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# MafK positively regulates NF- $\kappa$ B activity by enhancing CBP-mediated p65 acetylation

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Reactive oxygen species, produced by oxidative stress, initiate and promote many metabolic diseases through activation/suppression of redox-sensitive transcription factors. NF- $\kappa$ B and Nrf2 are important regulators of oxidation resistance and contribute to the pathogenesis of many diseases. We identified MafK, a novel transcriptional regulator that modulates NF- $\kappa$ B activity. MafK knockdown reduced NF- $\kappa$ B activation, whereas MafK overexpression enhanced NF- $\kappa$ B function. MafK mediated p65 acetylation by CBP upon LPS stimulation, thereby facilitating recruitment of p65 to NF- $\kappa$ B promoters such as IL-8 and TNF $\alpha$ . Consistent with these results, MafK-depleted mice showed prolonged survival with a reduced hepatic inflammatory response after LPS and D-GalN injection. Thus, our findings reveal a novel mechanism by which MafK controls NF- $\kappa$ B activity via CBP-mediated p65 acetylation.

Nuclear factor kappa B (NF- $\kappa$ B) is a transcription factor with multiple genetic targets and controls various cellular functions including immune inflammatory responses, cell adhesion, differentiation, apoptosis, stress-induced responses, survival, and progression of most chronic diseases<sup>1-3</sup>. NF- $\kappa$ B is directly activated in chronic inflammatory conditions such as cardiovascular, autoimmune, skin, and neurodegenerative diseases, as well as cancer<sup>4</sup>. NF- $\kappa$ B regulates the expression of over 500 genes involved in human diseases, and the NF- $\kappa$ B signalling pathway has become a target for pharmacological intervention<sup>5,6</sup>; however, no NF- $\kappa$ B blocker has been approved for human use. A major challenge is the development of NF- $\kappa$ B inhibitors based on their ability to target specific pathways or cells in different diseases, thereby avoiding undesired side effects<sup>7</sup>.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a basic leucine zipper redox-sensitive transcription factor that regulates antioxidant response element (ARE)-mediated induction of phase II detoxifying and antioxidant enzymes<sup>8,9</sup>. When challenged by oxidants or electrophiles, Nrf2 activates the transcription of over 100 cytoprotective and detoxification genes, including the antioxidants ferritin, glutathione-S-reductase (GSR), glutamyl cysteine ligase-modulator (GCLM), and glutamyl cysteine ligase-catalytic (GCLC), phase-I drug oxidation enzyme NAD(P)H:quinone oxidoreductase 1 (NQO1), and cytoprotective enzyme heme oxygenase-1 (HO-1)<sup>10-12</sup>. Nrf2 is normally retained in the cytoplasm by complex formation with Keap1<sup>13</sup>. In defending the body from oxidative, inflammatory, or toxic stress, Keap1 negatively regulates Nrf2 by enhancing the rate of Nrf2 proteasomal degradation and alters its subcellular distribution. Nrf2 translocates to the nucleus where it forms heterodimeric complexes with other transcription factors such as c-Jun, ATF3, ATF4, and small Mafs, and induces the expression of stress-preventing genes<sup>14,15</sup>.

The molecular mechanisms of oxidant signalling and antioxidant regulation involve various transcriptional factors<sup>16</sup>. In contrast to NF- $\kappa$ B, which plays a key role in oxidative stress and inflammation, Nrf2 is a master regulator of redox signalling and cellular resistance to oxidants; it may therefore ameliorate many diseases<sup>17-19</sup>. Recent evidence has suggested cross talk between Nrf2 and NF- $\kappa$ B under oxidative stress. In particular, the NF- $\kappa$ B p65 subunit represses the Nrf2-ARE pathway by competing for recruitment of the transcription co-activator CBP and HDAC3<sup>20</sup>. Moreover, NF- $\kappa$ B signalling inhibits the Nrf2-ARE pathway through interaction of p65 and Keap1<sup>21</sup>. However, definitive evidence of direct Nrf2 inhibition of NF- $\kappa$ B signalling has not been published.

In this study, we demonstrated that Nrf2 inhibits NF- $\kappa$ B signalling under oxidative stress. We identified the small maf protein MafK as a novel NF- $\kappa$ B-interacting protein *in vitro*. Indeed, MafK is a co-activator or repressor



of Nrf2 transcriptional activity and is therefore a positive regulator of NF- $\kappa$ B. This study establishes the important link between Nrf2-MafK and stress signal generation under oxidative stress via regulation of NF- $\kappa$ B activity.

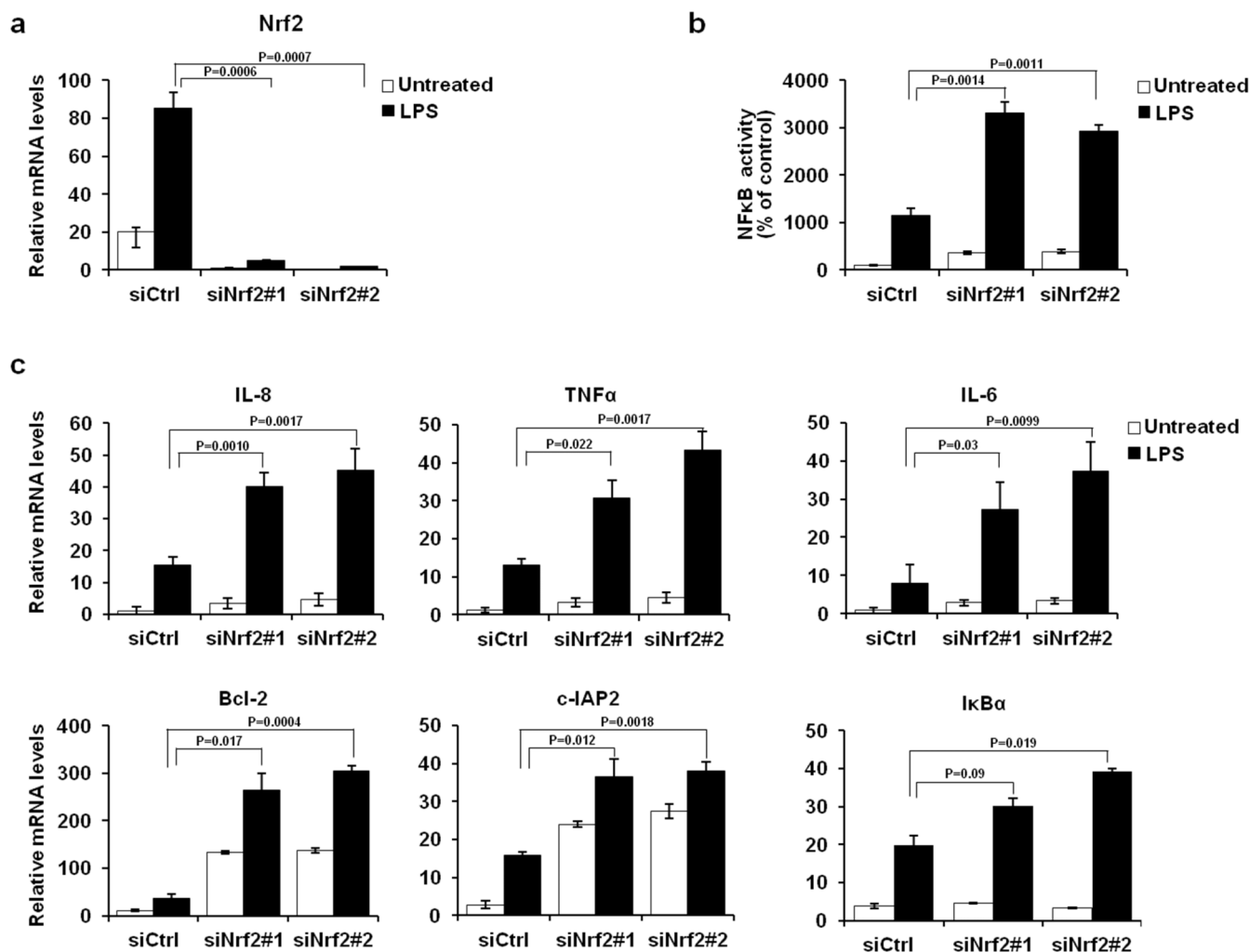
## Results

**Nrf2 negatively regulates NF- $\kappa$ B activity.** Although Nrf2 inhibits NF- $\kappa$ B signalling, the precise mechanism is not fully understood<sup>22</sup>. We transfected human hepatocellular liver carcinoma (HepG2) cells with NF- $\kappa$ B luciferase reporter plasmid and control or Nrf2-specific siRNA. Knockdown of Nrf2 significantly enhanced NF- $\kappa$ B transcriptional activity and NF- $\kappa$ B-dependent transcription in response to LPS (Fig. 1). Nrf2 overexpression reduced NF- $\kappa$ B transcriptional activity and NF- $\kappa$ B-dependent transcription (see Supplementary Fig. S1). These results suggested Nrf2 specifically blocks NF- $\kappa$ B activity.

