The Mastermind-like 1 (MAML1) Co-activator Regulates Constitutive NF-κB Signaling and Cell Survival

Baofeng Jin, Huangxuan Shen, Shuibin Lin, Jian-Liang Li, Zirong Chen, James D. Griffin, and Lizzi Wu

From the Department of Molecular Genetics and Microbiology, Shands Cancer Center, and the Interdisciplinary Center for Biotechnology Research, University of Florida, Gainesville, Florida 32610 and the Department of Medical Oncology, Dana-Farber Cancer Institute, Brigham and Women’s Hospital, and Harvard Medical School, Boston, Massachusetts 02115

Nuclear factor-κB (NF-κB)-based signaling regulates diverse biological processes, and its deregulation is associated with various disorders including autoimmune diseases and cancer. Identification of novel factors that modulate NF-κB function is therefore of significant importance. The Mastermind-like 1 (MAML1) transcriptional co-activator regulates transcriptional activity in the Notch pathway and is emerging as a co-activator of other pathways. In this study, we found that MAML1 regulates NF-κB signaling via two mechanisms. First, MAML1 co-activates the NF-κB subunit RelA (p65) in NF-κB-dependent transcription. Second, MAML1 causes degradation of the inhibitor of NF-κB (IκBα). Maml1-deficient mouse embryonic fibroblasts showed impaired tumor necrosis factor-α (TNFα)-induced NF-κB responses. Moreover, MAML1 expression level directly influences cellular sensitivity to TNFα-induced cytotoxicity. In vivo, mice deficient in the Maml1 gene exhibited spontaneous cell death in the liver, with a large increase in the number of apoptotic hepatic cells. These findings indicate that MAML1 is a novel modulator for NF-κB signaling and regulates cellular survival.

Nuclear factor-κB (NF-κB) signaling regulates diverse biological responses, including cell proliferation, survival, inflammation, and immunity (for review, see 1–4). Deregressed NF-κB signaling is associated with many disease states such as AIDS, asthma, arthritis, cancer, diabetes, muscular dystrophy, stroke, and viral infection. NF-κB consists of homo- or heterodimers of members of the Rel family: RelA (p65), RelB, c-Rel, p105 and its processing product p50, and p100 and its processing product p52. These proteins contain a Rel homology domain, a conserved 300-amino acid domain within their N termini that is responsible for DNA binding and homo- or heterodimerization. The common active forms of NF-κB are RelA/p50 or RelA/p52 heterodimers. In its inactive state, NF-κB remains sequestered in the cytoplasm by members of the inhibitor IκB family. Although the IκB family consists of IκBα, β, γ (p105), δ (p100), ε and Bcl-3, the best studied and major IκB protein is IκBα. NF-κB-based signaling results from a variety of stimuli, including T cell receptor signals, cytokines, and viral and bacterial products. In the canonical pathway, an IκB kinase (IKK) complex is activated upon response to these stimuli, and two kinases in this complex, IKKα and IKKβ, phosphorylate IκB. Phosphorylation triggers IκB for ubiquitination by the Skp/Cullin/F-box-containing ubiquitin ligase complex, leading to the degradation of IκB by the 26 S proteasome. NF-κB subsequently becomes liberated from its interaction with IκB, rapidly translocates to the nucleus, and binds to its cognate DNA-binding site in the promoter or enhancer regions of specific NF-κB target genes. Thus, the result of NF-κB activation triggered from a myriad of cellular activators is highly regulated gene expression.

The biological and pathogenic importance of NF-κB signaling emphasizes the need to control its action tightly, both physiologically and therapeutically. Indeed, research in recent years has produced significant insights into regulation of the NF-κB signaling pathway. These studies have revealed that NF-κB regulation occurs at multiple levels, including signal-induced kinase cascades leading to IκB degradation, regulation of NF-κB nuclear translocation, and interaction with other signaling pathways that modulate transcriptional activation of NF-κB target genes. The interaction of NF-κB with other signaling pathways is particularly interesting and complex. For example, Notch receptor-mediated signaling is a critical developmental signaling pathway and has complicated cross-communications with NF-κB (for review, see 5, 6).

