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Lemur tyrosine kinase 2 acts as a positive regulator of NF-κB activation and colon cancer cell proliferation

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ABSTRACT

Lemur tyrosine kinase 2 (LMTK2) belongs to both protein kinase and tyrosine kinase families. LMTK2 is less studied and little is known about its function. Here we demonstrate that LMTK2 modulates NF-κB activity and functions to promote colonic tumorigenesis. We found that LMTK2 protein was abundant in colon cancer cells and LMTK2 knockdown (LMTK2-KD) inhibited proliferation of colon cancer cells through inactivating NF-κB. In unstimulated condition, LMTK2 modulated NF-κB through inhibiting phosphorylation of p65 at Ser468. Mechanistically, LMTK2 phosphorylated protein phosphatase 1A (PP1A) to prevent PP1A from dephosphorylating p-GSK3β(Ser9). The p-GSK3β(Ser9) could not phosphorylate p65 at Ser468, which maintained the basal NF-κB activity. LMTK2 also modulated TNFα-activated NF-κB. LMTK2-KD repressed TNFα-induced IKKβ phosphorylation, IκBα degradation and NF-κB activation, implying that LMTK2 modulates TNFα-activated NF-κB via IKK. These results suggest that LMTK2 modulates basal and TNFα-induced NF-κB activities in different mechanisms. Animal studies show that LMTK2-KD suppressed colon cancer cell xenograft growth, decreased PP1A phosphorylation and increased p-p65(Ser468). Our results reveal the role and underlying mechanism of LMTK2 in colonic tumorigenesis and suggest that LMTK2 may serve as a potential target for chemotherapy of colon cancer.

1. Introduction

Lemur tyrosine kinase 2 (LMTK2), also known as serine/threonine-protein kinase KPI-2, belongs to both the protein kinase and the tyrosine kinase families. It contains N-terminus transmembrane helices and a long C-terminal cytoplasmic tail with serine/threonine/tyrosine kinase activity [1]. It complexes with protein phosphatase 1 catalytic subunit alpha (PP1A) and phosphorylates PP1A at Thr320, leading to inactivation of PP1A [1]. LMTK2 is a target of CDK5 [2] and has been shown to control Smad2 signaling by regulating PP1A and GSK3β [3]. LMTK2 was found to be a negative regulator of NGF-induced neuronal differentiation and plays a role in NGF-TrkA signaling [4]. It plays a critical role in endosomal membrane trafficking [5,6]. LMTK2 knockout mice are viable but infertile due to defective germ cell maturation [7]. LMTK2 was also found to play a role in cancer development. Mutations of LMTK2 were found in a few cancers [8–11]. LMTK2 was upregulated in prostate cancers and acted as a negative regulator of androgen receptor activity [12]. It was also demonstrated in cancer cells that LMTK2 was a determinant of cell sensitivity to apoptosis by regulating the expression of the BCL2 family members [13].

Studies about LMTK2 are less and its biological function remains largely unknown. We determined the possible role of LMTK2 in colon cancer cells. We found that LMTK2 was abundant in colon cancer cells. Knockdown of LMTK2 (LMTK2-KD) inhibited the proliferation of colon cancer cells through suppressing nuclear factor-κB (NF-κB). LMTK2 modulated unstimulated and TNF-induced NF-κB activities in different mechanisms. In unstimulated condition, LMTK2 modulated NF-κB activity through PP1/GSK3β/p65 pathway. In TNF-stimulated condition, LMTK2 modulated NF-κB through PP1/IKK pathway. The animal studies show that inhibition of LMTK2 expression inhibited xenograft growth of colon cancer cells in mice. Our results suggest that LMTK2 regulates positively colon tumor growth and thus it may serve as a target for chemotherapy of colon cancers.