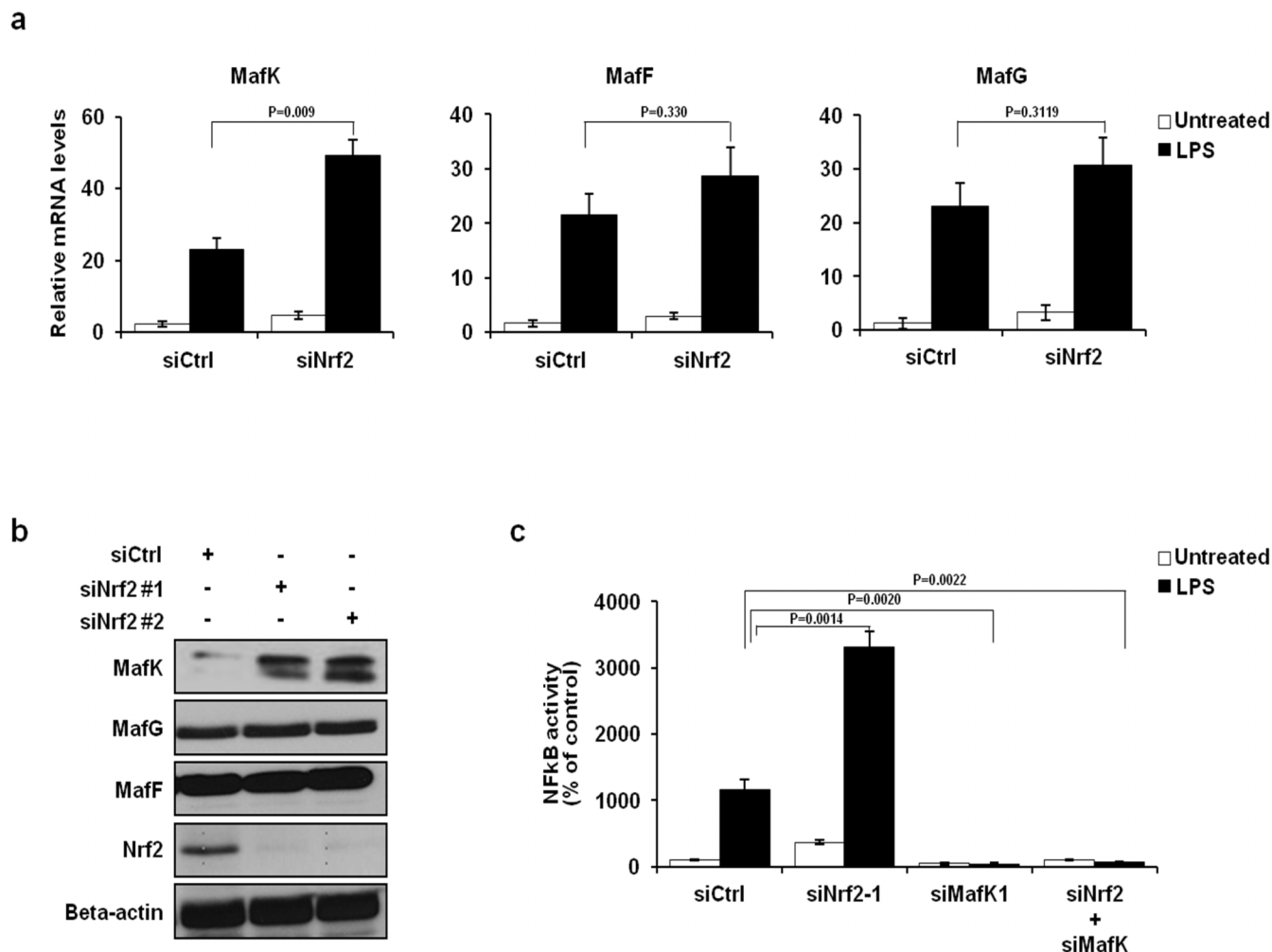
**Nrf2 deficiency upregulated expression of MafK.** Nrf2 forms heterodimers with small molecules, including small maf proteins, which are involved in co-activation or repression of transcriptional activity<sup>23</sup>. To identify novel potential regulators of NF- $\kappa$ B signalling under Nrf2 depletion, we investigated the influence of small maf proteins on Nrf2-regulated NF- $\kappa$ B activity. We analysed the

expression of small maf proteins and observed a significant increase in MafK protein levels in Nrf2-depleted HepG2 cells. Interestingly, MafK transcript levels increased upon Nrf2 depletion, although MafK dimerizes with Nrf2. However, there was no significant difference in the levels of MafG and MafF (Fig. 2a and 2b). Since MafK expression increased upon Nrf2 depletion, we asked whether increased MafK is responsible for enhanced NF- $\kappa$ B response after Nrf2 knockdown. MafK and Nrf2 were simultaneously depleted and NF- $\kappa$ B reporter assays were performed. Elevated NF- $\kappa$ B activity upon Nrf2 knockdown was rescued by MafK depletion, suggesting that MafK plays a key role in Nrf2-regulated NF- $\kappa$ B activity (Fig. 2c).

**MafK regulates NF- $\kappa$ B activity.** We hypothesized that MafK induced by Nrf2 inhibition may directly regulate NF- $\kappa$ B transcriptional activity. To identify the function of MafK in regulating NF- $\kappa$ B signalling, siMafK was simultaneously transfected into HepG2-NF- $\kappa$ B-luciferase reporter cells and NF- $\kappa$ B-dependent reporter activity was evaluated after induction with LPS. As shown in Figure 3a (left panel), LPS-induced activation of NF- $\kappa$ B was inhibited by MafK depletion in HepG2 cells. Similar results were obtained with HeLa cells (see Supplementary Fig. S2). In contrast, ectopic expression of MafK significantly enhanced LPS-induced NF- $\kappa$ B activation ( $p = 0.00026$ , Fig. 3a, right panel). p65 and p50, the



**Figure 1** | Nrf2 negatively regulates NF- $\kappa$ B activity. (a) Nrf2 knockdown by Nrf2 siRNA. HepG2 cells were transfected with control or Nrf2 siRNA. After 24 h, LPS (1  $\mu$ g/mL) was added and the cells were incubated for 24 h and harvested for real-time PCR. (b) Nrf2 knockdown increased NF- $\kappa$ B activity. The NF- $\kappa$ B-response-element-Luc reporter was cotransfected with Nrf2 siRNA into HepG2 cells. After 24 h, cells were treated with LPS. After 24 h, cells were lysed for the luciferase assay. (c) Nrf2 knockdown by Nrf2 siRNA upregulated the expression of NF- $\kappa$ B-responsive genes. For all panels, error bars represent SEM ( $n = 3$ ). P values were calculated using Student's  $t$ -test.



