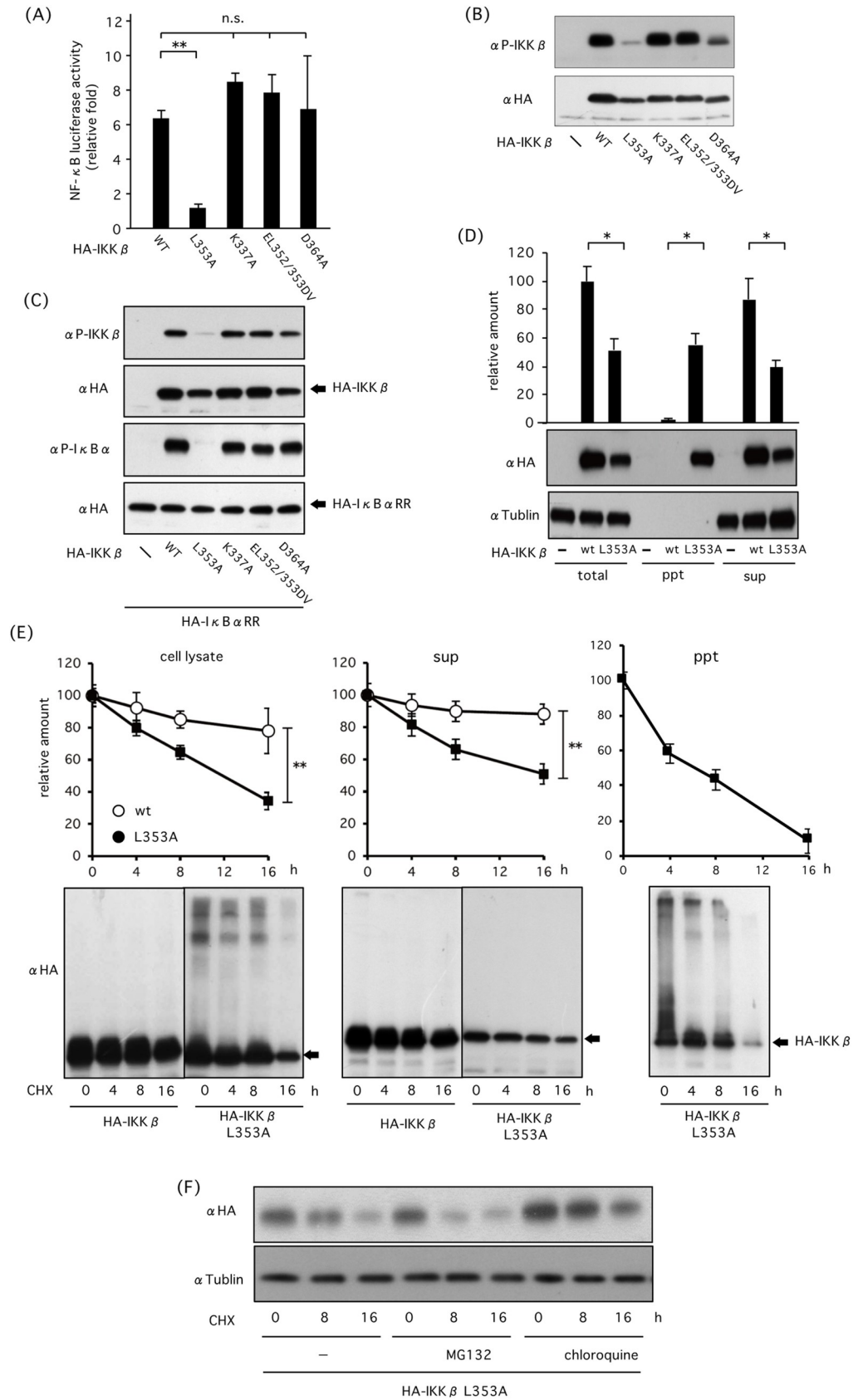
## Results

### Inhibition of Hsp90 promotes Keap1-mediated autophagic degradation of IKK $\beta$ through enhancing Keap1 binding

When cells were treated with GA, the decrease in IKK $\beta$  levels was slower in *Keap1*<sup>-/-</sup> MEF cells than in their wild-type counterparts, suggesting that Keap1 is partially involved in GA-induced IKK $\beta$  degradation (Fig 1A). Inhibitors of autophagy such as chloroquine and 3-MA, but not proteasome inhibitor MG132, delayed GA-induced degradation, indicating that autophagy mainly mediates GA-induced IKK $\beta$  degradation (Fig 1B). Misfolded proteins are known to form detergent-insoluble aggregates. GA induces translocation of transfected HA-IKK $\beta$  into detergent-insoluble fractions (Fig 1C). IKK $\beta$  is composed of KD, ULD, SDD, and NBD, and the Keap1 binding motif which includes two essential glutamate residues (E36 and E39) locates in the N-terminal of KD (Fig 1D). Immunoprecipitation assay revealed that GA increases binding of IKK $\beta$  to Keap1 (Fig 1E). Glu-to-Ala substitution mutations at E36 and E39 (E36A and E39A) decreased GA-mediated enhanced IKK $\beta$ -Keap1 association (Fig 1F). Besides, these mutations reduced the binding of co-expressed IKK $\alpha$  and NEMO/IKK $\gamma$  to Keap1. GA-mediated degradation of IKK $\beta$  mutants (E36A and E39A) were slower than that of wild type IKK $\beta$  (wt IKK $\beta$ ), suggesting that inhibition of Hsp90 promotes autophagic degradation of IKK $\beta$  mediated in part by binding to Keap1.

### Keap1-mediated IKK $\beta$ degradation is not inhibited by tBHQ

Cysteine residues in Keap1 are modified by electrophiles, such as tBHQ, or reactive oxygen species, leading to Keap1 dissociation from Nrf2. As a result, Nrf2 is stabilized and it subsequently translocates to the nucleus. To elucidate whether tBHQ stabilizes IKK $\beta$ , we investigated degradation of IKK $\beta$  in the presence or absence of tBHQ, in cells treated