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LETTER TO THE EDITOR

The E3 ubiquitin ligase MIB1 negatively regulates basal IκBα level and modulates NF-κB activation

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Dear Editor,

Nuclear factor (NF)-κB is a family of transcription factors, which are critically involved in regulation of divergent physiological and pathological processes, such as apoptosis, tumorigenesis and immune response. The NF-κB family consists of five members, p65 (RelA), RelB, c-Rel, p100 (the precursor of p52) and p105 (the precursor of p50). The combinations between them produce a series of homodimers and heterodimers, such as p65:p50, c-Rel:p50, p50:p50, and so on, which play central roles in transcriptional regulation of genes involved in a wide range of functions from adaptive and innate immune responses to development of immune cells [1, 2].

A wide range of stimuli induce NF-κB activation, such as various cytokines and certain components of microbes. The best studied examples of NF-κB activation are induced by the proinflammatory cytokines tumor necrosis factor (TNF)α and interleukin (IL)-1β. These cytokines signal activation of a serine/threonine kinase complex called IKK through various adapter proteins and E3 ubiquitin ligases [3, 4]. In fact, it is now well known that various NF-κB activation pathways converge at IKK, which consists of two kinase subunits IKKα and IKKβ, and a regulatory subunit IKKγ. Activated IKK phosphorylates IκBα (that is bound to the p65:p50 heterodimer) at Ser32 and Ser36. This phosphorylation enables IκBα to be recognized and ubiquitinated by a large ubiquitin ligase $SCF^{\beta TrCP}$ complex and degraded by the proteasomes [5-8]. This frees NF-κB, which is then translocated into the nucleus to drive transcription of downstream genes.

IκBα is the prototypic member of the IκB family, which also contains IκBβ and IκBε [1]. IκBα exists in an equilibrium between free and p65:p50-bound forms. The signal-induced cascades that result in the phosphorylation and thereby polyubiquitination and subsequently 26S proteasome-mediated degradation of IκBα have been well studied [9]. Several studies have gained insights into the mechanisms of free IκBα turnover under physiological conditions. It has been reported that the turnover of free IκBα is intrinsic, which is correlated with the unstructured and unfolded ANK repeats and C-terminal PEST sequences, and is mediated directly through the 20S proteasomes without the requirement of its ubiquitination [10, 11]. However, detailed mechanisms are still unknown for the control of IκBα level in unstimulated cells.

To identify additional proteins that regulate NF-κB activity, we performed unbiased expression screens for enzymes that can regulate NF-κB activity in reporter assays. From 288 clones that encode ubiquitinating and deubiquitinating enzymes, we identified the E3 ubiquitin ligase MIB1 as a positive regulator of NF-κB activation. Previously, it has been reported that MIB1 acts as an E3 ubiquitin ligase that targets Delta, the ligand of Notch-signaling pathway, for ubiquitination and subsequent endocytosis, which is essential for the activation of Notch signaling. To confirm the positive regulatory effect of MIB1 on NF-κB activation, we determined whether MIB1 could activate NF-κB in a dose-dependent manner. As shown in Figure 1A, MIB1 activated NF-κB in a dose-dependent manner in 293 cells in reporter assays. Moreover, MIB1 potentiated TNFα- and IL-1β-induced activation of NF-κB (Figure 1B). In contrast, overexpression of MIB1 had no marked effect on IFNγ-induced activation of the IRF1 promoter (Supplementary information, Figure S1). These data suggest that MIB1 plays a specific role in mediating NF-κB activation.