In this study, we reveal a novel function for Mastermind-like 1 (MAML1) in regulating the NF-κB signaling pathway based on cell culture-based studies and the analysis of Maml1-knockout (ko) mice. MAML1 belongs to a family of three MAML transcriptional co-activators (for review, see 7), which were originally identified as essential co-activators for Notch receptors (8–10). Excitingly, recent studies have indicated that MAML1 has Notch-independent activities (for review, see 11), co-activating other transcription factors, including the muscle transcriptional factor MEF2C (12), p53 (13), and β-catenin (14). Here, we found that MAML1 interacts with nuclear RelA (p65) to promote NF-κB-dependent transcription events. MAML1 also interacts with NF-κB inhibitor IκBα and causes...
its degradation. Maml1-deficient mouse embryonic fibroblasts (MEFs) showed impaired TNFα-induced NF-κB responses and enhanced TNFα-mediated cellular cytotoxicity. In vivo, Maml1-ko mice exhibited ongoing hepatocyte cell death, and hepatocytes from Maml1-ko mice were hypersensitive to TNFα-mediated cell death. Our combined data indicate that MAML1 is a novel modulator for NF-κB signaling and regulates cellular survival.

EXPERIMENTAL PROCEDURES

Mice and Histology—Maml1-ko mice were generated and genotyped as described (12). Experiments were performed according to a protocol approved by the IACUC committee of the University of Florida. For routine histological analysis, tissue samples were fixed in Bouin’s solution and paraffin-embedded. Tissue sections were then stained with hematoxylin and eosin. For immunofluorescence staining, tissue samples were fixed in 4% paraformaldehyde in phosphate-buffered saline and OCT-embedded. The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays were performed on frozen tissue sections using an In Situ Cell Death Detection kit, TMR red (Roche Applied Science).

Antibodies—Mouse anti-FLAG antibody (clone M2) and anti-β-actin antibody were from Sigma. Hemagglutinin (HA) (clone HA.11) was from Babco. IkBα (9242), phospho-IkBα Ser32 (9241), MAML1 (4608), NF-κB p65 (sc-109), GFP (sc-9996), anti-Myc (sc-40), goat anti-mouse IgG-horseradish peroxidase (sc-2302), and goat anti-rabbit IgG-horseradish peroxidase (sc-2301) were from Santa Cruz Biotechnology. Alexa Fluor 594 donkey anti-rabbit IgG (A-21207) and anti-mouse IgG (A-21203) were from Invitrogen.

Plasmids—GFP-p65, pNF-κB-luc, and pEF-RL were gifts from Dr. Warner C. Greene and Dr. Lin-feng Chen (15). DsRed-S32A/S36A mutant, under the control of IgG (A-21203) were from Invitrogen.

RESULTS

Maml1-deficient Mice Exhibit Increased Cell Death in the Liver—We previously showed that the Maml1-ko mice fail to thrive and die within 10 days after birth (12). We determined that these mice exhibit a muscular dystrophy-like defect (12) and are unable to generate a type of mature B cells, marginal zone B cells (18). To characterize other potential defects that account for growth retardation and early death in the Maml1-ko mice, we performed further histological analyses and observed multiple regions of cell death in the livers of Maml1-ko mice (Fig. 1A). In striking contrast, regions of cell death were not observed in livers from wild-type (WT) littermates. The lesions became more apparent after neonatal day 3 (P3), appearing to grow in severity until death of the mice, with an increase in size and the number of necrotic regions. These data suggested that liver failure could be a cause of death for these mice.

Certain hepatocytes within the lesions clearly exhibited enlarged cytoplasm in the hematoxylin and eosin-stained section, suggesting that they were necrotic. To investigate whether Maml1-ko hepatocytes might also be more susceptible to apoptosis, we performed TUNEL assays and found that there was a significant increase in the number of apoptotic (TUNEL-positive) cells in the Maml1-ko liver sections compared with that in the controls in both neonatal day 1 and day 3 (Fig. 1, B and C). The difference is not as dramatic at day 3 compared with the earlier day 1, suggesting that more apoptosis occurs at the earlier stage. These data indicate that Maml1 deficiency results in an increase in cell death in the liver, in part, due to an increase in apoptotic cells.

MAML1 as a Novel Regulator of NF-κB Signaling
The liver phenotypes of the Maml1-ko mice are reminiscent of several knock-out models that are defective in the NF-κB pathway. For instance, knock-out of the components of the NF-κB pathway including RelA (p65) (19) and IKKβ (20) caused severe cell death in the liver. The death of hepatocytes is believed to be caused by impaired NF-κB responses of these cells to endogenous production of TNF because the mice that lack both RelA (p65) and TNF genes are viable and have normal livers (21). Therefore, normal NF-κB activity is required to prevent TNFα-mediated apoptosis in hepatocytes. Based on the resemblance of the liver phenotypes of our Maml1-ko and the knockouts of NF-κB components, we thus investigated whether Maml1 plays any role in modulating NF-κB signaling and whether the ablation of the Maml1 gene results in defective NF-κB signaling and sensitizes the cells to TNFα-mediated cytotoxicity.