**Figure 2** | Nrf2 deficiency upregulated MafK expression. (a and b) Nrf2 deficiency upregulated the expression of MafK, but not of other small maf proteins. HepG2 cells were transfected with control or Nrf2 siRNA. After 24 h, LPS (1  $\mu$ g/mL) was added and the cells were incubated for 24 h and harvested for real-time PCR or western blot. (c) Elevated NF- $\kappa$ B activity upon Nrf2 knockdown is rescued by MafK depletion. HepG2 cells were transfected with NF- $\kappa$ B-driven reporter plasmid and siMafK or siNrf2 or siMafK and siNrf2. After 24 h, cells were treated with LPS and incubated for 24 h, then lysed for the luciferase assay. For all panels, error bars represent SEM ( $n = 3$ ). P values were calculated using Student's *t*-test.

subunits of NF- $\kappa$ B, were used as positive controls to verify that NF- $\kappa$ B mediates LPS-induced gene expression in HepG2 cells. Transfection with sip65 or sip50 reduced NF- $\kappa$ B activity (see Supplementary Fig. S3).

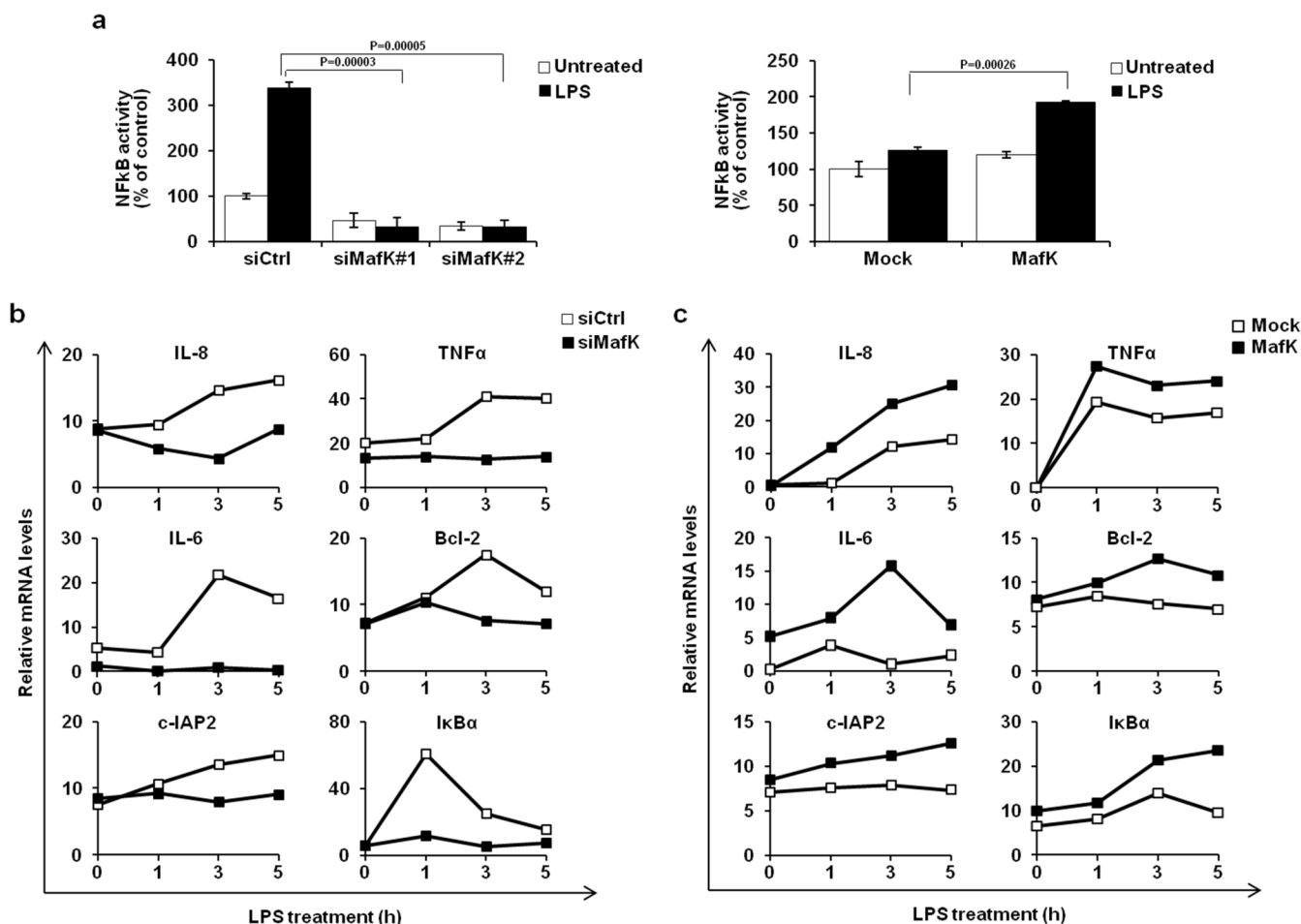
After identifying MafK as a regulator of NF- $\kappa$ B activation, we next sought to determine if MafK also regulates NF- $\kappa$ B-dependent transcription of several key proinflammatory mediators. MafK depletion inhibited LPS-stimulated mRNA expression of IL-8, TNF $\alpha$ , IL-6, Bcl2, c-IAP2, and I $\kappa$ B $\alpha$  in HepG2 and HeLa cells (Fig. 3b and Supplementary Fig. S2). To confirm the effect of MafK, we evaluated NF- $\kappa$ B target gene expression in LPS-stimulated cells with ectopically expressed MafK. As shown in Figure 3c, MafK overexpression enhanced LPS-induced mRNA expression of IL-8, TNF $\alpha$ , IL-6, Bcl2, c-IAP2, and I $\kappa$ B $\alpha$ . Depletion of p65 and p50 inhibited the LPS-induced expression of NF- $\kappa$ B target genes (see Supplementary Fig. S3). Together, these results suggest MafK is an important factor for NF- $\kappa$ B activation.

**MafK regulates NF- $\kappa$ B activation via p65 acetylation.** We next sought to determine how MafK enhances NF- $\kappa$ B-dependent signaling. Upon stimulation with an inducer such as LPS or TNF $\alpha$ , I $\kappa$ B $\alpha$  is phosphorylated, which results in the degradation and dissociation of I $\kappa$ B $\alpha$  from NF- $\kappa$ B, which translocates to the nucleus and induces expression of target genes<sup>24</sup>. Thus, we determined whether MafK

regulates LPS-induced NF- $\kappa$ B activation by altering phosphorylation and degradation of I $\kappa$ B $\alpha$ . As shown in Figure 4a, knockdown of MafK did not produce a significant inhibitory effect on LPS-induced I $\kappa$ B $\alpha$  degradation and phosphorylation. Similar results were obtained in LPS-stimulated HepG2 cells overexpressing MafK (see Supplementary Fig. S4).

As MafK did not affect cytosolic NF- $\kappa$ B signalling, we investigated the effect of MafK on nuclear translocation of p65 and found no significant difference between control and MafK-knocked down cells (Fig. 4b). Thus, MafK-mediated regulation of NF- $\kappa$ B occurs within the nucleus. Because DNA binding of the NF- $\kappa$ B complex is a critical event in transcriptional regulation<sup>25</sup>, we used EMSA to investigate the effect of MafK on DNA binding of NF- $\kappa$ B upon LPS stimulation. Overexpression of MafK induced binding between DNA and NF- $\kappa$ B (see Supplementary Fig. S5). In addition, we investigated the effect of MafK on LPS-induced DNA binding of NF- $\kappa$ B using chromatin immunoprecipitation (ChIP). Binding of p65 and acetyl-p65 to the IL-8 and TNF $\alpha$  promoters was weakened in MafK-knocked down cells (Fig. 4c). Our data suggest MafK regulates NF- $\kappa$ B-dependent transcription at its promoter region rather than at a point upstream in the NF- $\kappa$ B signalling pathway.

