HSP70/DNAJA3 chaperone/cochaperone regulates NF-κB activity in immune responses

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Abbreviations: HEK, human embryonic kidney cells; HRP, horseradish peroxidase; HSP, heat shock protein; IKK. IκB kinase; IP, immunoprecipitation; mAb, monoclonal antibody; NF-κB, nuclear factor kappa B; PAGE, polyacrylamide gel electrophoresis; PES, 2-phenylethynesulfonamide; RNAi, RNA interference; SDS, sodium dodecyl sulfate; siRNA, small interference RNA; TLR, Toll-like receptor

Abstract

Nuclear factor kappa B (NF-κB) controls the transcription of various genes in response to immune stimuli. Our previous study revealed that the Droj2/DNAJA3 cochaperone contributes to the NF-κB pathway in *Drosophila* and humans. In general, the cochaperone is associated with the 70-kDa heat shock protein (HSP70) chaperone and the complex supports the folding of diverse target proteins. The cochaperone/chaperone functions in the NF-κB pathway, however, are not clearly understood. Here, we report that HSP70 proteins are involved in activating canonical NF-κB signaling during immune responses. In human cultured cells, HSP70 inhibitor destabilized the IKKβ/IκBα/NF-κB p65 complex and dampened the phosphorylation of NF-κB p65 in response to flagellin stimulation. We identified HSPA1A and HSPA8 as the HSP70 family proteins that physically interact with DNAJA3, and established their requirement for the phosphorylation of NF-κB p65. Furthermore, as in flies with knockdown of Droj2, flies with knockdown of Hsc70-4, a *Drosophila* homolog of HSPA8, were more susceptible to infection. Our results suggest that the chaperone/cochaperone complex regulates NF-κB immune signaling in an evolutionarily conserved manner.

Keywords: HSP70; DNAJA3; immune response; NF-κB; IKKβ/IκBα/NF-κB p65 complex

Highlights

- HSP70s and DNAJA3 regulate NF-κB activity by stabilizing IKKβ/IκBα/NF-κB p65

- HSPA1A and HSPA8 are associated with DNAJA3 in human cells

- Hsc70-4, a *Drosophila* homolog of HSPA8, is required for fly immunity

1. Introduction

Nuclear factor kappa B (NF-κB) is a transcription factor with key roles in innate and adaptive immune systems [1,2]. NF-κB regulates the expression of target genes such as cytokines, growth factors, and effector enzymes [2]. Because the immune system must be precisely controlled to maintain the physiological homeostasis of organism, NF-κB activity is fine-tuned by various factors.

The intracellular signaling mechanisms of the NF-κB pathway have been studied extensively. Activation of the NF-κB pathway is initiated by extracellular stimuli, such as pathogen-associated microbial patterns and cytokines. In mammals, these stimuli are recognized by Toll-like receptors (TLRs) and cytokine receptors, and signals are transmitted into cells through the signaling cascade [3–5], where they reach the NF-κB complex comprising NF-κB, IκB, and IκB kinase (IKK). In a steady state, IκB protein retains the NF-κB dimer in the cell cytoplasm and prevents its nuclear localization [6,7]. Under stimulating conditions, the signal induces the phosphorylation of IKK [3–5], and IKK in turn phosphorylates IκB protein, leading to its Lys48-linked ubiquitination [6–8]. Ubiquitinated IκB is degraded via the 26S proteasome, and this degradation ultimately releases NF-κB, which enters the nucleus [9]. Concomitantly, IKK and other kinases phosphorylate the NF-κB p65 subunit at the transactivation domain, and this seemingly optional step is essential for the optimal induction of NF-κB target genes [10].

The regulatory mechanisms of NF-κB signaling are evolutionarily preserved among vertebrates and invertebrates. The fruit fly *Drosophila melanogaster* has two distinct NF-κB pathways, the Toll and Imd pathways, which correspond to the TLR and tumor necrosis factor signaling pathways, respectively, in mammals [11]. Both signaling pathways are essential for fly innate immunity to combat against pathogen infection [12].