NF-κB-dependent Transcription Is Regulated by MAML1 Co-activation—To determine whether MAML1 regulates NF-κB signaling, we first investigated whether MAML1 affects NF-κB target gene expression by monitoring the activities of a luciferase reporter containing six copies of artificial NF-κB-responsive elements as readouts (Fig. 2A). We found that in U20S cells, the NF-κB-responsive reporter was dramatically activated by MAML1 (M1) expression without further stimulation with cytokines, and the activation increased in a dose-dependent manner (Fig. 2B). To determine the specific domain(s) of the MAML1 co-activator required for NF-κB activation, we tested two MAML1-truncated mutants (Fig. 2, B and C). One is M1(Δ71–301)nls, lacking residues 71–301 but containing an added nuclear localization sequence. This mutant was unable to activate the NF-κB-responsive reporter. Because this mutant contains a p300/CREB-binding protein-binding site (8), MAML1-mediated p300 binding may be critical for MAML1 activa-
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MAML1, we first determined the possibility of in vivo interactions of MAML1 and NF-κB transcription factors by examining potential co-localization of MAML1 with NF-κB transcription factors p65, p50, and c-Rel. We found that when co-expressed with MAML1, only RelA (p65) dramatically changed its subcellular localization from the cytoplasm to the nuclear dots (Fig. 3A) and co-localized with MAML1 (supplemental Fig. 1). The specific MAML1 and RelA (p65) interaction was further supported by immunoprecipitation studies showing that MAML1 and RelA (p65) co-immunoprecipitate at endogenous and exogenous levels (Fig. 3, B and C). We further showed that the MAML1 C-terminal TAD domain 303–1016 amino acids might be required for binding to RelA (p65) because the MAML1(1–302) mutant that lacks this domain was unable to bind to RelA (p65) (Fig. 3D and supplemental Fig. 2). Importantly, MAML1 cooperates with RelA (p65) in the activation of the NF-κB responsive reporter (Fig. 3E). In contrast, the mutant MAML1(1–302), a mutant lacking RelA (p65) binding but retaining p300 binding, acted as a dominant negative to inhibit RelA (p65)-induced NF-κB promoter (Fig. 3F), possibly competing away a crucial transcriptional co-activator for NF-κB signaling, the p300 transcriptional co-activator (22). Moreover, MAML1 was able to promote the ability of RelA (p65) when fused to the Gal4 DB to activate a luciferase reporter that contains Gal4-binding sites in the promoter (Fig. 3G), indicating a co-activator role of MAML1 for RelA (p65). All of these data together indicate that MAML1 interacts with RelA (p65) and co-activates RelA (p65)-mediated transcription, providing one molecular mechanism for MAML1-mediated NF-κB transcriptional activation.

**MAML1 Interacts with IκBα and Causes the Degradation of IκBα—**Signal-stimulated phosphorylation and ubiquitination of the inhibitor IκBα is a key process for the liberation and nuclear translocation of NF-κB, leading to subsequent target gene transcription. Also, IκBα has a nuclear function as a repressor of NF-κB proteins (23–25). We showed that MAML1 is predominantly a nuclear protein (supplemental Fig. 4). Here, we tested whether MAML1 functionally interacts with IκBα in the nucleus. We transfected GFP-tagged IκBα into 293T cells and treated cells with the vehicle control DMSO or leptomycin B, an inhibitor of nuclear export. We found that IκBα normally is localized to the cytoplasm but is retained in the nucleus when treated with leptomycin B (Fig. 4A), indicating that IκBα is a protein shuttling between the cytoplasm and the nucleus. When co-expressed with MAML1, IκBα exhibited nuclear localization and co-localized with MAML1 in the nuclear speckles even without the leptomycin B treatment (Fig. 4A), suggesting that MAML1 might interact with nuclear IκBα and helps retain IκBα in the nucleus through their interaction. Indeed, the interaction of MAML1 and IκBα appears to be direct, as shown by GST pulldown assay (Fig. 4B).