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2. Materials and Methods

2.1. Cell culture

Human colon cancer cells RKO and HCT116 were grown in DMEM/M5A medium. Human normal colon epithelial CCD841 cells were maintained in RPMI 1640 medium. Human colon cancer cells SW480 and SW620 were grown in L15 medium. Human colon cancer cells Caco2 was grown in MEM medium. Human colon cancer cells HT29 was grown in M5A medium. All medium were supplemented with 10% fetal bovine serum, 100 u/mL penicillin, and 100 μg/ml streptomycin. The cells were cultured at 37 °C in an incubator containing 5% CO_2.

2.2. Antibodies

The IκBα(#4812), p-IκBα (Ser32) (#2859), p65 (#8242), p-p65(Ser536) (#3033), p-p65(Ser468) (#3039), GSK3β(#12456), p-GSK3β(Ser9) (#5558), p-PP1A (Thr320) (#2581), PP1A (#2582), Rb (#9313), p-Rb (#9307), p-IKKα/β(#2697), IKKβ(#2678) and A20 (#5630) antibodies were products of Cell Signaling Technology. IL-8 antibody was purchased from Sino Biological (10098-T36). LMTK2 antibody (SAB4500900) was obtained from Sigma-Aldrich. β-actin (M20011), HA (M20003) and Flag (M20008) antibodies were from Abmart. Cyclin D1 (No.556470) and Ki67(#16667) antibodies were from BD and Abcam, respectively.

2.3. Quantitative RT-PCR (qPCR)

qPCR was performed as described [14]. GAPDH was used as internal control. The primers are as follows:

LMTK2: 5′CCGAACACAAACAGCAGAGA3′(F), 5′GCCTCCCAGACATACTCGAA3′(R);

CCND1: 5′CGCCCTCCGTATCTTACTTC3′(F), 5′CTCTTCGCACTTCTGCTCCT3′(R);

IL-8: 5′AGGTGCAGTTTTGCCAAGGA3′(F), 5′TTTCTGTGTTGGCGCAGTGT3′(R);

A20: 5′GCGTTCAGGACACAGACTTG3′(F), 5′GCAAAGCCCCGTTTCAACAA3′(R);

IκBα: 5′CTGAGCTCCGAGACTTTCGAGG3′(F), 5′CACGTGTGGCCATTGTAGTTGG3′(R);

GAPDH: 5′CCATCTTCCAGGAGCGAGATC3′(F), 5′GCCTTCTCCATGGTGGTGAA3′(R).

2.4. Short hairpin RNA (shRNA) and small interference RNA (siRNA)

To inhibit the expression of human LMTK2, siRNA oligos or shRNA knockdown vectors were employed. To construct the LMTK2 hairpin siRNA (shLMTK2) expression cassette, complementary DNA nucleotides of LMTK2 RNA interference were synthesized, annealed and inserted into pLKO.1. The LMTK2 targeting sequences are, 5′CCGGCAGGTACAAGGAGGATTATATCTCGAGATATAATCCTCCTTGTACCTGTTTTTG3′, 5′AATTCAAAAACAGGTACAAGGAGGATTATATCTCGAGATATAATCCTCCTTGTACCTG3’. The scrambled control sequences are,

5′CCGGCCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGGTTTTTG3′,

5′AATTCAAAAACCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGG3’. The sequences of siLMTK2 and control siRNA oligos are as follows.

siLMTK2-1: 5′GCAGAGGUCUUCACACUUUTT3’;

siLMTK2-2: 5′GCGUAGAAGCGAUUCCCUATT3’;

Control: 5′UUCUCCGAACGUGUCACGUTT3'.

2.5. Constructs

The vectors encoding p65 and IKKβ were as described [14]. The AP-1 and NF-κB luciferase reporter plasmids were as described [15]. The PP1A vector was as described [16]. The vector encoding Flag-tagged human LMTK2 (LMTK2-Flag) was constructed by PCR, cloned into pcDNA3.0 and confirmed by sequencing. The vector encoding Flag-tagged LMTK2(V1355AF1357A) was constructed by site mutagenesis.