with GA, and found that tBHQ did not prevent GA-induced IKK $\beta$  degradation (Fig 2A). Co-transfection assay revealed that tBHQ prevented Keap1-induced Nrf2 degradation, but not IKK $\beta$  degradation (Fig 2B). tBHQ promoted Nrf2 activity and prevented Keap1-mediated suppression of Nrf2 (Fig 2C). However, it did not inhibit Keap1-mediated suppression of IKK $\beta$ -induced NF- $\kappa$ B activation (Fig 2D). Treatment of cells with GA suppressed TNF $\alpha$ -induced NF- $\kappa$ B



**Fig 3. Effect of L353A mutation in the ULD of IKKβ on structural stability or degradation of IKKβ.** (A) Effect of different HA-IKKβ mutations on IKKβ-induced NF-κB activation in terms of NF-κB luciferase activity. HEK293 cells were transfected with NF-κB reporter and HA-IKKβ mutants. After 18 h, luciferase activities were measured. (B) Effect of HA-IKKβ mutants on IKKβ autophosphorylation. HEK293 cells were transfected with HA-IKKβ mutants and phosphorylation of IKKβ was detected by western blot analysis. (C) Effect of HA-IKKβ on IKKβ-induced IκBα phosphorylation. HEK293 cells were transfected with HA-IKKβ mutants together with HA-IκBαRR, and phosphorylation of IκBα was detected by western blot analysis. (D) Role of L353A mutation in HA-IKKβ translocation into detergent insoluble or insoluble fractions of HEK293 cells. Cells were transfected with wild type or mutated HA-IKKβ(L353A), and expression levels were investigated in total cell lysates, detergent soluble and insoluble fractions. (E) Effect of L353A mutation on IKKβ degradation in different fractions. HEK293 cells were transfected with wild type or mutated HA-IKKβ(L353A) mutant. After incubation with 10 μg/ml CHX, expression levels were investigated in total cell lysates, detergent soluble and insoluble fractions. (F) Autophagy mediates degradation of L353A mutant. HEK293 cells transfected with HA-IKKβ or HA-IKKβ(L353A) mutants were incubated with CHX in the presence or absence of MG132 and chloroquine. \* P < 0.05, \*\* P < 0.01.