Our previous study demonstrated that an evolutionarily conserved cochaperone, Droj2/DNAJA3, contributes to the NF-κB pathway in *Drosophila* and humans [13]. The NF-κB activation under flagellin stimulation is suppressed in DNAJA3 knockdown human cells, and Droj2 knockdown flies are highly susceptible to bacterial infection. Droj2/DNAJA3 belongs to the HSP40/DNAJ cochaperone family associated with the 70-kDa heat shock protein (HSP70) chaperone. The HSP40 cochaperone stimulates ATP hydrolysis of HSP70 and facilitates the delivery of substrates to HSP70 [14]. The chaperone/cochaperone complex is involved in refolding nascent and damaged proteins, and promotes proteasomal degradation of ubiquitinated proteins [15]. HSP40 and HSP70 families have several members (49 and 13 in humans, respectively), and their combinations are thought to determine the specificity of substrate proteins. The biological functions of chaperone/cochaperone complexes are diverse, and their roles in the NF-κB pathway have been studied [16]. A clear picture of NF-κB pathway regulation, however, has remained elusive and whether or not the mechanism is evolutionarily conserved is yet unclear. In the present study, we investigated the roles of HSP40 and HSP70 in the regulation of NF-κB activity in humans and *Drosophila*.

2. Materials and Methods

2.1 Cell culture

HEK293 cells (human embryonic kidney cells) were maintained at 37°C in Dulbecco's modified Eagle's medium with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/mL amphotericin.

For expression of exogenous DNAJA3, cells on a 6-well plate were transfected with 2.5 μg of pcDNA3/DNAJA3-FLAG using Lipofectamine LTX (ThermoFisher). After 24 h of plasmid transfection, cells were lysed for immunoprecipitation.

For knockdown of endogenous DNAJA3, cells were transfected with small interference RNA (siRNA) against DNAJA3 (Qiagen, SI04218606; 10 pmol on 6-well plate, 40 pmol on a 100-mm dish) using Lipofectamine RNAiMAX. For knockdown of HSP70s, siRNAs against HSPA1A (Qiagen, SI00442974), HSPA8 (Qiagen, SI04269083), and HSPA9 (Qiagen, SI02654813) were used. After 36 h of siRNA transfection, cell lysates were prepared. In addition, cells were treated with 2-phenylethynesulfonamide (PES; MilliporeSigma) for 1 h or with flagellin (300 ng/ml) (Invivogen) for 15 min before preparing the cell lysates.

2.2 Immunoprecipitation assay

Anti-IKKβ (D30C6) rabbit monoclonal antibodies (mAb, Cell Signaling Technology) and anti-FLAG M2 mouse mAb (MilliporeSigma) were used for this assay. Cell lysates with lysis buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% Nonidet P-40 supplemented with protease inhibitor mixture) were incubated with the antibodies for 2 h at 4°C, and then with Dynabeads M280 (ThermoFisher) for 1 h at 4°C. The bead-captured proteins were eluted with sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris-HCl, 200 mM β-mercaptoethanol, 2%

SDS, 0.0125% bromophenol blue, and 10% glycerol) at 95°C for 5 min. The proteins were analyzed on SDS–polyacrylamide gel electrophoresis (PAGE) with silver-staining (ATTO Corp). DNAJA3-interacting proteins were characterized by liquid chromatography-tandem mass spectrometry performed in APROSCIENCE Co., Ltd.

2.3 Western blotting analysis

Samples were resolved on 8%-10% SDS-PAGE. Western blotting was performed as described previously [13]. The protein bands were visualized using the Luminata Forte Western horseradish peroxidase (HRP) substrate (Millipore) according to the supplier's protocol, and the images were captured by ImageQuant LAS 4000 mini (GE Healthcare). The following antibodies were used: Tid-1 (RS13) mouse mAb (Cell Signaling Technology), IκBα (L35A5) mouse mAb (Cell Signaling Technology), phospho-IκB-α (Ser-32, 14D4) rabbit mAb (Cell Signaling Technology), NF-κB p65 (D14E12) XP rabbit mAb (Cell Signaling Technology), phospho-NF-κB p65 (Ser-535, 93H1) rabbit mAb (Cell Signaling Technology), IKKβ (D30C6) rabbit mAb (Cell Signaling Technology), phospho-IKKα (Ser-176)/IKKβ (Ser-177) (C84E11) rabbit mAb (Cell Signaling Technology), anti-actin antibody (ab179467, Abcam), anti-rabbit IgG and HRP-linked whole antibody (GE Healthcare), anti-mouse IgG and HRP-linked whole antibody (GE Healthcare), and mouse anti-rabbit IgG (Light-Chain Specific) (D4W3E) mAb, as well as HRP conjugate (Cell Signaling Technology).