2.6. Transient transfection of cells

Transient transfection of the cells was performed using Lipofectamine 2000 (Invitrogen) per the manufacturer's instructions. Briefly, DNA was incubated with Lipofectamine 2000 in serum-free Opti-MEM medium (Invitrogen) for 30 min. This solution was then added to the cells and allowed to incubate at 37 °C for 6 h. The normal cell culture medium substituted for the medium with Lipofectamine, and cells were cultured as described above.

2.7. Cell proliferation and cell cycle analysis

Cells were plated into a 12-well plate at 2×10^4/well. After growth, the cells were harvested and cell number was counted under a microscope as described [17]. To analyze cell cycle, the cells were stained with propidium iodide and subjected to fluorescence-activated cell sorting (FACS) analysis in a BD Biosciences FACScan [17].

2.8. Determination of xenograft growth

Growth of xenograft was performed as described [14]. The male nude mice (5-week-old) were obtained from Shanghai Experimental Animal Center and grown in pathogen-free conditions. Cancer cells (1×10^6) were implanted at each flank of the mice subcutaneously. Tumor volumes were measured and calculated as described [15]. Animals were housed in specified pathogen-free conditions. All procedures were approved by the Institutional Animal Care and Use Committee at the Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, CAS.

2.9. Statistic analysis

Statistical analysis was conducted using the unpaired two-tailed Student's *t*-test or two-way analysis of variance (ANOVA) with GraphPad Prism 5.0. Data are means ± sem. $P < 0.05$ is considered statistically significant.

3. Results

3.1. LMTK2 promotes cell proliferation

Before investigating the possible role of LMTK2 in cell proliferation, we determined the expression level of LMTK2 in human colon epithelial cell lines, including colon cancer SW480, SW620, Caco2, RKO, HCT116, HT29 cells and normal colon epithelial CCD841 cells. The immunoblotting results show that all these cells produced LMTK2 (Fig. 1A). The colon cancer cells produced abundant LMTK2 as compared to CCD841 cells. Next we determined the effect of LMTK2 knockdown (LMTK2-KD) on cell proliferation. HCT116 and RKO cells were examined. We found that LMTK2-KD suppressed significantly the proliferation of HCT116 (Fig. 1B) and RKO cells (Fig. 1C). Flow cytometry assay results indicate that LMTK2-KD resulted in cell growth arrest at G1 phase (Fig. 1D). We also determined the effect of overexpression of LMTK2 on cell proliferation. CCD841 and RKO cells were examined and the results show that overexpression of LMTK2 promoted proliferation of these cells (Fig. 1E). Flow cytometry assay indicates that overexpression of LMTK2 enhanced the amount of the cells at S phase (Fig. 1F). These results suggest that LMTK2 plays a positive role in proliferation of colon epithelial cells.

Cyclin Dl, which is encoded by the *CCND1* gene, plays a critical role in controlling the transition from G1 to S phase in the cell cycle [18,19].