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activation, and tBHQ did not prevent GA-mediated NF-κB suppression (Fig 2E). Co-transfection with IKKβ significantly activated NF-κB, and GA suppressed IKKβ-induced NF-κB activation (Fig 2F). GA-mediated NF-κB suppression was not prevented by tBHQ in IKKβ-transfected cells. However, GA did not suppress NF-κB-activation in cells transfected with RelA, suggesting that GA specifically suppressed IKKβ activity (Fig 2G). Western blot analysis revealed that GA suppressed TNF-induced IKKβ phosphorylation, and tBHQ did not inhibit GA-mediated IKKβ suppression (Fig 2H). These results indicate that Hsp90 inhibition leads to Keap1-mediated autophagic degradation that is not inhibited by tBHQ.

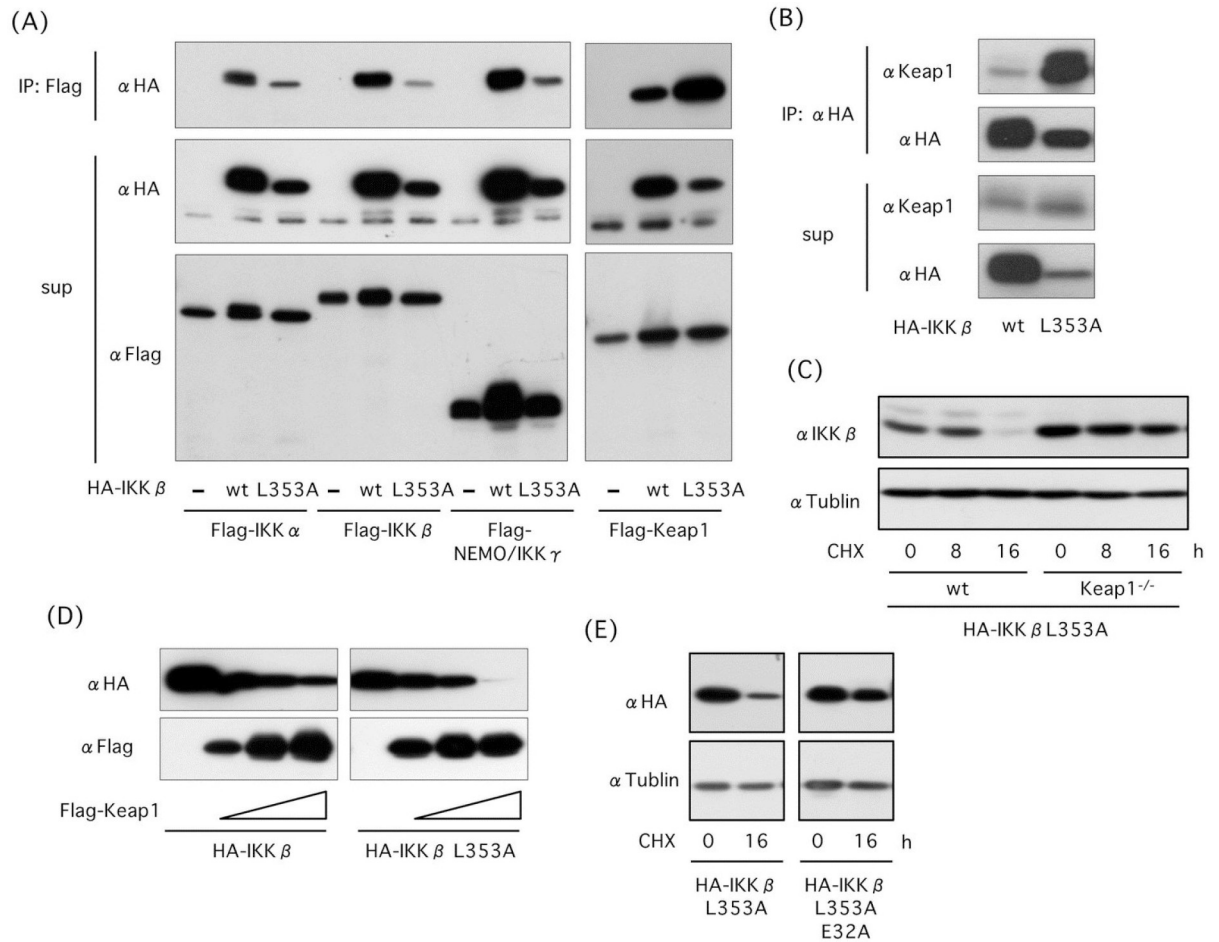
### L353A mutation destabilizes IKKβ and leads its autophagic degradation