Previous studies demonstrate that MIB1 is an E3 ubiquitin ligase, which contains a zinc finger domain at its N-terminus, ankyrin-repeats domain in the middle and a Ring finger domain at its C-terminus [12]. It has been reported that the C-terminal Ring finger domain of MIB1 is important for its E3 enzyme activity. To determine which domains of MIB1 are critical for the activation of NF-κB, we made several deletion mutants. Reporter assays indicated that deletion of the N-terminal zinc finger domain reduced the ability of MIB1 to activate NF-κB, whereas deletion of the C-terminal Ring finger domain of MIB1 completely abolished its ability to activate NF-κB (Figure 1C). These results suggest that the C-terminal Ring finger domain is required for the ability of MIB1 to

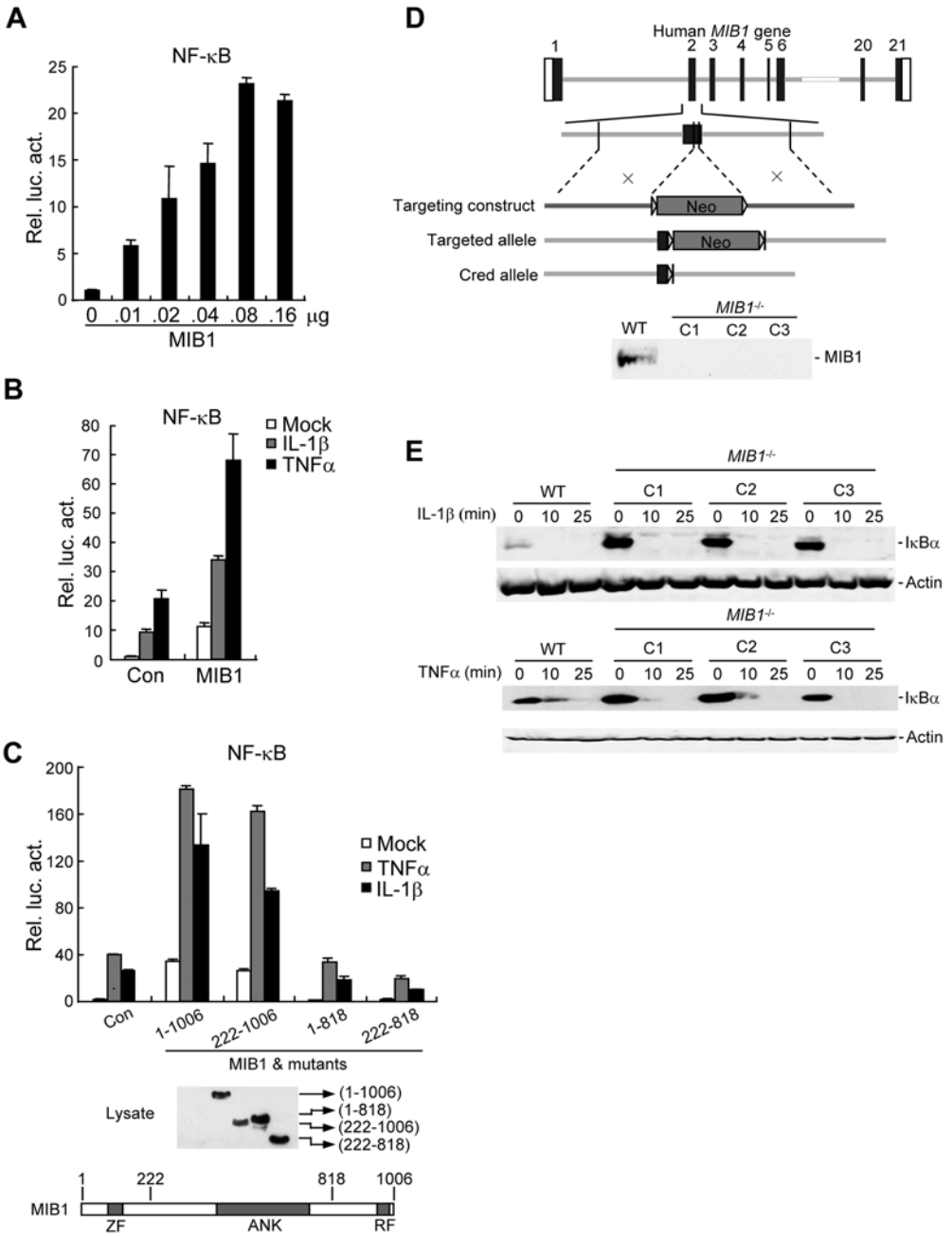

Figure 1 MIB1 mediates NF-κB activation and negatively regulates IκBα level. **(A)** MIB1 activates NF-κB in a dose-dependent manner in 293 cells. 293 cells (1×10^5) were transfected with NF-κB luciferase reporter plasmid (0.02 μg) and the indicated amount of expression plasmids for MIB1. Luciferase assays were performed 24 h after transfection. **(B)** Effect of MIB1 on IL-1β- and TNFα-induced activation of NF-κB. 293 cells (1×10^5) were transfected with NF-κB luciferase reporter plasmid (0.02 μg) and a control or the expression plasmid for MIB1 (0.1 μg). At 20 h after transfection, cells were left untreated or treated with TNFα or IL-1β (20 ng/ml) for 8 h before luciferase assays were performed. **(C)** Effect of MIB1 and their mutants on IL-1β- and TNFα-induced activation of NF-κB. 293 cells (1×10^5) were transfected with NF-κB luciferase reporter plasmid (0.02 μg), and a control or expression plasmids for MIB1 or its mutants (0.1 μg). At 20 h after transfection, cells were left untreated or treated with TNFα or IL-1β (20 ng/ml) for 8 h before luciferase assays were performed. Expression of MIB1 and its mutants was examined by immunoblot analysis with anti-Flag. **(D)** Strategy for site-specific gene disruption for the generation of human MIB1 knockout cells. The site targeted by the construct is in exon 2. The disrupted positive clones were analyzed by immunoblot with anti-MIB1. **(E)** Immunoblot analysis of IκBα in wild-type and MIB1 knockout HCT116 cells. Wild-type or MIB1 knockout HCT116 cells (4×10^5) were treated with TNFα (20 ng/ml) or IL-1β (20 ng/ml) for the indicated time, or left untreated. The cell lysates were analyzed by immunoblot with the indicated antibodies.