Post-translational modifications, particularly acetylation, play a critical role in NF- $\kappa$ B activation by enhancing the DNA-binding activity of p65<sup>26,27</sup>. Because MafK may recruit CBP<sup>20</sup>, we



**Figure 3 | MafK regulates NF- $\kappa$ B-dependent gene expression.** (a) Regulation of NF- $\kappa$ B activity after MafK knockdown or overexpression. The NF- $\kappa$ B-response-element-Luc reporter was cotransfected with MafK siRNA or MafK plasmids into HepG2 cells. After 24 h, cells were treated with LPS and incubated for 24 h, then lysed for the luciferase assay. Expression of firefly luciferase reporter was normalized to *Renilla* luciferase expression. (b) Cells were transfected with MafK or control siRNA for 48 h. Cells were treated with LPS (1  $\mu$ g/mL) followed by measurement of NF- $\kappa$ B-dependent gene expression in lysates after 1, 3, and 5 h. (c) Cells were transfected with MafK or control plasmids for 48 h. Cells were treated with LPS and NF- $\kappa$ B-dependent gene expression was measured after 1, 3, and 5 h.

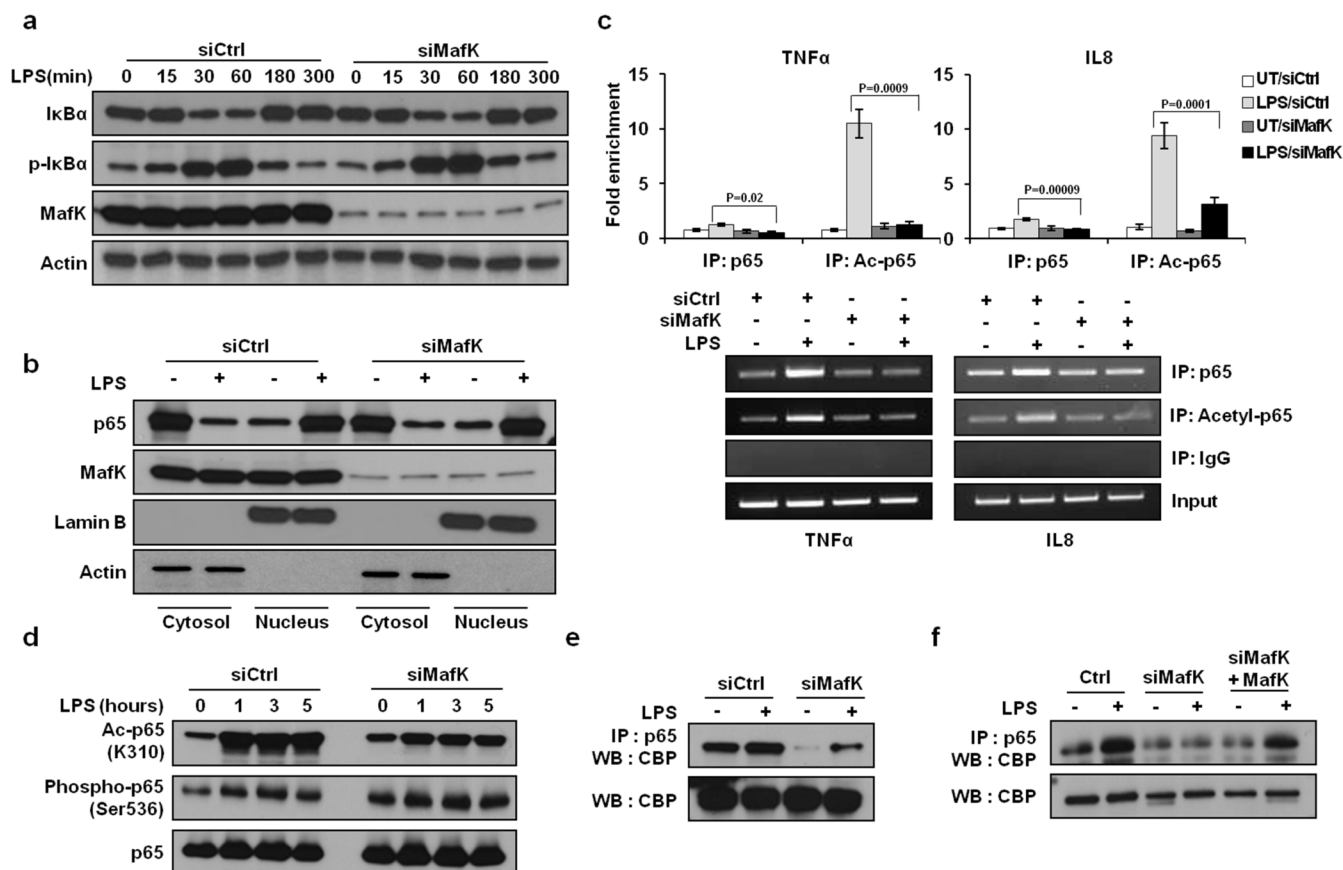
hypothesized that MafK regulates the DNA-binding activity of NF- $\kappa$ B by increasing acetylation of p65 at lysine 310 (K310). Indeed, p65 acetylation at K310 was markedly reduced by MafK ablation. Acetylation of p65 is dependent on p65 phosphorylation<sup>28</sup> and in this study, p65 acetylation was decreased, and phosphorylation at Ser536 remained unaffected in MafK-knocked down cells (Fig. 4d). Similar results were obtained in LPS-stimulated HepG2 cells overexpressing MafK (see Supplementary Fig. S6). CBP/p300 acetyltransferase plays a major role in p65 acetylation<sup>29</sup>, and we observed increased p65-CBP binding after LPS stimulation; this process is essential for NF- $\kappa$ B enhancosome assembly. Interestingly, binding between p65 and CBP was reduced in the absence of MafK and overexpression of MafK triggered p65-CBP interaction (Fig. 4e, 4f and Supplementary Fig. S6). We also confirmed that p65 acetylation by MafK involves the histone acetyltransferase (HAT) activity of CBP/p300, which increased in MafK-overexpressing HepG2 cells. However, HAT activity did not increase in MafK-overexpressing HepG2 cells treated with the CBP inhibitor C464 (see Supplementary Fig. S6). These data demonstrate that MafK regulates the DNA-binding activity of NF- $\kappa$ B by enhancing p65 acetylation and recruiting CBP.

**MafK interacts with p65 and regulates NF- $\kappa$ B activation.** MafK regulates NF- $\kappa$ B activation and subsequent inflammatory responses

by mediating DNA-binding activity of the NF- $\kappa$ B complex. The ability of MafK to regulate p65 activity through posttranslational regulation led us to use immunoprecipitation analysis to characterize the interaction between MafK, p65, and CBP. Exogenously expressed MafK and p65 interacted in HepG2 cells (Fig. 5a, left panel, and Supplementary Fig. S7), as did endogenous MafK and p65 (Fig. 5b, left panel). In contrast, p50 did not interact with MafK (Fig. 5a and 5b, right panels). Interestingly, MafK also interacted with CBP (Fig. 5c). The ability of MafK to interact with p65 and CBP led us to examine whether MafK controls complex formation between p65 and CBP. HepG2 cells were transfected with p65 and CBP with or without MafK and treated with LPS; western blotting of the immunoprecipitates showed that p65 interacts with CBP (Fig. 5d). These interactions increased in the presence of MafK, suggesting MafK may enhance p65-CBP complex formation, thereby increasing p65 acetylation.