Structural destabilized mutant of IKKβ was studied to elucidate whether structural destabilization per se induces autophagic degradation. The ULD interacts with KD and SDD in a highly organized manner that is required for a critical interaction with IκBα and kinase activity [8]. We generated several Ala substitution mutants in the ULD and found that Leu-to-Ala substitution mutation at Leu353 abolishes IKKβ-induced NF-κB activation (Fig 3A), which was consistent with previous reports [8, 25]. Moreover, autophosphorylation was reduced in L353A mutant (Fig 3B). Co-transfection of HEK293 cells with HA-IKKβ mutants and ubiquitination-resistant HA-IκBαRR mutant revealed that L353A mutant hardly phosphorylate IκBα (Fig 3C). L353A mutant was localized to the detergent-insoluble fraction (Fig 3D). L353A mutant degraded more rapidly than wt IKKβ, and the rate of disappearance was faster in the detergent-insoluble fraction than in the detergent-soluble fraction (Fig 3E). Degradation of L353A mutant was partially prevented by chloroquine but not by MG132, suggesting that autophagy mainly mediates degradation of structural destabilized L353A mutant (Fig 3F).

### L353A mutation increases Keap1 binding

To elucidate whether Keap1 is involved in degradation of L353A mutant, we investigated the interaction of Keap1 to L353A mutant. Immunoprecipitation assay of transfected cells revealed that binding of Keap1 to IKKβ is increased in L353A mutant, whereas binding of IKKα, IKKβ and NEMO/IKKγ to IKKβ were not changed (Fig 4A). Additionally, the increased interaction of endogenous Keap1 to L353A mutant was detected in cells (Fig 4B). The reduction in the amount of L353A mutant was slower in *Keap1*<sup>-/-</sup> than in wt MEFs under CHX treatment, suggesting that Keap1 is involved in L353A degradation (Fig 4C). Co-transfection assay revealed that Keap1 degraded L353A mutant more effectively than wt IKKβ (Fig 4D). We generated expression plasmids encoding double mutants of L353A/E36A and L353A/E39A. L353A/E36A was expressed effectively, whereas L353A/E39A was hardly expressed in cells. Then we compared degradation ratio of L353A/E36A to L353A and found that L353A/E36A is more stable