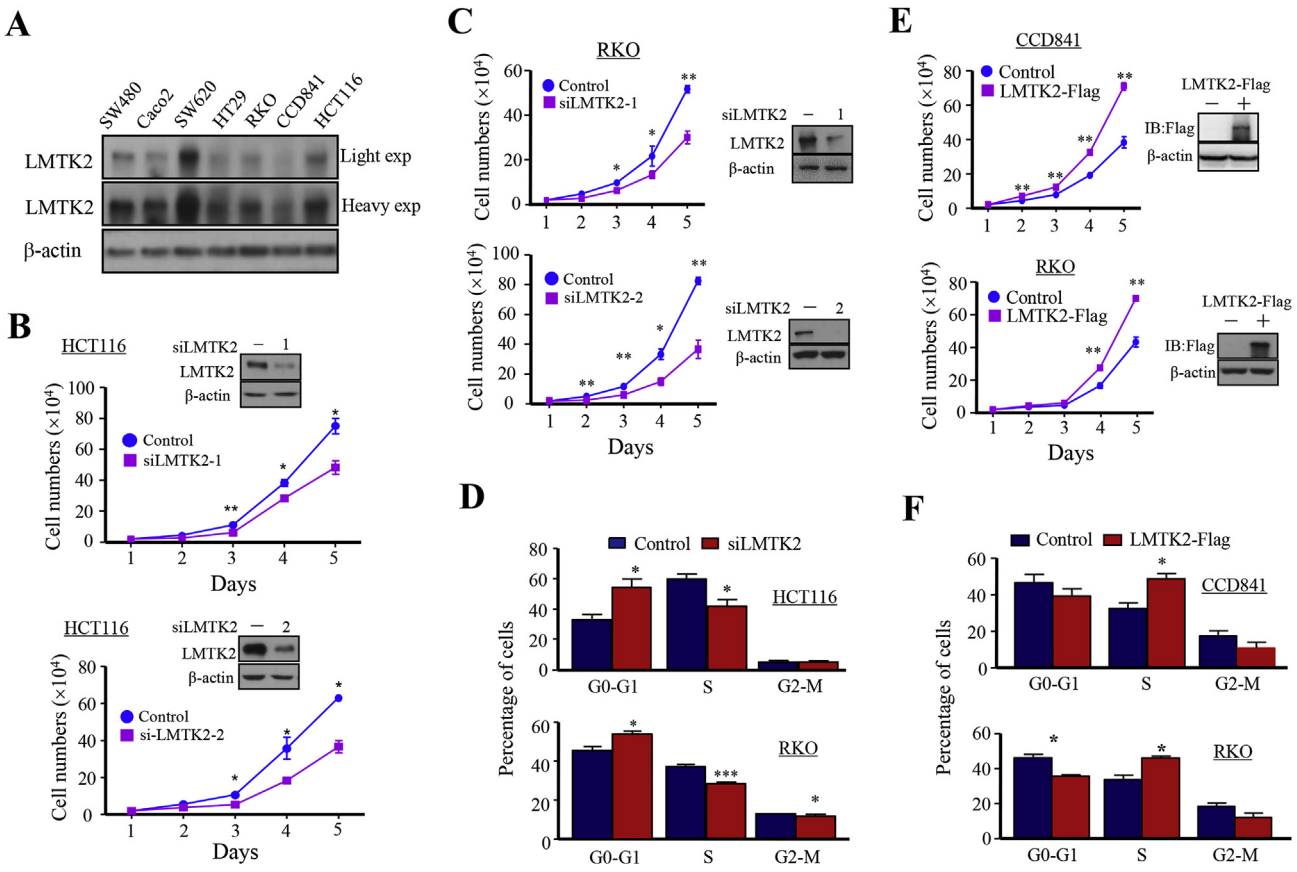

Fig. 1. LMTK2 regulates cell proliferation. (A) Expression of LMTK2 in colon epithelial cells. (B, C) LMTK2-KD inhibited proliferation of colon cancer HCT116 (B) and RKO (C) cells. The cells were transfected with control or LMTK2 siRNA oligos. After 24 h, the cells were collected and split into 12 well plates at 2×10^4/well and cell proliferation was determined as described in Methods. (D) LMTK2-KD induced cell cycle G1 arrest of HCT116 and RKO cells. The cells were transfected as indicated. After 48 h, the cells were subjected to FACS analysis as described in Methods. (E) Overexpression of LMTK2 promoted proliferation of CCD841 and RKO cells. The cells were transfected with control or LMTK2-Flag vector. After 24 h, the cells were split into 12 well plate at 2×10^4/well and cells proliferation was determined as described above. (F) Overexpression of LMTK2 promoted cell cycle G1-S transition. The cells were treated as in E. After 24 h, the cells were subjected to FACS analysis. Data are mean ± SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