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**FIGURE 1.** Maml1-deficient mice exhibit increased cell death in the liver. A, regions of cell death are present specifically in the Maml1-null livers. Frozen liver sections from Maml1-ko and their wild-type (wt) control littersmates at neonatal day 6 (P6) were stained with hematoxylin and eosin. The upper panels are low magnifications, and lower ones are high magnifications. More than 10 ko mice and their control littermates at various ages were examined. B, the number of apoptotic cells increases in the Maml1-null livers. TUNEL assays were performed on the frozen tissue sections from Maml1-ko and their wt control littersmates at neonatal day 1 (P1) and day 3 (P3). The average number of apoptotic cells are presented based on triplicate staining sections from liver samples of at least two mice/group in two independent experiments. Error bars, S.E.

**FIGURE 2.** MAML1 enhances NF-κB-dependent transcription. A, a diagram for a NF-κB luciferase reporter containing six copies of responsive NF-κB binding sites (pNF-κB-luc) is shown. B, MAML1 expression activates a NF-κB-responsive reporter in a dose-dependent manner, and the MAML1 mutants that lack either the p300 binding domain or the C-terminal TAD fail to activate NF-κB transcription. U2OS cells were transfected with 5 ng of Renilla luciferase plasmid as internal control, 0.2 μg of pNF-κB-luc luciferase reporter, and increasing amounts of expression plasmids encoding FLAG-tagged full-length (FL) or truncated MAML1. Cell lysates were prepared at 44–48 h after transfection for luciferase assays, and the pNF-κB-luc reporter activities are expressed as fold activation relative to cells not expressing MAML1. The data presented were pooled from three independent experiments. Error bars, S.E.

**FIGURE 3.** MAML1 cooperates with RelA (p65) to Activate NF-κB-dependent Transcription—To determine the mechanisms underlying the transcriptional activation of NF-κB signaling by MAML1, we first determined the possibility of in vivo interactions of MAML1 and NF-κB transcription factors by examining potential co-localization of MAML1 with NF-κB transcription factors p65, p50, and c-Rel. We found that when co-expressed with MAML1, only RelA (p65) dramatically changed its subcellular localization from the cytoplasm to the nuclear dots (Fig. 3A) and co-localized with MAML1 (supplemental Fig. 1). The specific MAML1 and RelA (p65) interaction was further supported by immunoprecipitation studies showing that MAML1 and RelA (p65) co-immunoprecipitate at endogenous and exogenous levels (Fig. 3, B and C). We further showed that the MAML1 C-terminal TAD domain 303–1016 amino acids might be required for binding to RelA (p65) because the MAML1(1–302) mutant that lacks this domain was unable to bind to RelA (p65) (Fig. 3D and supplemental Fig. 2). Importantly, MAML1 cooperates with RelA (p65) in the activation of the NF-κB responsive reporter (Fig. 3E). In contrast, the mutant MAML1(1–302), a mutant lacking RelA (p65) binding but retaining p300 binding, acted as a dominant negative to inhibit RelA (p65)-induced NF-κB promoter (Fig. 3F), possibly competing away a crucial transcriptional co-activator for NF-κB signaling, the p300 transcriptional co-activator (22). Moreover, MAML1 was able to promote the ability of RelA (p65) when fused to the Gal4 DB to activate a luciferase reporter that contains Gal4-binding sites in the promoter (Fig. 3G), indicating a co-activator role of MAML1 for RelA (p65). All of these data together indicate that MAML1 interacts with RelA (p65) and co-activates RelA (p65)-mediated transcription, providing one molecular mechanism for MAML1-mediated NF-κB transcriptional activation.

**MAML1 Interacts with IκBα and Causes the Degradation of IκBα—**Signal-stimulated phosphorylation and ubiquitination of the inhibitor IκBα is a key process for the liberation and nuclear translocation of NF-κB, leading to subsequent target gene transcription. Also, IκBα has a nuclear function as a repressor of NF-κB proteins (23–25). We showed that MAML1 is predominantly a nuclear protein (supplemental Fig. 4). Here, we tested whether MAML1 functionally interacts with IκBα in the nucleus. We transfected GFP-tagged IκBα into 293T cells and treated cells with the vehicle control DMSO or leptomycin B, an inhibitor of nuclear export. We found that IκBα normally is localized to the cytoplasm but is retained in the nucleus when treated with leptomycin B (Fig. 4A), indicating that IκBα is a protein shuttling between the cytoplasm and the nucleus. When co-expressed with MAML1, IκBα exhibited nuclear localization and co-localized with MAML1 in the nuclear speckles even without the leptomycin B treatment (Fig. 4A), suggesting that MAML1 might interact with nuclear IκBα and helps retain IκBα in the nucleus through their interaction. Indeed, the interaction of MAML1 and IκBα appears to be direct, as shown by GST pulldown assay (Fig. 4B).
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A