2.4 Reporter Gene Assay

The reporter assay was performed as described previously [13]. Briefly, HEK293 cells were transfected with ELAM (endothelial leukocyte-adhesion molecule)-NF-κB luciferase and

pRL-TK using Lipofectamine LTX. Twenty-four hours after reporter transfection, cells were treated with PES for 1 h and then stimulated with flagellin (300 ng/ml) for 6 h. After stimulation, relative luciferase activity was measured using the Dual-Glo luciferase assay system (Promega).

2.5 Drosophila infection experiments

The c564-Gal4; tub-Gal80^{ts} females were crossed with the UAS-GFP RNA interference (RNAi; control) males or the UAS-HSC70-4 RNAi (NIG#4264R-1 and R-2) males. The progenies were developed at 18°C, and the newly emerged adults were incubated at 29°C for 3 days. The bacterium *Erwinia carotovora carotovora 15* (*Ecc15*) was cultured in lysogeny broth medium at 30°C, and the bacterial suspensions in saline (OD = 1) were injected into female flies (70 nL each) using the Nanoject device (Drummond Scientific). The number of dead flies at 29°C were counted every day. The *relish*[E20] mutant was used as a positive control. Data were analyzed on R platform, and survival rates were fitted to a Kaplan-Meier model. Statistical tests for multiple and pairwise comparisons were performed using the Log-rank test and Tukey's HSD post hoc test.

3. Results

3.1 Knockdown of DNAJA3 destabilized the IKKβ/IκBα/NF-κB p65 complex

We previously demonstrated that DNAJA3 contributes to the NF-κB pathway [13]. To further characterize the roles of DNAJA3, we analyzed the NF-κB complex in HEK293 cells. Western blot analysis confirmed that the siRNA treatments knocked down DNAJA3 (Fig. 1A). Under this condition, NF-κB p65 protein levels were slightly decreased compared with that in control cells, but no change was observed in the IKKβ and IκBα protein levels. Co-immunoprecipitation experiments showed that the interactions of IκBα and NF-κB p65 with IKKβ were significantly attenuated in the DNAJA3 knockdown cells (Fig. 1A, B). This suggests that the DNAJA3 cochaperone is involved in stabilizing the IKKβ/IκBα/NF-κB p65 complex.

3.2 Inhibition of HSP70s destabilized the IKKβ/IκBα/NF-κB p65 complex

We hypothesized that the DNAJA3/HSP70 chaperone/cochaperone complex modifies NF-κB activity. To test this hypothesis, we examined whether HSP70 inhibition impaired NF-κB activity. HEK293 cells transfected with the NF-κB reporter were treated with the HSP70 inhibitor PES (also known as pifithrin-μ), and then stimulated with the TLR5 ligand flagellin. We found that the NF-κB reporter activity stimulated by flagellin was suppressed by PES in a concentration-dependent manner (Fig. 2A). Next, we analyzed the IKKβ/IκBα/NF-κB p65 complex under this condition. Co-immunoprecipitation experiments revealed that the interactions of IKKβ with IκBα and NF-κB p65 were inhibited by PES treatment (Fig. 2B, C). Consequently, the phosphorylation of NF-κB p65 induced by flagellin stimulation was suppressed by PES treatment, whereas the phosphorylation of IκBα was not impaired (Fig. 2D, E). These findings suggest that HSP70s regulate NF-κB activity through stabilizing the IKKβ/IκBα/NF-κB p65

complex.