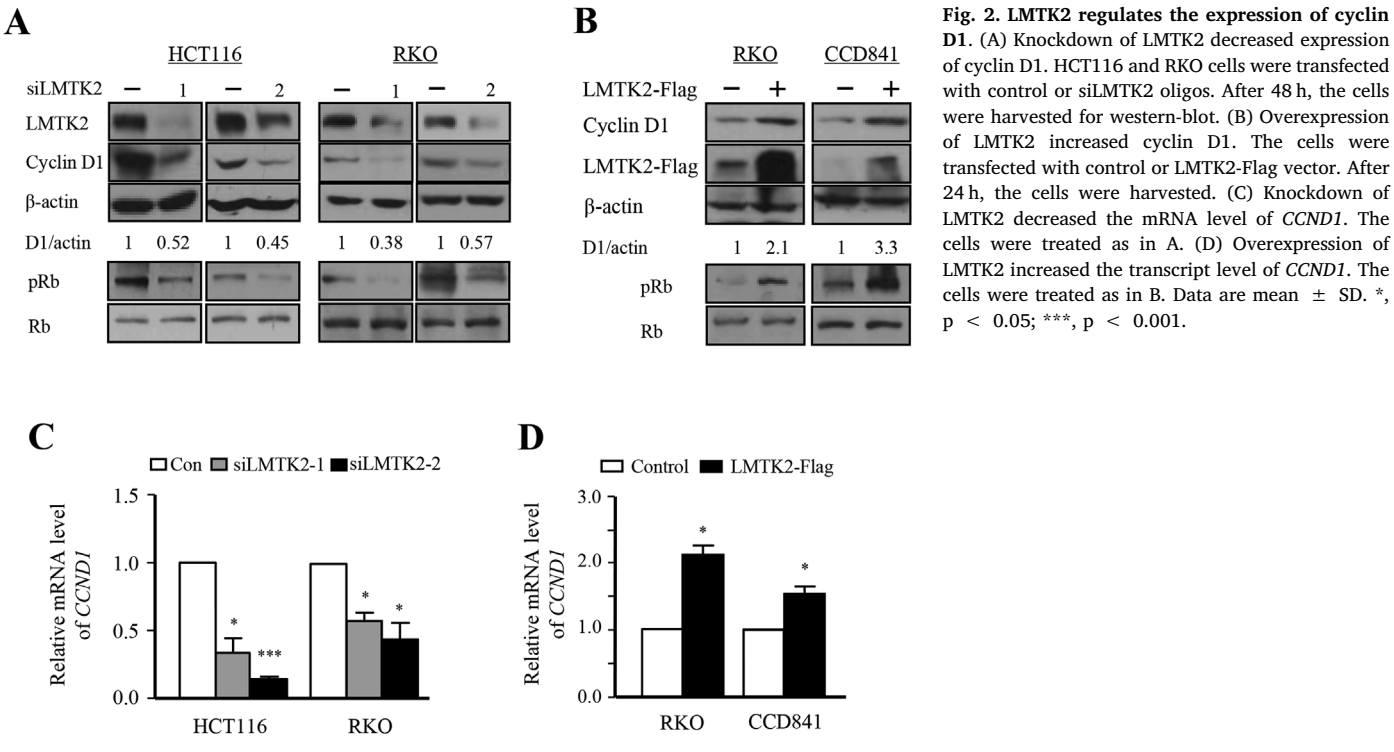

Fig. 2. LMTK2 regulates the expression of cyclin D1. (A) Knockdown of LMTK2 decreased expression of cyclin D1. HCT116 and RKO cells were transfected with control or siLMTK2 oligos. After 48 h, the cells were harvested for western-blot. (B) Overexpression of LMTK2 increased cyclin D1. The cells were transfected with control or LMTK2-Flag vector. After 24 h, the cells were harvested. (C) Knockdown of LMTK2 decreased the mRNA level of *CCND1*. The cells were treated as in A. (D) Overexpression of LMTK2 increased the transcript level of *CCND1*. The cells were treated as in B. Data are mean ± SD. *, $p < 0.05$; ***, $p < 0.001$.