GFP-RelA

GFP-RelA+MAML1

B

Input | IP | IgG | MAML1

|      |    |    |

C

GFP-RelA M2 IP  WCL

| -  | +  | +  |

FLAG-MAML1

| -  | +  | +  |

GFP blot

|      |    |

M2 blot

|      |    |

D

FLAG-MAML1 GFP-RelA

| +  | +  | +  | +  |

Vec  Δ71-301 FL  1-302

GFP blot

|      |    |    |

M2 blot

|      |    |

M2 IP

|      |    |

GFP blot

|      |    |

10% WCL input

E

pNF-κB-luc

Fold activation

| 0 ug M1 | 0.25 ug M1 | 0.5 ug M1 | 1 ng M1 |

| 0 ng RelA | 0.1 ng RelA | 0.5 ng RelA |

F

pNF-κB-luc

Fold activation

| 0 ug M1 | 0.25 ug M1 | 0.5 ug M1 | 1 ug M1 |

| 0 ng RelA | 0.1 ng RelA | 0.5 ng RelA |

G

pGal4-luc

Fold activation

| 0 ug M1 | 0.1 ug M1 | 0.2 ug M1 | 0.5 ug M1 |

| DB only | 0.1 ng DB-RelA | 0.5 ng DB-RelA |
Because IκBα is able to repress the function of NF-κB proteins in the nucleus, we next determined whether the interaction of MAML1 and IκBα altered IκBα expression levels. To evaluate the effect of MAML1 on endogenous IκBα protein levels, we established a stably transduced HeLa cell line with MAML1 retroviruses to achieve MAML1 overexpression (Fig. 4C). We found that increased MAML1 expression results in reduced level of endogenous IκBα (Fig. 4C). To determine the effect of MAML1 expression on exogenous IκBα expression levels, we co-transfected 293T cells with HA-tagged IκBα and various forms of FLAG-tagged MAML1 and analyzed IκBα protein levels by Western blotting. We found that the protein levels of transfected and endogenous IκBα were reduced significantly in the presence of MAML1 co-expression (compare

**FIGURE 3.** MAML1 interacts with RelA (p65) and cooperates with RelA (p65) to activate NF-κB-responsive transcription. A, MAML1 causes redistribution of RelA (p65) from the cytoplasm to the nucleus. 293T cells were cultured on poly-D-lysine-treated coverslips and transfected with GFP-tagged RelA (p65) with or without the co-transfection of Flag-tagged MAML1 (M1). Cell lysates were prepared for immunoprecipitation with M2 beads (anti-FLAG). Both whole cell lysates (WCL) and immunoprecipitates were separated on SDS-polyacrylamide gels and analyzed by Western blotting for GFP-tagged RelA (p65) and MAML1. B, MAML1 and RelA (p65) co-immunoprecipitate. 293T cells were co-transfected with various combinations of expression constructs expressing GFP-tagged RelA (p65) and/or FLAG-tagged MAML1 as indicated. For each transfection, the total plasmid DNA was kept constant with the backbone vectors. Cell lysates were prepared for immunoprecipitation with M2 beads (anti-FLAG). Both whole cell lysates (WCL) and immunoprecipitates were separated on SDS-polyacrylamide gels and analyzed by Western blotting for GFP-tagged RelA and FLAG-tagged MAML1. C, the C-terminal region (amino acids 303–1016) of MAML1 is required for RelA degradation, and both the p300 binding domain and the TAD of MAML1 are required for this activity. 293T cells were transfected with HA-tagged IκBα and FLAG-tagged full-length (FL) or truncated MAML1, and then the expression levels of IκBα and MAML1 were detected by Western blot analyses with IκBα and anti-FLAG antibodies. D, β-Actin expression was used as a loading control. E, MAML1 fails to cause the degradation of IκBα α, and MAML1-induced IκBα degradation is blocked by a proteasome inhibitor. 293T cells were transfected with either WT or the SR form of HA-tagged IκBα in the presence or absence ofFLAG-tagged full-length MAML1. Cells were split into two groups on the second day and treated with MG132 or the vehicle control DMSO for 8 h. IκBα, MAML1, and β-actin expression was determined by Western blot analyses using anti-HA, anti-FLAG, and anti-β-actin antibodies. The relative band intensities of IκBα were quantitated relative to β-actin expression levels. F, MAML1 promotes IκBα ubiquitination. HeLa cells were co-transfected with HA-tagged IκBα, FLAG-tagged MAML1, and Myc-tagged ubiquitin (Ub) and treated with MG132 on the second day for 8 h before cell lysates were harvested. IκBα was subsequently immunoprecipitated (IP) with HA antibodies and blotted for anti-Myc antibodies to detect polyubiquitinated IκBα species. The total lysates were also blotted for anti-HA, anti-FLAG (M2), and β-actin antibodies.