**MafK deficiency triggered TNF $\alpha$ -induced apoptosis.** The functions of TNF $\alpha$  occur through TNF receptor triggering, which initiates either a prosurvival pathway through activation of NF- $\kappa$ B or a proapoptotic pathway through caspase activation<sup>41</sup>. MafK regulates NF- $\kappa$ B activation by modulating p65 acetylation after LPS stimulation; MafK would also regulate NF- $\kappa$ B and p65 acetylation in TNF $\alpha$ -induced cells. We treated MafK-depleted HepG2 cells with TNF $\alpha$





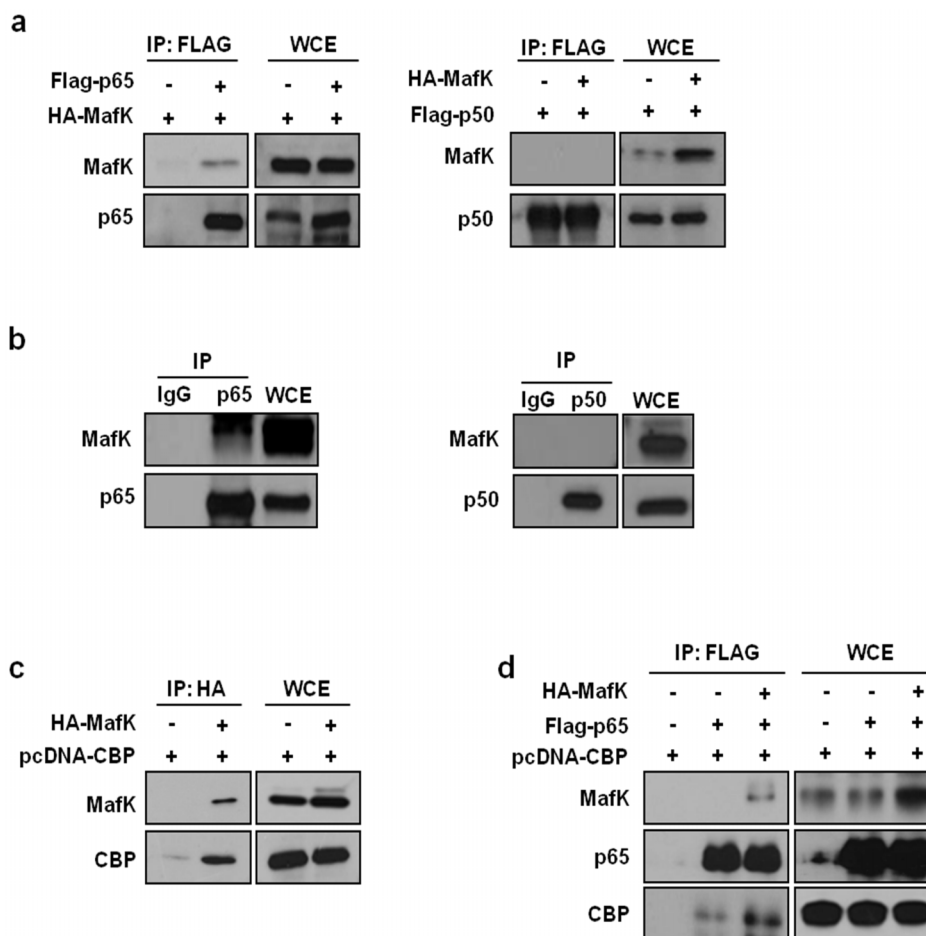
**Figure 4 | MafK deficiency inhibits NF- $\kappa$ B DNA binding.** (a) HepG2 cells were transfected with MafK or control siRNA and after 48 h, cells were treated with LPS (1  $\mu$ g/mL) followed by measurement of I $\kappa$ B $\alpha$  degradation and phosphorylation in lysates after 15, 30, 60, 180, and 300 min. (b) HepG2 cells were transfected with MafK or control siRNA and after 48 h, cell were stimulated with LPS for 30 min. Nuclear and cytoplasmic fractions were analysed by immunoblotting. (c) HepG2 cells were stimulated with LPS for 6 h, followed by chromatin immunoprecipitation using anti-p65, anti-Acetyl-p65, and normal rabbit IgG (control) antibodies. Chromatin immunoprecipitation was analysed using primers specific to the IL-8 and TNF $\alpha$  promoter regions. (d) MafK deficiency suppressed p65 acetylation but not affected phosphorylation. HepG2 cells were transfected with MafK or control siRNA, then stimulated with LPS as indicated time. (e) HepG2 cells were transfected with MafK or control siRNA for 48 h, and then treated with LPS and incubated for 6 h, collected, and lysed in immunoprecipitation lysis buffer. Whole-cell lysates were immunoprecipitated with human anti-p65 antibody and probed by western blotting with rabbit anti-CBP antibodies. (f) HepG2 cells were transfected with MafK siRNA, or MafK siRNA and MafK plasmid. After 48 h, cells were treated with LPS and incubated for 6 h; the cells were collected and lysed in immunoprecipitation lysis buffer. Whole-cell lysates were immunoprecipitated with human anti-p65 antibody and probed by western blotting with rabbit anti-CBP antibody.

and found that TNF $\alpha$  treatment reduced NF- $\kappa$ B activity and expression of NF- $\kappa$ B-dependent genes in MafK-depleted cells (see Supplementary Fig. S8, and Fig. 6a). MafK deficiency did not alter TNF $\alpha$ -induced I $\kappa$ B $\alpha$  degradation and phosphorylation (see Supplementary Fig. S8). To rule out general defects in NF- $\kappa$ B activation in MafK-depleted cells, we treated the cells with IL-1 $\beta$ , another proinflammatory cytokine. IL-1 $\beta$ -induced NF- $\kappa$ B activation was intact in MafK-knockdown cells (see Supplementary Fig. S8). We also confirmed that TNF $\alpha$ -induced p65 acetylation was reduced in MafK-depleted cells (see Supplementary Fig. S8). These findings suggest that MafK plays a role in TNF $\alpha$ -induced NF- $\kappa$ B activation.

TNF $\alpha$  activates NF- $\kappa$ B signalling by forming TNFR complex I, leading to cell survival<sup>30,31</sup>, and triggers caspase-8-dependent extrinsic apoptosis via complex II. Thus, the balance between TNF $\alpha$ -induced survival and cell death is an interesting issue. To study the role of MafK in TNF $\alpha$ -induced apoptosis, we treated control and MafK-knockdown cells with TNF $\alpha$ . Apoptosis was observed in MafK-knockdown cells 6 h after TNF $\alpha$  treatment, while there was no death of control cells, as indicated by microscopy and intracellular ATP content (Fig. 6b and 6c). Caspase inhibition by z-Vad-fmk, a pan-caspase inhibitor, completely abrogated the death of

MafK-depleted cells upon TNF $\alpha$  stimulation, indicating the involvement of caspase-dependent apoptosis. To confirm that the cell death was indeed caspase-dependent apoptosis, we analysed caspase-3 and caspase-8 cleavage by immunoblotting. MafK-knockdown cells treated with TNF $\alpha$  exhibited cleavage of both caspases, while control cells did not. No caspase-3 activation or caspase-8 cleavage was observed in control cells (Fig. 6d). These data suggest MafK depletion induces caspase-8-dependent apoptosis upon TNF $\alpha$  stimulation by suppressing the induction of NF- $\kappa$ B-dependent pro-survival genes.

**MafK knockdown mice exhibit defective NF- $\kappa$ B signalling.** To study the ability of transient MafK siRNA to affect NF- $\kappa$ B-dependent transcription *in vivo*, mice were treated with 600 pmol PEI-complexed MafK siRNA or a PEI-complexed unrelated siRNA negative control. After 3 days, MafK siRNA injection efficiently reduced MafK mRNA in the liver (Fig. 7a). We next evaluated the ability of MafK-knockdown mice to mount a response to endotoxins, an NF- $\kappa$ B signalling-dependent function. MafK-knockdown and control mice were treated with LPS after sensitization with D-galactosamine (GalN)<sup>32</sup> followed by hourly monitoring. A Kaplan-Meier plot suggested that mice depleted for MafK by siRNA (siMafK) are resistant to endotoxic shock (Fig. 7b). We also confirmed there



**Figure 5 | MafK interacts with p65.** (a) Interaction between exogenous MafK and p65 or p50. HepG2 cells were transfected with pcDNA3-FLAG-p65 or pcDNA3-FLAG-p50 and pcDNA3-HA-MafK. Transfectants were harvested and whole-cell extracts (WCE) were immunoprecipitated with  $\alpha$ -FLAG antibody, followed by western blotting with MafK, p65, and p50 antibodies. (b) Interaction between endogenous MafK and p65 or p50. Cells were harvested and whole-cell extracts were immunoprecipitated with p65 or p50 antibodies (c) Interaction between exogenous MafK and CBP. HepG2 cells were transfected with pcDNA3-CBP and pcDNA3-HA-MafK. Transfectants were harvested and whole cell extracts were immunoprecipitated with  $\alpha$ -HA antibody, followed by western blotting with MafK and CBP antibodies. (d) MafK enhances binding between p65 and CBP. HepG2 cells were transfected with pcDNA3-FLAG-p65 and pcDNA3-CBP with or without pcDNA3-HA-MafK. After transfection (48 h), the cells were lysed, immunoprecipitated with anti-FLAG, and immunoblotted with anti-MafK, anti-p65, or anti-CBP.