activate NF-κB, while the N-terminal Zinc finger domain of MIB1 contributes to its full activity.

To investigate the mechanisms by which MIB1 activates NF-κB, we attempted to generate MIB1 knockout cells, utilizing a recently reported method [13]. Following these procedures, we generated three independent *MIB1* gene knockout clones in human colon cancer HCT116 cells. As expected, MIB1 expression was completely deficient in these clones, as analyzed by western blot with anti-MIB1 antibody (Figure 1D).

Since degradation of IκBα is a key event in activation of NF-κB, we determined whether the level of IκBα was influenced by MIB1 deficiency. Surprisingly, the level of IκBα was dramatically upregulated in MIB1-deficient cells in comparison to their wild-type counterparts (Figure 1E). Treatment of cells with IKK inhibitor BMS-345541 [14] reversed the upregulation of IκBα level caused by MIB1 deficiency (Supplementary information, Figure S2), suggesting that the effect of MIB1 on IκBα level needs IKK activity. Very interestingly, TNFα or IL1β treatment caused degradation of the upregulated IκBα in MIB1-deficient cells as well as in their wild-type counterparts (Figure 1E). In contrast, the amounts of other tested proteins that are related to NF-κB activation, including IκBβ, IKKα, IKKγ, p65, p50, p100 and p52, were not changed by MIB1 deficiency (Supplementary information, Figure S3). These data suggest that MIB1 negatively regulates the stability of IκBα under endogenous conditions.

We further examined the roles of MIB1 in TNFα- or IL1β-induced gene transcription. We found that basal transcription of *IκBα*, a downstream target gene induced by TNFα or IL1β, was not markedly affected in MIB1-deficient cells in comparison to their wild-type counterparts (Supplementary information, Figure S4), suggesting that the elevated level of IκBα in MIB1-deficient cells is caused by a posttranscriptional mechanism. Interestingly, TNFα- or IL1β-induced transcription of *IκBα* gene was increased in MIB1-deficient cells in comparison to their wild-type counterparts. Similar observations were found for other TNFα- or IL1β-induced downstream genes, such as *cIAP1*, *ICAM1*, *TNFα*, *Rantes* and *CXCL10* (Supplementary information, Figure S4). These results suggest that MIB1 plays a negative regulatory role in TNFα- or IL1β-induced transcription of downstream genes.