**FIGURE 4.** MAML1 interacts with IκBα and causes IκBα degradation. A, MAML1 and IκBα co-localize in the nucleus. 293T cells were transfected with GFP-tagged IκBα with or without the co-transfection of Flag-tagged MAML1 (M1). At 24 h after transfection, cells were treated with either DMSO or leptomycin B (LMB) at 10 ng/ml overnight. Cells were finally fixed and photographed. B, MAML1 interacts with IκBα in a GST pulldown assay. GST-IκBα or GST was used to pull down the in vitro translated 35S-MAML1. C, MAML1 overexpression results in reduced expression levels of endogenous IκBα. Stable HeLa cells transduced with MAML1 retroviruses (MAML1: HeLa_pLNCX_MAML1) and the control cells (Vec: HeLa_pLNCX vector) were subjected to Western blot analyses for endogenous IκBα, MAML1, and β-actin expression. Here, MAML1 antibody recognizes both endogenous and exogenous MAML1. D, MAML1 causes IκBα degradation, and both the p300 binding domain and the TAD of MAML1 are required for this activity. 293T cells were transfected with HA-tagged IκBα and FLAG-tagged full-length (FL) or truncated MAML1, and then the expression levels of IκBα and MAML1 were detected by Western blot analyses with IκBα and anti-FLAG antibodies. β-Actin expression was used as a loading control. E, MAML1 fails to cause the degradation of IκBα α, and MAML1-induced IκBα degradation is blocked by a proteasome inhibitor. 293T cells were transfected with either WT or the SR form of HA-tagged IκBα in the presence or absence ofFLAG-tagged full-length MAML1. Cells were split into two groups on the second day and treated with MG132 or the vehicle control DMSO for 8 h. IκBα, MAML1, and β-actin expression was determined by Western blot analyses using anti-HA, anti-FLAG, and anti-β-actin antibodies. The relative band intensities of IκBα were quantitated relative to β-actin expression levels. F, MAML1 promotes IκBα ubiquitination. HeLa cells were co-transfected with HA-tagged IκBα, FLAG-tagged MAML1, and Myc-tagged ubiquitin (Ub) and treated with MG132 on the second day for 8 h before cell lysates were harvested. IκBα was subsequently immunoprecipitated (IP) with HA antibodies and blotted for anti-Myc antibodies to detect polyubiquitinated IκBα species. The total lysates were also blotted for anti-HA, anti-FLAG (M2), and β-actin antibodies.
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We transfected 293T cells with various combinations of MAML1 and WT or SR IkBα for 24 h and then treated cells with MG132, a proteasome inhibitor (or the vehicle control DMSO) for 8 h. We found that MAML1 significantly reduced the expression of WT IkBα (compare lane 3 with lane 2 in Fig. 4E) but not SR IkBα (compare lane 5 with lane 4 in Fig. 4E). Therefore, Ser22 and Ser36 sites of IkBα are critical for MAML1-mediated IkBα degradation, suggesting a possible role for MAML1 in inducing IkBα phosphorylation. When cells were treated with MG132, IkBα expression was not significantly reduced by MAML1 compared with DMSO treatment (compare lane 8 with lane 3 in Fig. 4E), indicating that MAML1-induced IkBα degradation is through a proteasome-mediated pathway.

Finally, we tested whether MAML1 promotes IkBα ubiquitination. Indeed, when HeLa cells were co-transfected with HA-tagged IkBα, FLAG-tagged MAML1, and Myc-tagged ubiquitin and then treated with MG132, polyubiquitinated IkBα species were readily detected after IkBα immunoprecipitation (Fig. 4F, lane 3), indicating that MAML1 promotes IkBα ubiquitination. The above data combined indicate that MAML1 enhances IkBα ubiquitination and degradation, which would lead to enhanced activities of nuclear NF-κB.