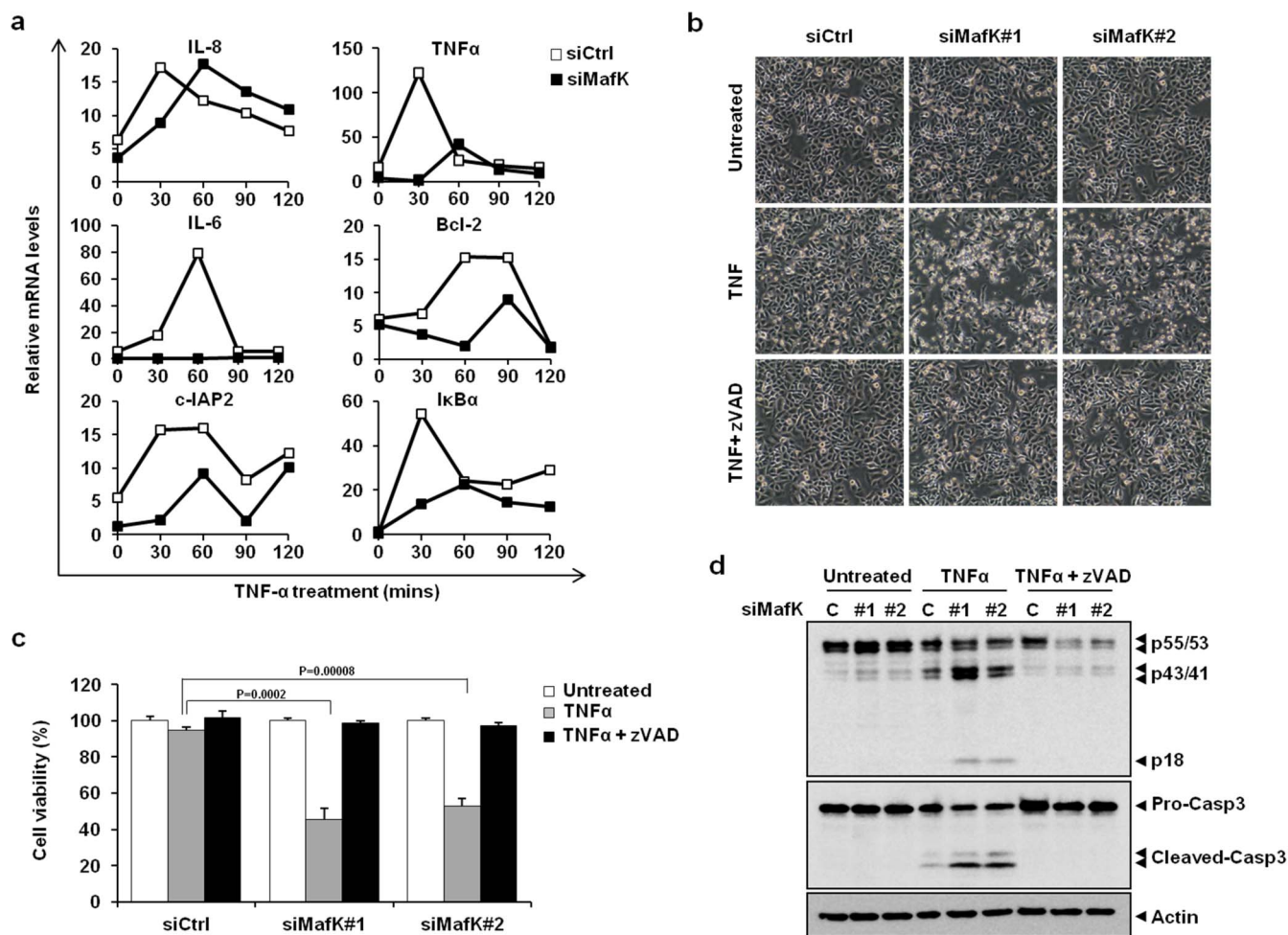
was no off-target effect of PEI-complex alone (see Supplementary Fig. S9). To investigate whether MafK depletion also results in inflammatory responses, we evaluated the expression of various inflammatory cytokines in MafK-knockdown mice injected with LPS. Transcript expression of most NF- $\kappa$ B dependent cytokines was downregulated in the livers of MafK-deficient mice (Fig. 7c). Cytokine expression was also reduced in LPS-induced MafK knock-down mice (Fig. 7d). Consistent with these results, MafK depletion improved liver toxicity in LPS-induced mice (see Supplementary Fig. S9). We conclude that functional MafK regulates NF- $\kappa$ B-dependent signalling *in vivo*.

## Discussion

The importance of NF- $\kappa$ B to anti-oxidative activity has been well established. Activated NF- $\kappa$ B occurs in several types of oxidative stress and promotes inflammation by inducing the expression of genes that mediate cell proliferation, survival, and oxidative stress<sup>33–35</sup>. Nrf2 is also a key regulator of anti-oxidative activity via ARE-mediated gene expression of Phase II-detoxifying and antioxidant enzymes<sup>8–12</sup>. Many details of the molecular mechanisms of Nrf2 and NF- $\kappa$ B transcriptional activity and signalling have been explained<sup>17–19</sup>. However, it is unclear how the transcriptional activity

of NF- $\kappa$ B is regulated via interaction of Nrf2 and related molecules such as small maf proteins. In this study, we provide direct evidence that MafK regulates NF- $\kappa$ B-dependent signalling in HepG2 cells and in an *in vivo* mouse model. Interestingly, MafK expression induced NF- $\kappa$ B-dependent signalling, which enhanced p65 acetylation, thereby increasing the DNA-binding activity of NF- $\kappa$ B and the inflammatory response. We demonstrated that MafK enhances LPS-induced NF- $\kappa$ B activation by inducing p65 acetylation through CBP. This result may provide novel insights into the role of MafK in regulating NF- $\kappa$ B and NF- $\kappa$ B-dependent inflammatory responses (Fig. 8).

Of particular interest in this study is the identification of MafK as a novel regulator of NF- $\kappa$ B-dependent inflammatory responses *in vitro* and *in vivo*. MafK has been reported to be an activator or repressor of Nrf2 transcriptional activity<sup>11,36</sup>. Nrf2-small maf homodimers repress ARE-driven gene transcription; small maf homodimers compete with Nrf2 for ARE, thereby repressing ARE-driven gene transcription<sup>37</sup>. Therefore, we propose participation of MafK in NF- $\kappa$ B regulation as a transcriptional activator. As a transcription factor, MafK has the potential to interact with co-repressors and co-activators and is involved in many signalling pathways that regulate cell cycle genes<sup>38</sup>. However, there has been no report demonstrating a



**Figure 6** | MafK deficiency triggers TNF $\alpha$ -induced apoptosis. (a) HepG2 Cells were transfected with MafK or control siRNA for 48 h. Cells were treated with TNF- $\alpha$  (10 ng/mL) and NF- $\kappa$ B-dependent gene expression was measured after 30, 60, 90, and 120 min. (b) HeLa cells were transfected with MafK or control siRNA. After 48 h, triplicate samples were treated with TNF $\alpha$  for 3 h. Cells were examined by phase-contrast microscopy and photographed using a SPOT digital camera and software. (c) HeLa cells were transfected with MafK or control siRNA. After 48 h, triplicate samples were treated with TNF $\alpha$  and zVAD for 6 h. Cell death was quantified by measuring intracellular ATP. (d) HeLa cells were transfected with MafK or control siRNA. After 48 h, triplicate samples were treated with TNF- $\alpha$  and zVAD for 3 h. Cell lysates were immunoblotted with anti-caspase3 and anti-caspase-8 antibodies.