We found that in HCT116 and RKO cells knockdown of LMTK2 decreased the protein level of cyclin D1 (Fig. 2A). We determined the phosphorylation of cyclin D1's downstream target Retinoblastoma (Rb), a key regulator of entry into cell division [20]. The results show that phosphorylation of Rb was decreased upon LMTK2-KD (Fig. 2A). Moreover, we found that overexpression of LMTK2 increased cyclin D1 protein and increased phosphor-Rb (Fig. 2B). Regulation of expression of cyclin D1 is usually at a transcriptional level. So, we next determined the effect of knockdown of LMTK2 on the transcript level of *CCND1*. Quantitative PCR results show that LMTK2-KD decreased and the mRNA level of *CCND1* (Fig. 2C). In agreement with the results, overexpression of LMTK2 increased mRNA level of *CCND1* (Fig. 2D). These results suggest that LMTK2 modulates the expression of cyclin D1 at a transcriptional level.

3.2. LMTK2 modulates NF-κB activity in unstimulated condition

Transcriptional regulation of expression of *CCND1* is regulated by a few transcriptional factors such as AP-1 [21] and NF-κB [22]. We asked whether LMTK2 regulated the expression of *CCND1* through AP-1 or NF-κB. To this end, we employed AP-1 and NF-κB promoter luciferase reporter plasmids in our work. LMTK2-KD had little effect on AP-1 reporter activities (Fig. 3A), implying that LMTK2 does not regulate *CCND1* expression through AP-1. Interestingly, LMTK2-KD inhibited significantly the NF-κB reporter activities (Fig. 3B). We then determined the effect of overexpression of LMTK2 on NF-κB activities and found that forced expression of LMTK2 enhanced NF-κB reporter activities (Fig. 3C). These data imply that LMTK2 might regulate the expression of *CCND1* through NF-κB. To confirm this, we determined whether overexpression of NF-κB p65 subunit could reverse cyclin D1 expression inhibited by LMTK2-KD. The results show that overexpression of p65 reversed cyclin D1 protein level decreased by LMTK2-KD (Fig. 3D). As expected, overexpression of p65 reversed *CCND1* mRNA level abated by LMTK2-KD (Fig. 3E). The results suggest that LMTK2 modulates the expression of cyclin D1 through NF-κB. We also determined the effect of LMTK2 on expression of other NF-κB downstream genes such as *IL-8*, *IκBα* and *A20*. The results show that LMTK2-KD decreased the transcription levels of these genes (Fig. 3F).

3.3. LMTK2 regulates NF-κB activity through PP1A-GSK3β-p65 in unstimulated condition

In our subsequent experiments, we determined the molecular mechanism underlying the regulation of NF-κB activity by LMTK2. The inhibitor of kappa B α (IκBα) inhibits NF-κB by keeping NF-κB proteins sequestered in an inactive state in the cytoplasm [23]. However, we found that LMTK2-KD had little effect on the protein levels of IκBα (Fig. 4A). The results suggest that, in unstimulated condition, LMTK2 does not modulate NF-κB activity through IκBα.

It is known that glycogen synthase kinase 3beta (GSK3β) phosphorylates NF-κB p65 subunit at Ser468, which leads to inactivation of NF-κB [24]. We therefore determined whether LMTK2 regulated NF-κB through GSK3β. We found that LMTK2-KD increased p-p65(Ser468) (Fig. 4B) and decreased p-GSK3β(Ser9) (Fig. 4C). Dephosphorylation of GSK3β at Ser9 leads to activation of this kinase [25]. The results suggest that LMTK2-KD induces p-p65(Ser468) through activating GSK3β. To verify this, we employed LiCl, the GSK3β inhibitor in our work. Inhibition of GSK3β with LiCl prevented LMTK2-KD from inducing p-p65(S468) (Fig. 4D). LiCl also reversed the NF-κB activities inhibited by LMTK2-KD (Fig. 4E). Together, these results suggest that LMTK2 modulates NF-κB through GSK3β.