Impaired NF-κB Responses—To determine whether Maml1 deficiency results in defective NF-κB responses, we first introduced the NF-κB-responsive promoter reporter into WT and Maml1−/− MEFs and treated cells with TNFα. We found that although WT MEFs showed dose-dependent activation of the NF-κB-responsive promoter in response to TNFα treatment, Maml1−/− MEFs had significantly decreased activation (Fig. 5E), indicating that MAML1-induced IkBα degradation is through a proteasome-mediated pathway.

To examine the biochemical changes in the NF-κB pathway directly, we examined the levels of p-IκBα and total IκBα at various time points of TNFα-induced NF-κB responses. By Western blot analysis, we found that in WT MEFs, p-IκBα lev-
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Because Maml1-deficient mice exhibited a large degree of cell death in the liver, which was correlated with the higher levels of apoptotic cells (Fig. 1), we wanted to determine whether hepatocytes are more sensitive to TNFα treatment due to Maml1 deficiency. We isolated primary hepatocytes from Maml1-ko and their WT control littermates at neonatal day 1 and determined the sensitivity of these cells to TNFα-induced cytotoxicity by treating cells with various concentrations of TNFα and then quantifying the live cells using trypan blue exclusion assays. We found that Maml1-null cells were more prone to TNFα-induced cell death as indicated by a significant increase in dead cells in the Maml1-ko samples (Fig. 6A). Similar responses were also found in the Maml1-ko MEFs (Fig. 6B) and HeLa cells with retroviral-based short hairpin RNA-mediated MAML1 knockdown (Fig. 6C). These data indicate that the loss or reduced MAML1 expression results in defective NF-κB signaling and sensitizes the cells for TNFα-mediated cytotoxicity. On the other hand, we found that MAML1-overexpressing HeLa cells after transduced with retroviral-based MAML1 showed significant reduced cell death compared with cells transduced with an empty vector (Fig. 6D). These data indicate that increased MAML1 expression has protective activity against TNFα-induced cell death.

DISCUSSION

The present study demonstrates that MAML1 is a novel regulator for constitutive NF-κB signaling. We found that MAML1 effectively enhances NF-κB transcription via two mechanisms. First, MAML1 co-activates RelA (p65)-mediated transcription. Second, MAML1 enhances the degradation of IκBα. The regulatory function of MAML1 in NF-κB signaling is supported by its ability to regulate cell survival because Maml1 deficiency results in enhanced cell death in the liver, which is correlated with defective NF-κB responses and enhanced sensitivity to TNFα-induced cytotoxicity in primary hepatocytes and MEFs.

Previously, it was shown that NF-κB is required for hepatocyte survival under stress-inducing conditions. For instance, a normal NF-κB response is necessary to protect hepatocytes from endogenous TNF injury in vivo. This is supported by the evidence that loss of the RelA (p65) subunit results in massive hepatocyte apoptosis with embryonic lethality between embryonic days 14 and 15 (19). TNFα treatment triggers apoptosis and/or necrosis of hepatocytes in vivo (26–28). However, mice...
that are deficient for both RelA (p65) and TNF genes are viable and have normal livers (21), indicating that RelA(p65)-mediated antia apoptotic signals prevent cell death from TNF injury in vivo. Also, the induction of NF-κB is required for liver regeneration (29, 30) because loss of NF-κB activities after partial hepatectomy resulted in massive hepatocyte apoptosis. We showed in this study that Maml1 deficiency resulted in increased cell death in the livers and isolated primary Maml1-null hepatocytes and MEFs have impaired NF-κB response and are sensitive to TNFα cytotoxicity. Moreover, using cell culture models where MAML1 has been either overexpressed or knocked down, we found that MAML1 expression levels are inversely related to cell death induced by TNFα treatment. However, the exact downstream mechanisms by which MAML1 regulates TNFα-induced cell death are unclear at the present. In light of the role of MAML1 in TNFα-induced cell death and its ability to regulate NF-κB signaling, it will be important in the future to dissect the detailed molecular mechanisms and assess the contribution of the NF-κB pathway in mediating MAML1 function in cell death pathway. Nonetheless, our data indicate that MAML1 is a novel regulator for constitutive NF-κB signaling events, and the impaired NF-κB response due to the Maml1 deficiency may provide direct explanations for the enhanced cell death observed in the livers of the Maml1-ko mice.