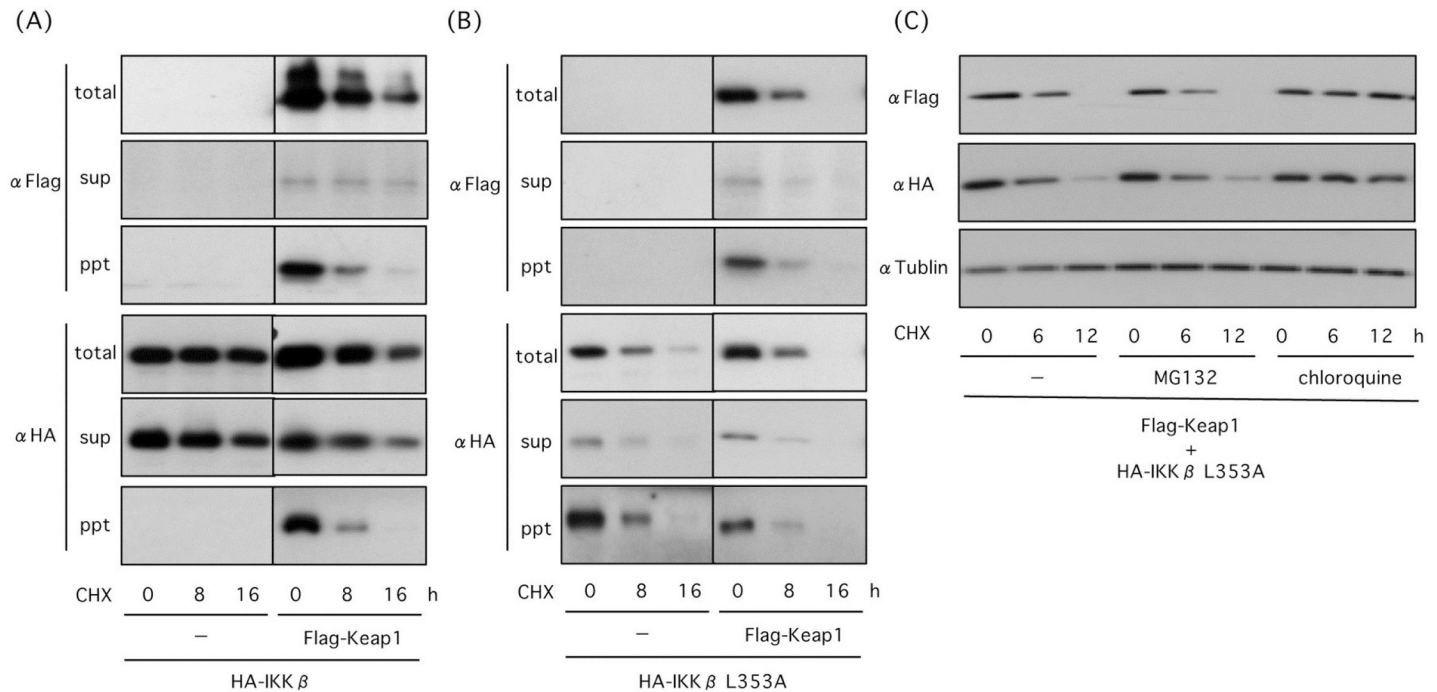
**Fig 4. Effect of L353A mutation on association of IKKβ with Keap1 and autophagic degradation.** (A) Effect of L353A mutation on IKKβ-Keap1 binding. Cells were transfected with wild type or mutated HA-IKKβ(L353A) together with Flag-IKKα, Flag-IKKβ, Flag-NEMO/IKKγ, and Flag-Keap1. After 18 h, cell lysates were immunoprecipitated with an anti-Flag agarose beads, and association of IKKβ with IKKα, IKKβ, NEMO/IKKγ, and Keap1 was investigated. (B) Effect of L353A mutation on association of IKKβ with endogenous Keap1. HEK293 cells transfected with HA-IKKβ or HA-IKKβ(L353A) were immunoprecipitated with an anti-HA antibody. (C) Role of Keap1 in IKKβ(L353A) degradation. After transfection of WT and *Keap1*<sup>-/-</sup> MEF cells with mutated HA-IKKβ(L353A), cells were treated with CHX and degradation of HA-IKKβ(L353A) was investigated by western blot analysis. (D) Effect of L353A mutation on Keap1-mediated IKKβ degradation. HEK293 cells were transfected with HA-IKKβ or HA-IKKβ(L353A) together with Flag-Keap1, and expression levels were investigated in cell lysates by western blot analysis. (E) Involvement of Keap1-binding motif in degradation of L353A mutant. After 18 h transfection with HA-IKKβ(L353A) and double mutant HA-IKKβ(L353A/E36A), HEK293 cells were incubated with CHX. Expression of transfected IKKβ proteins was detected by western blot with an anti-HA antibody.