Previous studies have demonstrated that there are two forms of IκBα in resting cells. The majority of them bind to p65:p50 heterodimers, the rest exists in free form. As shown in Supplementary information, Figure S5A, the amount of p65-bound IκBα was markedly raised in *MIB1*$^{-/-}$ cells, compared with that in wild-type cells. Moreover, the degradation rate of IκBα in MIB1-deficient cells as measured by treatment with protein synthesis inhibitor cycloheximide was lower in comparison to the wild-type cells (Supplementary information, Figure S5A). These results suggest that MIB1 deficiency leads to increased association of IκBα with NF-κB. Because the amount of p65-bound IκBα is higher in MIB1-deficient cells than in the wild-type controls, however, the ratio of p65-bound IκBα to free form is not changed by MIB1 deficiency.

To further investigate the relationship between MIB1 and IκBα, we performed coimmunoprecipitation experiments. As shown in Supplementary information, Figure S5B, MIB1 interacted with IκBα in the mammalian overexpression system. Furthermore, MIB1 enhanced the ubiquitination of IκBα, whereas a deletion mutant lacking the C-terminal Ring finger domain failed to do so (Supplementary information, Figure S5C). Moreover, the ubiquitination of endogenous IκBα was decreased in MIB1-deficient cells in comparison with the wild-type cells upon MG132 treatment (Supplementary information, Figure S5D). These results suggest that MIB1 is associated with IκBα and partially mediates its ubiquitination. Currently, we are not able to demonstrate endogenous association between MIB1 and IκBα. One possibility is that this association is indirect or of low affinity in cells. We are also not able to demonstrate an interaction between p65 and MIB1 in both the overexpression system and at endogenous level. It is possible that MIB1 is only associated with free but not p65-bound IκBα, or the sensitivity of the coimmunoprecipitation experiments is not sufficient to detect such an interaction.

The ability of MIB1 to regulate the level of IκBα is mediated by its E3 ubiquitin ligase activity for several reasons. First, the Ring finger domain of MIB1 was required for its ability to activate NF-κB; second, MIB1 was capable of causing IκBα ubiquitination when overexpressed; third, MIB1 deficiency caused decreased ubiquitination of IκBα. However, currently we do not know whether MIB1 directly or indirectly ubiquitinates IκBα, primarily due to our failure to obtain full-length recombinant active MIB1 for *in vitro* ubiquitination assays. In our experiments, we found that knockout of MIB1 only partially inhibited the ubiquitination of IκBα. The simplest explanation is that MIB1 is not the unique enzyme that targets IκBα for ubiquitination.

In our experiments, we found that MIB1 deficiency caused upregulation of total IκBα level as well as p65-bound IκBα. Since free and p65-bound IκBα exist in equilibrium, we cannot distinguish whether free or p65-bound IκBα, or both are upregulated by MIB1 deficiency. Nevertheless, upregulation of IκBα caused by MIB1 de-

ficiency decisively demonstrates that MIB1 plays an important role in keeping IκBα at a proper level in unstimulated cells. Previously, it has been demonstrated that MIB1 is upregulated in various cancers, it is possible that MIB1 contributes to tumorigenesis through activation of NF-κB, a transcription factor critically involved in cell survival and antiapoptotic response. Our studies also indicate that MIB1 negatively regulates TNFα- and IL1β-induced NF-κB target gene activation. However, the mechanism of this action is unknown at this time. Taken together, our studies suggest that MIB1 plays opposite roles in regulating basal and induced NF-κB activation. Our findings may provide new clues to the complicated regulatory networks of NF-κB activation, as well as its regulation of physiological and pathological processes such as tumorigenesis and inflammation. Experimental materials and methods are described in the Supplementary information, Data S1.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)