3.3 DNAJA3 interacted with some HSP70 family proteins

The human HSP70 family has 13 members, and PES inhibits HSP70 [17,18]. Therefore, we sought to identify the HSP70 family proteins interacting with DNAJA3 *in vivo*. For this purpose, Flag-tagged DNAJA3 protein was transiently expressed in HEK293 cells, and its interacting proteins were co-immunoprecipitated from the cell lysate using an anti-Flag antibody (Fig. 3A). The interacting proteins were separated by SDS–PAGE and the ~70-kDa proteins were analyzed by mass spectroscopy (Fig. 3A, asterisk). We identified HSPA1A, HSPA8, and HSPA9 proteins of the HSP70 family and other proteins, including ARALAR1, ARALAR2, PABPC1, XRCC6, AIFM1, DDX3X, LMNA, and SYNCRIP, in this fraction.

To elucidate the involvement of these HSP70 family proteins in the NF-κB pathway, we knocked down HSPA1A, HSPA8, and HSPA9 in HEK293 cells, and examined the phosphorylation of the IκBα and NF-κB p65. Phosphorylation of NF-κB p65 following flagellin stimulation was suppressed by HSPA1A and HSPA8 siRNA treatment (Fig. 3B). Phosphorylation of IκBα, however, was not impaired by any siRNA treatment (Fig. 3B). These results suggest that some HSP70s (HSPA1A and HSPA8) regulate the activity of the IKKβ/IκBα/NF-κB p65 complex, especially at the step of NF-κB p65 phosphorylation. At present, we cannot exclude the possibility that HSP70s contribute to other steps of NF-κB activation.

3.4 A Drosophila homolog of HSPA8 is required for innate immunity.

To investigate the evolutionarily conserved role of HSP70 family proteins in the NF-κB

pathway, we examined the functions of HSC70-4, a *Drosophila* homolog of HSPA8, in innate immunity. HSC70-4 was knocked down in the fat body, the core tissue of fly immunity, using a Gal4-induced RNAi system [13]. Gram-negative bacterium (*Ecc15*) was injected into the body cavity, and fly survival was measured. As a control experiment, *relish* mutant flies defective for the Imd pathway succumbed rapidly after *Ecc15* infection, but control flies did not (Fig. 4A). Both of two HSC70-4 knockdown lines (R1 and R2) were susceptible to *Ecc15* infection (Fig. 4A), but their survival rates differed remarkably, probably because of variable knockdown efficiency. One knockdown line (R1) showed a slight and gradual decrease in survival even after saline injection (Fig. 4B), suggesting that HSC70-4 knockdown confers sensitivity to stress conditions. Thus, our results indicate that HSC70-4, like Droj2, plays important roles in host defense in fly immunity [13]. We suggest that the HSP70/HSP40 chaperone/cochaperone complex regulates NF-κB activity in an evolutionarily conserved manner.

4. Discussion

Chaperone/cochaperone complexes have several functions in protein homeostasis and signaling [15,16], and previous studies suggested that such a complex has a role in NF-κB signaling. The HSP90 chaperone and Cdc37 cochaperone are essential for stabilizing the IKK signalosome, the IKKα/IKKβ/IKKγ complex in the tumor necrosis factor-stimulated NF-κB pathway [19]. HSP70 binds to NEMO (IKKγ) and consequently suppresses NF-κB signaling during cell apoptosis [20]. Here, we suggest that HSP70s and DNAJA3 stabilize the IKKβ/IκBα/NF-κB p65 complex to activate NF-κB signaling during innate immune responses. Thus, several chaperone/cochaperone complexes contribute to distinct steps of the NF-κB pathway in a context-dependent manner.

Progressive reactions of the IKKβ/IκBα/NF-κB p65 complex are involved in NF-κB signaling: phosphorylation of each component, proteasomal degradation of IκBα, and translocation of NF-κB to the nucleus. We found that the HSP70 inhibitor suppressed the transcriptional activity of NF-κB p65 in response to flagellin stimulation. Under this condition, the IKKβ/IκBα/NF-κB p65 complex became unstable and NF-κB p65 phosphorylation was suppressed. Therefore, we suggest that HSP70 proteins control the activation of NF-κB by stabilizing the IKKβ/IκBα/NF-κB p65 complex and subsequently phosphorylating NF-κB p65. Furthermore, we found that an HSP70 inhibitor did not impair IκBα phosphorylation after flagellin stimulation. This suggests that IκBα phosphorylation proceeds without HSP70 chaperone, but is not sufficient for activating the NF-κB pathway. The dynamic interactions of the chaperone/cochaperone with the IKKβ/IκBα/NF-κB p65 complex during NF-κB activation require further analysis.