<https://doi.org/10.1371/journal.pone.0203978.g004>

than L353A (Fig 4E). These results indicate that structural destabilization of IKKβ increases its interaction to Keap1, leading to autophagic degradation.

### Keap1 is subjected to autophagic degradation

Keap1 is degraded by autophagy in response to oxidative stress [26, 27]. To elucidate whether Keap1 is degraded with IKKβ, we transfected cells with plasmids encoding wild type or mutated HA-IKKβ(L353A) together with or without Flag-Keap1. Co-expression of Keap1 induced translocation of wild type IKKβ into the detergent-insoluble fraction and its degradation, and Keap1 is degraded concomitant with IKKβ (Fig 5A). Similarly, Keap1 is degraded concomitant with HA-IKKβ(L353A) when cells were co-transfected with Flag-Keap1 and HA-IKKβ(L353A) (Fig 5B). Degradation of Keap1 was suppressed by chloroquine, but not by



**Fig 5. Keap1 is degraded with IKK $\beta$  by autophagy.** (A) Expression of Keap1 promoted IKK $\beta$  degradation and Keap1 is degraded concomitant with IKK $\beta$ . HEK293 cells were transfected with wild type HA-KK $\beta$  and Flag-Keap1. After 18 h of transfection, 10  $\mu$ g/ml CHX was added to cell culture. Then cell lysates were prepared following incubation for 8 and 16 h. Expression levels of proteins were investigated in total cell lysates, detergent soluble and insoluble fractions. (B) Keap1 was degraded concomitant with IKK $\beta$ (L353A) mutant. HEK293 cells were transfected with HA- IKK $\beta$ (L353A) together with Flag-Keap1 and were treated with CHX. (C) Keap1 was degraded by autophagy. HEK293 cells transfected with Flag-Keap1 and HA- KK $\beta$ (L353A) were incubated with CHX in the presence or absence of MG132 and chloroquine.

<https://doi.org/10.1371/journal.pone.0203978.g005>

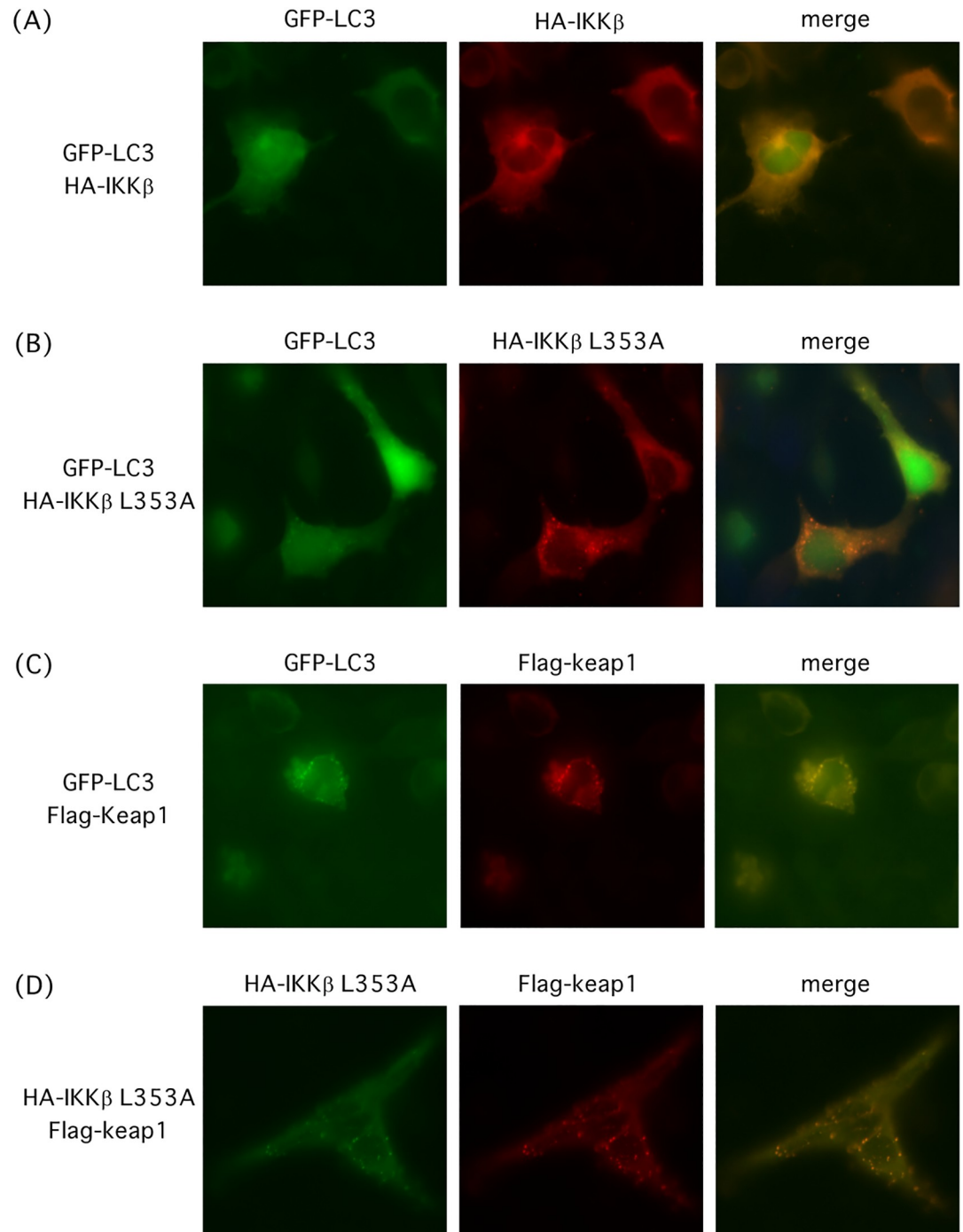
MG132, in cells co-transfected with HA-IKK $\beta$ (L353A) (Fig 5C). These results suggest that Keap1 is degraded concomitant with IKK $\beta$  by autophagy.