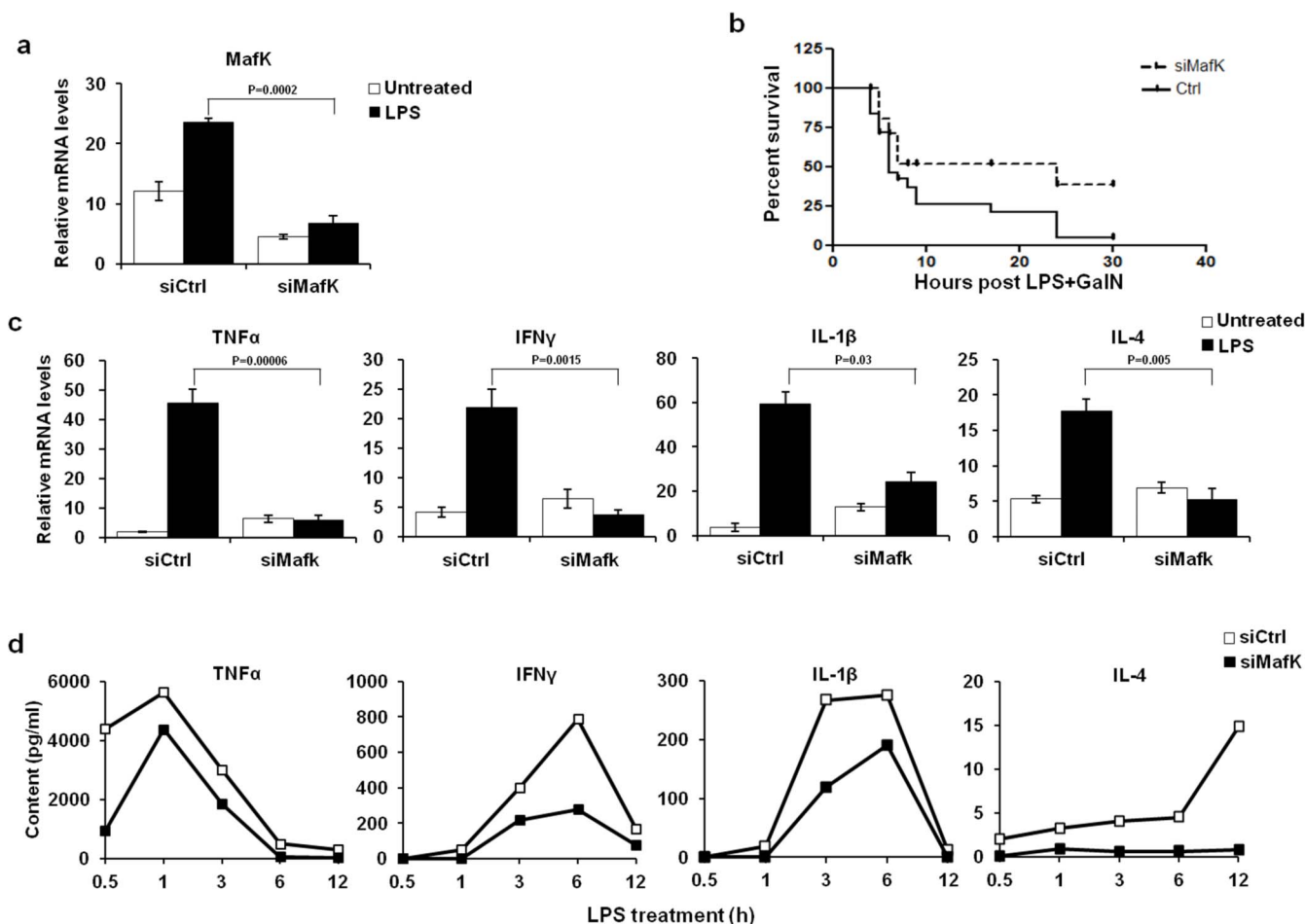
role for MafK in regulating NF- $\kappa$ B activity and the subsequent inflammatory response. In this study, we show that MafK regulates NF- $\kappa$ B activation and inflammatory response *in vitro* and *in vivo*.

A major finding of this study is that MafK enhances the DNA-binding activity of NF- $\kappa$ B by interacting with and enhancing acetylation of p65. The heterodimer of p65 and p50 subunits regulates a variety of physiologic and pathologic processes, including proliferation, differentiation, survival, tumorigenesis, and inflammation<sup>1–3</sup>. In the inactive state, NF- $\kappa$ B resides in the cytoplasm and forms a multi-protein complex with the inhibitory subunit, I $\kappa$ B. Upon activation by external stimuli, the inflammatory signal converges on and activates a set of I $\kappa$ B kinases, which phosphorylate NF- $\kappa$ B, targeting it for proteasomal degradation. Once released from the I $\kappa$ B $\alpha$  complex, NF- $\kappa$ B translocates to the nucleus, where it binds DNA and promotes transcription of proinflammatory mediators such as cytokines, and adhesion molecules, thereby playing a critical role in the inflammatory response<sup>24</sup>. Our studies indicate that MafK does not regulate NF- $\kappa$ B activation independent of I $\kappa$ B $\alpha$  phosphorylation or degradation (Fig. 4a), but is dependent on DNA-binding inhibition of the NF- $\kappa$ B complex (Fig. 4c). In contrast, the NF- $\kappa$ B complex must undergo additional posttranslational modifications. Among all known post-translational modifications of p65, acetylation is critical to controlling the duration and strength of NF- $\kappa$ B and its various

biological functions including DNA binding and transactivation<sup>39,40</sup>. Our data demonstrate that MafK directly binds p65 and enhances p65 acetylation (Fig. 4d and Fig. 5a).

We hypothesized that which MafK induced by Nrf2 inhibition may be directly regulate NF- $\kappa$ B transcriptional activity; we confirmed that a functional role of Nrf2 for the MafK-dependent NF- $\kappa$ B transcriptional activity and involvement for the p65 and MafK complex. Although Nrf2 failed to interact with p65, depletion of Nrf2 enhanced p65 acetylation without affecting I $\kappa$ B $\alpha$  degradation (see Supplementary Fig. S10). These results were insufficient to define the function of Nrf2 in MafK-dependent NF- $\kappa$ B transcriptional activity, but suggest Nrf2 indirectly regulates NF- $\kappa$ B possibly via MafK suppression. Further studies are needed to address how Nrf2 contributes to MafK-dependent NF- $\kappa$ B activity.

In conclusion, this study represents an initial effort to demonstrate that MafK functions as an activator of NF- $\kappa$ B, thereby regulating NF- $\kappa$ B transcriptional regulatory activity and cellular antioxidant capacity. NF- $\kappa$ B plays important roles in a number of human diseases, but whether its role depends on Nrf2 regulation of cellular antioxidant capacity requires further investigation. These results also suggest that inactivation of the MafK pathway may represent an effective genetic approach against oxidative stress-induced human diseases.



**Figure 7 | MafK-knockdown mice exhibit defective NF- $\kappa$ B signalling.** (a) Effects of MafK depletion on mRNA levels in the liver. Mice were transfected with the indicated MafK siRNAs for 72 h and liver samples were analysed by real-time PCR. (b) MafK-deficient mice were resistant to endotoxic shock. The mice were sensitized with GalN (700 mg/kg) followed by LPS (50  $\mu$ g/kg) challenge and monitored hourly. The survival curve is presented as the percentage of survivors over time post-challenge. (c) MafK-knockdown mice are defective in NF- $\kappa$ B-dependent gene expression. MafK-knockdown mice were sensitized with LPS (15 mg/kg) challenge and gene expression was analysed. The indicated genes were measured by real-time PCR of liver RNA. Values are representative of at least three independent experiments. (d) MafK-knockdown mice were sensitized with LPS (15 mg/kg) challenge and cytokine expression was analysed. The indicated cytokines in blood were measured by ELISA.