In this study, we identified HSPA1A, HSPA8, and HSPA9 as the partners associated with

DNAJA3 in HEK293 cells. Indeed, knockdown of HSPA1A and HSPA8, but not HSPA9, suppressed the phosphorylation of NF-κB p65 in response to flagellin stimulation. These observations are consistent with the fact that HSPA1A and HSPA8 are cytosolic HSP70 isoforms, while HSPA9 is the mitochondrial isoform [18]. Further studies should be performed to clarify whether HSPA1A and HSPA8 act redundantly on NF-κB activation. It is also important to investigate the involvement of other HSP70 family proteins at all steps of NF-κB activation. These future studies would reveal the biological roles of chaperone/cochaperone complexes for fine-tuning the NF-κB signaling in immune systems.

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Figure legends

Fig. 1 Western blotting analysis of the IKKβ/IκBα/NF-κB p65 complex under DNAJA3 knockdown.

(A) HEK293 cells were transfected with DNAJA3 or control (non-target) siRNA. Cell lysates (Input) were immunoprecipitated with anti-IKKβ antibody (IP). Western blotting analysis was performed using anti-IKKβ, IκBα, NF-κB p65, DNAJA3, and α-tubulin antibodies. (B) Quantifications of the band intensity in A. The relative values normalized by the intensity of control cells are shown. Similar results were obtained from two or three independent experiments (data not shown).

Fig. 2. Effects of the HSP70 inhibitor on NF-κB activity and the IKKβ/IκBα/NF-κB p65 complex.

(A) Luciferase reporter assay in HEK293 cells transfected with NF-κB-Luc, treated with PES, and stimulated with flagellin. Values represent the mean ± S.E. of triplicate samples. (B) Western blot analysis of cell lysates after the PES-treatment. The following antibodies were used: anti-IKKβ, anti-IκBα, anti-NF-κB p65, and anti-actin. (C) Quantifications of the band intensity in B. The relative values normalized by the intensity of control (DMSO-treated) cells are shown. (D) Cell lysates (Input) were immunoprecipitated with anti-IKKβ antibody (IP). Western blotting analysis was performed using the following antibodies: anti-phospho-IKKα/β, IKKβ, phospho-IκBα, IκBα, phospho-NF-κB p65, NF-κB p65, HSP70, and actin antibodies. (E) Quantification of the band intensity in D. The relative values normalized by the intensity of control cells are shown. Similar results were obtained from two or three independent experiments

(data not shown).

Fig. 3 Identification of DNAJA3-interacting proteins .

(A) SDS-PAGE gels with silver staining for analyzing DNAJA3-interacting proteins. HEK293 cells were transfected with pcDNA3 or DNAJA3-FLAG constructs. Cell lysates were immunoprecipitated with anti-FLAG antibody. The bands around 70 kDa molecular mass (asterisk) were extracted and analyzed by liquid chromatography-tandem mass spectrometry. (B) Western blot analysis of cell lysates after treatments of siRNAs against MyD88, HSPA1A, HSPA8, and HSPA9, and non-target siRNA (control). The following antibodies were used : anti-phospho-IKKα/β, anti-IKKβ, anti-phospho-IκBα, anti-IκBα, anti-phospho-NF-κB p65, anti-NF-κB p65, anti-DNAJA3, anti-HSP70, and anti-actin. Similar results were obtained from two or three independent experiments (data not shown).

Fig. 4 Effects of *Drosophila* HSC70-4 knockdown on the host defense.

(A) Survival after injection of *Ecc15* suspensions was measured in control (n = 65), HSC70-4 knockdown (KD) R1 (n = 70), KD R2 (n = 68), and *relish* mutant (n = 61) flies. (B) Survival after injection of saline was measured in control (n = 60), HSC70-4 KD R1 (n = 69), KD R2 (n = 64), and relish mutant (n = 57) flies. Survival rates marked with different letters (a, b, c, d) represent statistical significant differences (Log-rank test, and Tukey's HSD post hoc test, p-value < 0.05).