### Structural destabilized IKK $\beta$ colocalizes with autophagic machinery proteins in cells

Atg8/LC3 protein plays an essential role in autophagosome maturation and is widely used as an autophagosome marker. To confirm the role of autophagic/lysosomal machinery in degradation of structural destabilized IKK $\beta$ , we investigated cellular localization of IKK $\beta$ , Keap1, and autophagosome. HA-IKK $\beta$  appeared to be dispersed throughout the cytoplasm (Fig 6A). Whereas, although HA-IKK $\beta$ (L353A) was dispersed throughout the cytoplasm, some portion of HA-IKK $\beta$ (L353A) proteins formed small punctuate structures and colocalized with GFP-LC3 in the cytoplasm (Fig 6B). The majority of Flag-Keap1 proteins was dispersed throughout the cytoplasm, and some portion of Flag-Keap1 formed small punctuate structures and colocalized with GFP-LC3 (Fig 6C). HA-IKK $\beta$ (L353A) and Flag-Keap1 were colocalized in the small punctuate structures in the cytoplasm (Fig 6D). These results suggest that structural destabilized IKK $\beta$  interacts with Keap1 in the autophagic machinery protein complex and is degraded by autophagy.

### Discussion

The IKK $\beta$ /NF- $\kappa$ B and Keap1/Nrf2 signaling pathways play an essential role in cellular signaling in response to inflammatory stimuli and oxidative stress. It has been reported that



**Fig 6. Colocalization of L353A mutant of IKK $\beta$  with LC3 and Keap1 in the cytoplasm.** (A) HA-IKK $\beta$  was dispersed in the cytoplasm. HEK293 cells were transfected with plasmids encoding wild type HA-IKK $\beta$  and GFP-LC3. (B) HA-IKK $\beta$ (L353A) was colocalized with GFP-LC3 in small punctuate structures. HEK293 cells were transfected with HA-IKK $\beta$ (L353A) and GFP-LC3. (C) Flg-Keap1 was colocalized with GFP-LC3 in small punctuate structures. HEK293 cells were transfected with Flg-Keap1 and GFP-LC3. (D) HA-IKK $\beta$ (L353A) was colocalized with Flg-Keap1 in small punctuate structures. HEK293 cells were transfected with HA-IKK $\beta$ (L353A) and Flg-Keap1. Following transfection, cells were incubated for 18 h and subjected to immunostaining.