## Methods

**Cells and reagents.** Human liver cancer HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal bovine serum (FBS; Gibco) unless otherwise noted. Cells were maintained at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. Antibodies for immunoblotting included  $\beta$ -actin, Nrf2, MafK, MafF, MafG, phosphorylated p65 (Ser 536), phosphorylated I $\kappa$ B $\alpha$  (Ser 32), acetylated p65 (K310), and I $\kappa$ B $\alpha$  were from Abcam; p65 and CBP were from SantaCruz Biotechnology; Lamin B, Caspase-3, and Caspase-8 were from Cell Signalling. All antibodies were diluted 1 : 1000. TNF- $\alpha$  (Sigma) was used at 10 ng/mL and LPS (Sigma) was used at 1  $\mu$ g/mL. Dimethyl sulphoxide was from Sigma.

**Plasmid.** pcDNA-HA-MafK was constructed from pOTB7-MafK and subcloned into pcDNA-HA. pOTB7-MafK was purchased from Open Biosystems. The NF- $\kappa$ B response element reporter vector was purchased from Promega.

**Transfection of siRNA.** Human siRNA oligonucleotides were purchased from Integrated DNA Technologies. HepG2 cells were transfected with siRNA oligos by Lipofectamine RNAi max (Invitrogen). Twenty-four hours post-transfection, cells were treated with LPS (1  $\mu$ g/mL), and were then harvested. The siRNA sense oligo sequences were as follows: Control, 5'-UUCUCCGAACGUGUCACGUTT-3'; siNrf2-1, 5'-GGAACGUGAUGCUUCGCAATT3'-; siNrf2-2, 5'-AGGGAAG-UUUG GUCAAUCATT-3'; siMafK-1, 5'-GCCATACGCGUUUGTTCAUTT-3'; and siMafK -2, 5'-GGATCCGAAUGTTGGGACT-3'.

**Luciferase assay.** HepG2 cells were added to a 6-well plate. The NF- $\kappa$ B response element reporter was cotransfected with the *Renilla* reporter and the siRNA plasmids by using Lipofectamine 2000 (Invitrogen). Cells were lysed with passive lysis buffer (Promega) and relative luciferase activity was determined by measuring firefly

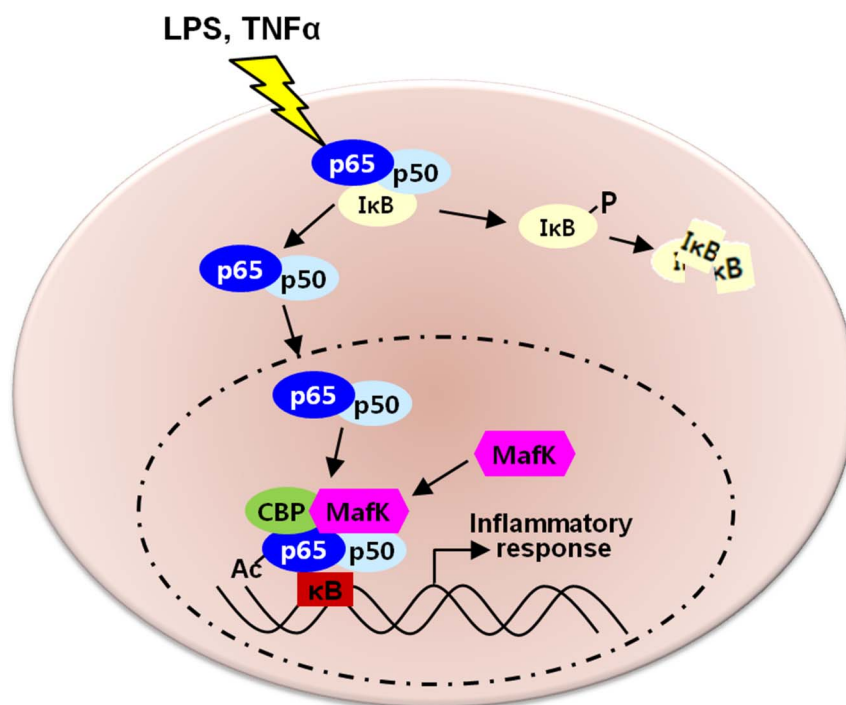
luciferase activity and normalizing it to *Renilla* luciferase activity with the Dual-Luciferase Reporter Assay System (Promega).

**Quantitative real-time PCR.** RNA was isolated using the RNeasy Plus kit (Qiagen). cDNA was prepared using the AMV Reverse Transcriptase synthesis kit (Promega) according to the manufacturer's protocol. Quantitative PCR (qPCR) was performed with the QuantiTect SYBR Green PCR kit (Qiagen). qPCR was performed on a Qiagen Rotorgene Q (Qiagen) using the comparative Ct quantification method.  $\beta$ -actin was amplified as an internal control. Primer sequences are available on request.

**Nuclear and cytoplasmic fractionation.** Cells were collected in ice-cold PBS, resuspended in hypotonic lysis buffer (10 mM HEPES at pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, protease and phosphatase inhibitors), and incubated on ice for 4 min. Cells were pelleted at 2000  $\times$  g for 3 min and the cytoplasmic fraction was removed to new tubes. The pellet was washed once with hypotonic buffer. The nuclear fraction was lysed in immunoprecipitation lysis buffer (10 mM Tris at pH 8, 170 mM NaCl, 0.5% NP40 and protease inhibitors) on ice for 30 min. The lysate was clarified by centrifugation and 1 mg of each fraction was used for immunoprecipitation.

**Immunoblotting.** Cells were lysed in RIPA buffer (150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 7.5, and 2 mM EDTA) on ice for 30 min. After centrifugation at 4°C for 20 min (12,000  $\times$  g), the supernatant was collected. Protein concentrations were determined by BCA assay (GenDEPOT). Equal amounts of extract were separated by 12.5% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). The membranes were blotted with antibody and detection was performed with an ECL system (Pierce) according to the manufacturer's instructions.





**Figure 8 | A model based on our studies.** (a) In response to NF-κB-activating stimuli, IκB proteins are degraded by IKK-mediated phosphorylation, a reaction that is not modulated by MafK. However, the MafK-CBP interaction is essential for acetylation of the p65 subunit. Modified p65 is an efficient inducer of target gene transcriptional expression.

**ChIP.** ChIP was performed with HepG2 cells treated with LPS for 60 min. Briefly, the cells were fixed with 1% formaldehyde and whole-cell lysates were sonicated to generate fragments of 200 to 500 bp. The sonicated lysates were used for ChIP with anti-p65, anti-acetyl-p65, or IgG control antibodies. After washing, the protein-DNA crosslinks were reversed; the DNA was eluted in 100 μl and used as a PCR template. Quantitative PCR was used to evaluate the ChIP eluates for binding to NF-κB-dependent promoters. GAPDH served as the negative control promoter.

**Endotoxin challenge and Kaplan-Meier survival curve.** Control and MafK knockdown-mice were sensitized with GalN (700 mg/kg) for 20 min and challenged with intraperitoneal injection of 50 μg/kg LPS in PBS; survival was monitored every hour for 30 h.

**Cell death assay.** Cells were added to duplicate 12-well plates and incubated for 24 h, then treated with TNF-α and z-VAD for 6 h. Cell viability was measured with Promega CellTiter Glo. Cell death is presented as the percentage of dead cells in each well.

**Statistical analyses.** Statistical analyses were performed with SPSS statistical software (version 12.0). The data represent means ± SEM from three independent experiments except where indicated. Statistical analyses were performed by student's *t*-test at a significance level of *P* < 0.05.

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## Author contributions

J.S. and K.-A.H. conceived the study and designed the experiments. Y.-J.H. and E.-W.L. performed most of the experiments. All authors including H.-R.K. and Y.-C.J. analysed the data and discussed the results. K.-A.H. supervised the project and wrote the manuscript with the help of Y.-J.H., E.-W.L., and J.S.

## Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

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