Figure

Figure 1

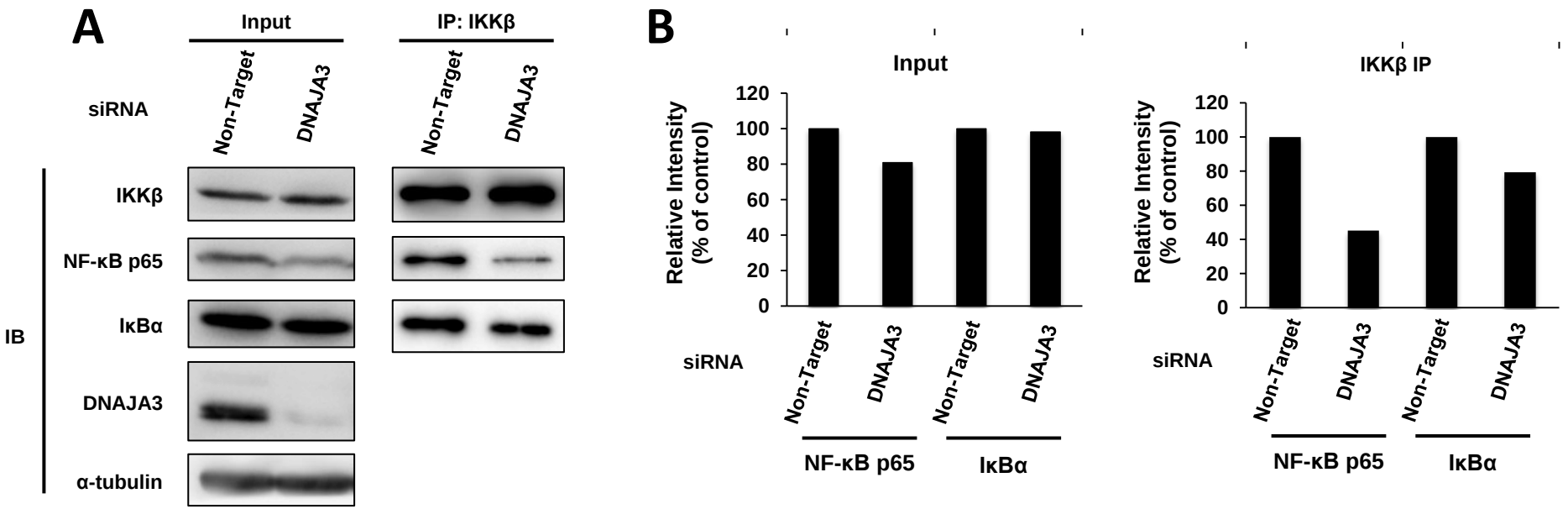

Figure 2