Mechanistically, we found that MAML1 enhances NF-κB-dependent transcription via functional interactions with IkBα and RelA (p65). In unstimulated cells, the NF-κB complex is inhibited by IkBα proteins, which inactivate NF-κB by trapping it in the cytoplasm. Phosphorylation of serine residues on the IkB proteins by IkB kinase marks them for destruction via the ubiquitination pathway, thereby allowing activation and nuclear translocation of the NF-κB complex. Also, IkBα was previously shown to have a nuclear function as a repressor of NF-κB proteins (23–25). Here, we found that MAML1 interacts with IkBα in the nucleus, leading to IkBα ubiquitination and degradation. Moreover, SR IkBα, which is a phosphorylation-defective mutant and can cause cells to be unresponsive to stimuli, is resistant to MAML1-induced degradation. All of these data support that a role for MAML1 in IkBα phosphorylation and subsequent ubiquitination.

Currently, the mechanism underlying MAML1 regulation of IkBα is unclear; however, MAML1 was shown to cause phosphorylation of its interacting partners including Notch, p300, and MEF2C, although the responsible kinases are not yet defined (8, 12). We hypothesize that MAML1 might interact with certain IkBα kinase(s) in the nucleus which are capable of phosphorylating IkBα and affecting IkBα stability. MAML1-induced IkBα phosphorylation and degradation could lead to enhanced NF-κB signaling. Therefore, it will be important in the future to determine whether the MAML1-containing complex has such IkBα kinase activities and if so, what specific kinase(s) contribute to IkBα phosphorylation and degradation.

Moreover, we identified a second mechanism that accounts for MAML1-mediated promoted activities of NF-κB signaling. MAML1 also regulates NF-κB at the transcriptional level and is able to activate NF-κB-dependent transcription via its interaction with RelA (p65). This was further demonstrated by the ability of MAML1 to promote Gal4 DB-RelA fusion in activating a Gal4-responsive promoter. Several co-activators have been identified for NF-κB signaling, and the most well studied among them is p300/CREB-binding protein (1–4). We found that MAML1 co-activator activities appear to be greater than p300 in the reporter assays (supplemental Fig. 5). One interesting possibility is that p300 recruitment might be required for MAML1-enhanced NF-κB activities because deletion of the p300 binding domain from the MAML1 co-activator results in its inability to activate NF-κB. Also, MAML1(1–302) containing the p300 binding domain can function as a dominant negative to block RelA (p65)-induced NF-κB-dependent transcription, and one potential mechanism could be by competing away p300. It remains to be determined regarding the relationship of the MAML1 co-activator and other co-activators including p300/CREB-binding protein in NF-κB-mediated transcription.

MAML1 belongs to a family of defined transcriptional co-activators for the Notch pathway that enhance Notch signaling through interactions with Notch and CSL. More recently, its co-activator activities for other transcription factors were also revealed, including MEF2C, p53, β-catenin, as well as NF-κB in this study. How the MAML1 exerts such diverse activities is currently unknown. A growing body of evidence supports the idea of complicated cross-talks between Notch and NF-κB pathways (for review see 5, 6). The complex interactions between two pathways could result in either synergistic or antagonistic effects of these two pathway activities (16, 31–33). It was shown that Notch signaling regulates the transcription of the components in the NF-κB pathway, including a member of the NF-κB family of transcription factors, NF-κB2 (p100) (34) and IkBα (35). Conversely, NF-κB affects Notch signaling by regulating the expression of Notch ligand Jagged1 as well as Notch targets, showing a synergistic interaction of two pathways during marginal zone B cell development and T cell receptor activation (16, 33, 36). The mechanistic interactions of these two pathways and the functional outcomes require further elucidation in defined cellular contexts. Because MAML1 has roles in both the Notch and NF-κB pathways, it potentially represents another layer of regulation for cross-talks between these two pathways.

In summary, we identified a novel role for MAML1 in the regulation of the NF-κB pathway, and this regulatory activity is important in cellular survival. Mechanistically, MAML1 promotes NF-κB-dependent transcription via mediating IkBα degradation and co-activating RelA (p65). The exact biological roles for MAML1 regulation of NF-κB in other tissues besides the liver or other processes in vivo are not yet clear, but this study reveals important functional implications for MAML1 in light of the critical role of NF-κB in human malignancies and innate immune response.

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