<https://doi.org/10.1371/journal.pone.0203978.g006>

activation of NF- $\kappa$ B antagonizes Nrf2-mediated transcription by either inhibition of Nrf2-DNA binding [28] or depriving CREB binding protein (CBP) from Nrf2 and facilitating the interaction of recruited histone deacetylase 3 (HDAC3) at the Nrf2-DNA binding site [29]. Nrf2-deficient MEFs show greater activation of NF- $\kappa$ B in response to lipopolysaccharide (LPS) stimulus [30], and Nrf2 suppresses NF- $\kappa$ B-induced transcriptional upregulation of proinflammatory cytokines, including IL-6 and IL-1 $\beta$  in macrophages [31]. Indeed, NF- $\kappa$ B activation is suppressed in tBHQ-treated cells in which Nrf2 is activated (Fig 2D, 2E and 2F). Thus, cellular responses to inflammatory stimuli and oxidative stress is regulated by crosstalk between the IKK $\beta$ /NF- $\kappa$ B signaling and the Keap1/Nrf2 signaling pathways.

Although Keap1 is involved in structurally destabilized IKK $\beta$  degradation, the contribution of Keap1 to degradation is partial; thus, other factors would be involved in IKK $\beta$  degradation as well. It has been reported that *Drosophila* NEMO/IKK $\gamma$  has an LC3-interacting region (LIR) or Atg8-interacting motif (AIM) in the N-terminal region which recruits the IKK complex to the autophagosome, and acts as a selective autophagy receptor that mediates the degradation of the IKK complex [32]. Whereas human NEMO/IKK $\gamma$  lacks functional LIR/AIM and does not act as a selective autophagy receptor for degradation of the IKK complex. Instead, NEMO/IKK $\gamma$  is ubiquitinated in human and could also interact with selective autophagy receptors via ubiquitin [33, 34]. In this study, we found that structurally destabilized IKK $\beta$  interacts with Keap1 and is degraded by autophagy. Thus, several mechanisms would be involved in degradation of the IKK complex in cells under proteotoxic stress.

Cells are challenged by various extrinsic and intrinsic insults which provoke proteotoxic stress under conditions of injury, inflammation, and oxidative stress. As disturbance of proteome homeostasis is associated with a broad range of human diseases, maintenance of proteome integrity is essential to maintain cellular fitness and functions. To protect cells from proteotoxic stress, cells mobilize heat shock proteins including Hsp90 that unfold proteins and proteolysis systems that remove damaged proteins. In this study, it was found that Hsp90 inhibition, which causes proteotoxic stress, enhances interaction of IKK $\beta$  with Keap1. These findings also suggest that cells respond to proteotoxic stress by regulating IKK $\beta$ /NF- $\kappa$ B and Keap1/Nrf2 signaling pathways. The Keap1/Nrf2 and IKK $\beta$ /NF- $\kappa$ B signaling pathways have interesting features concerning the proteolytic mechanisms such as autophagy and UPS. Keap1 suppresses Nrf2 activation by UPS, whereas it is degraded by autophagy in response to oxidative stress [26, 27]. IKK $\beta$  induces NF- $\kappa$ B activation through degradation of I $\kappa$ B $\alpha$  by UPS, whereas it is degraded by autophagy in response to proteotoxic stress. Furthermore, IKK $\beta$  contributes to the induction of autophagy in an NF- $\kappa$ B-independent manner [35], and Keap1 interacts with p62, a selective autophagy receptor, which binds to Atg8/LC3 protein, resulting in autophagy [36, 37]. There is a plausibility that oxidative stress and proteotoxic stress induce selective degradation of Keap1 and IKK $\beta$  by autophagy and provoke specific cellular responses.

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