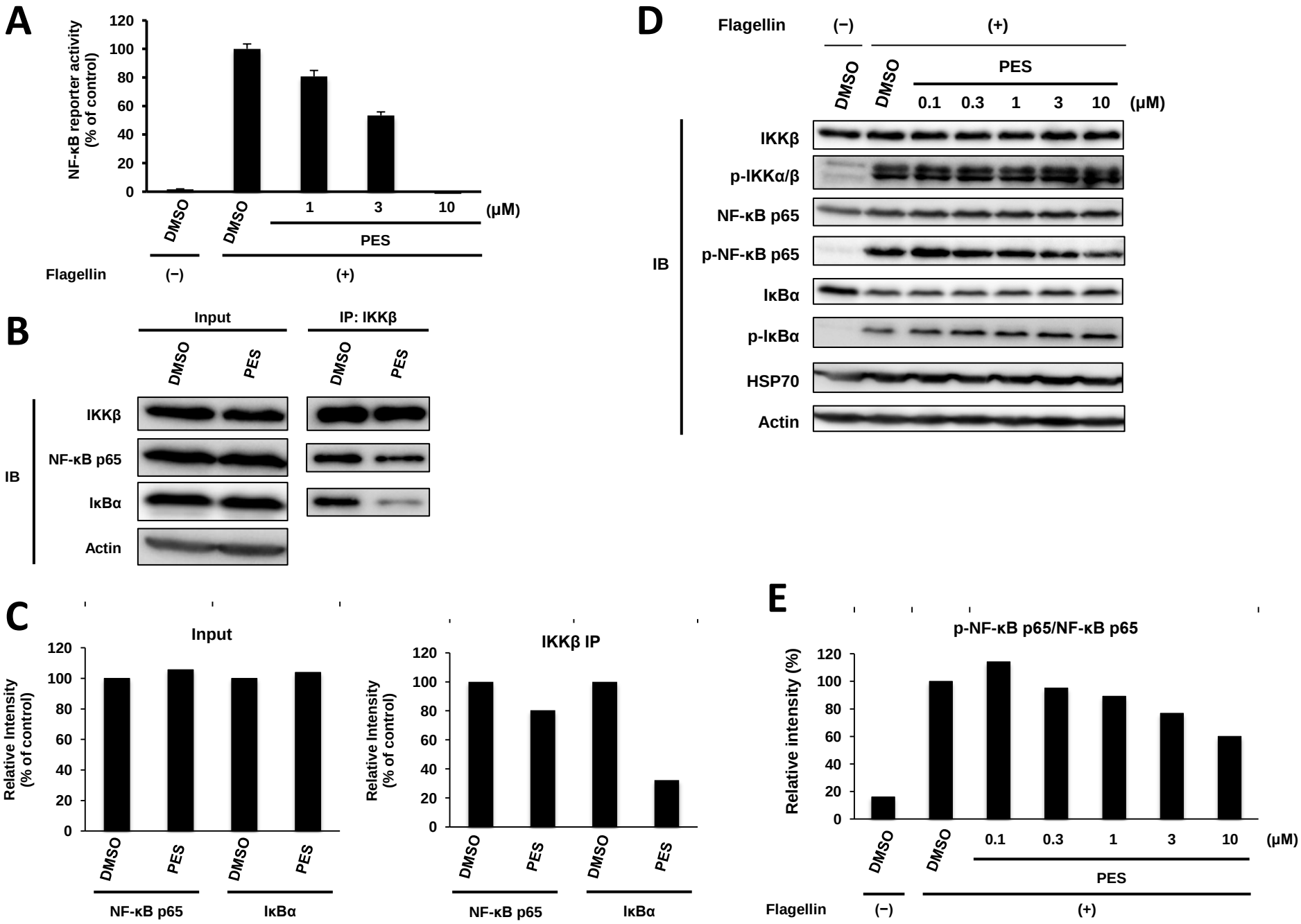
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Figure 3

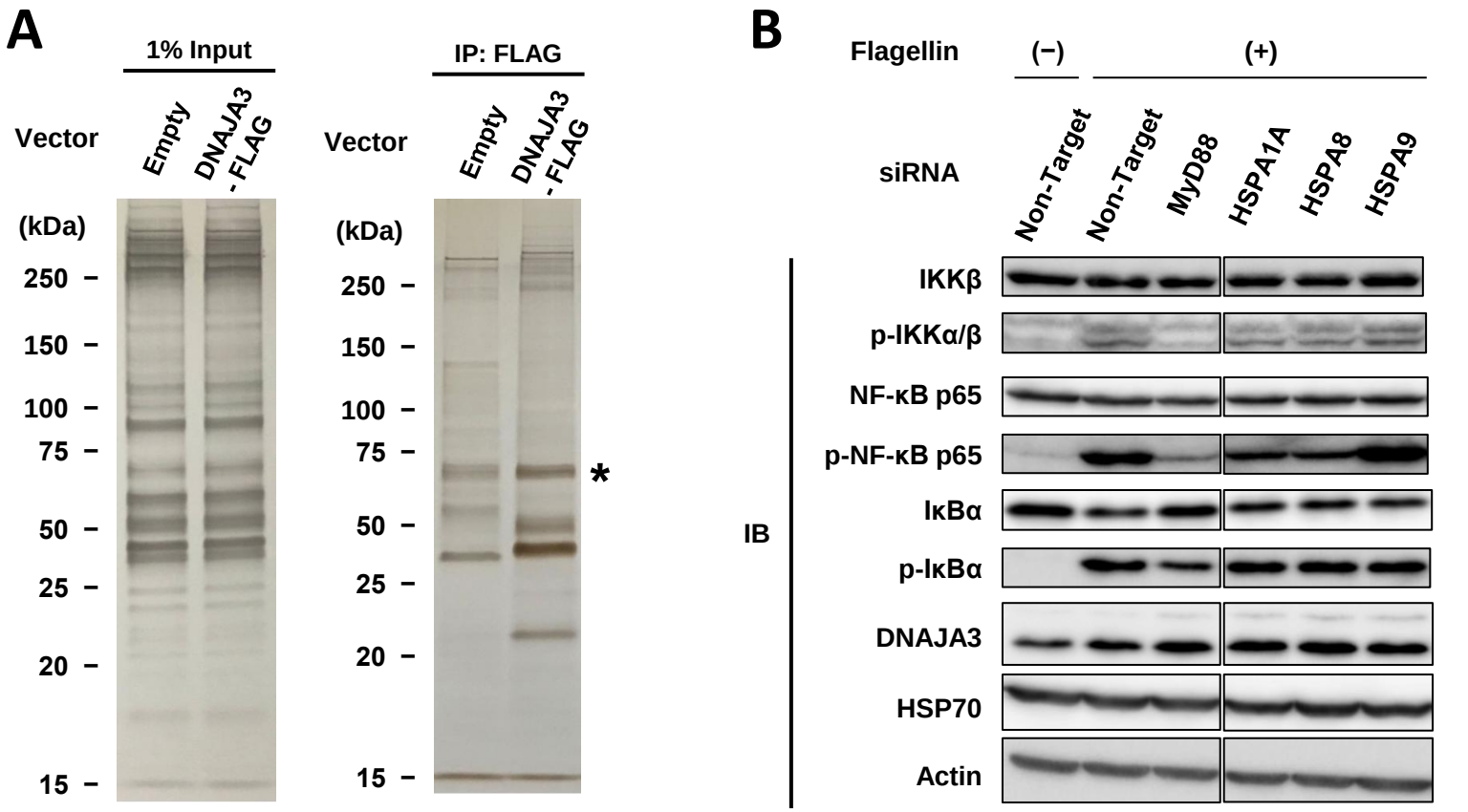
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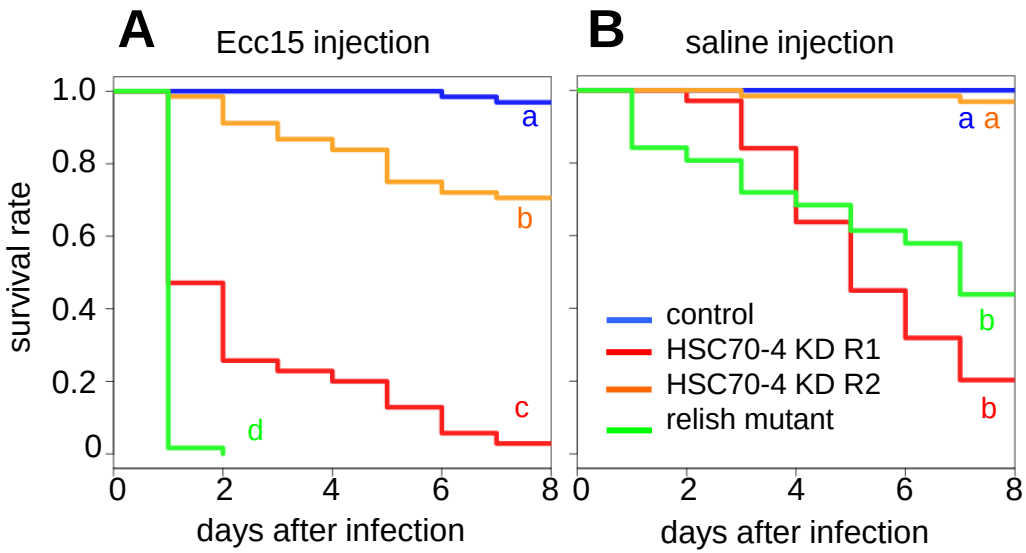
Figure 4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control
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HSC70-4 KD R2
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