The protein phosphatase 1 (PP1) activates GSK3β through dephosphorylating GSK3β [26]. The PP1 catalytic subunit alpha (PP1A) is a substrate of LMTK2 and LMTK2 phosphorylates PP1A at Thr320, leading to inactivation of PP1 [1]. We presumed that LMTK2 might increase p-GSK3β(Ser9) through phosphorylating and inactivating PP1A. As a matter of fact, Manser et al. [3] reported that LMTK2 induced p-GSK3β(Ser9) through inactivating PP1 in Hela cells. In our

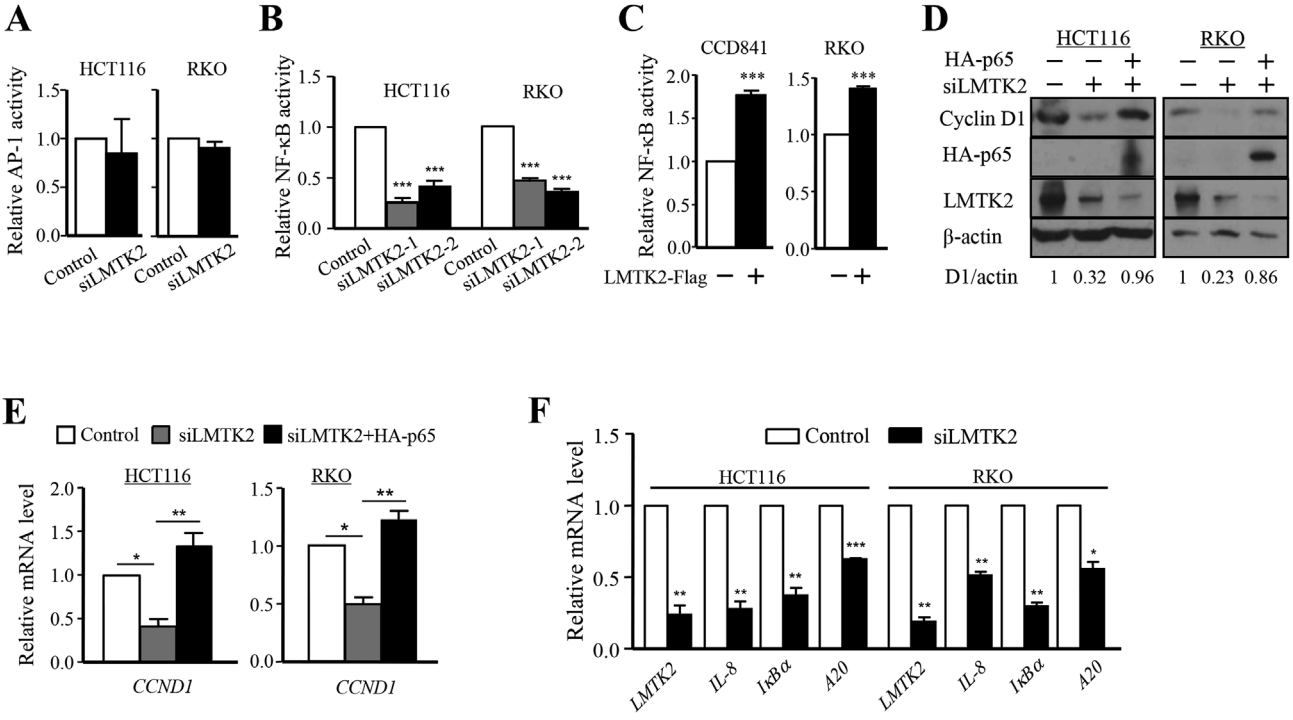

Fig. 3. LMTK2 regulates expression of cyclin D1 through NF-κB. (A) HCT116 and RKO cells were transfected with AP-1 luciferase reporter and siLMTK2 oligos. After 48 h, the cells were harvested for luciferase assay. (B) The cells were transfected with NF-κB luciferase reporter and siLMTK2 oligos. After 48 h, the cells were harvested for luciferase assay. (C) Overexpression of LMTK2 increased NF-κB activity. (D) Overexpression of p65 reversed the protein level of cyclin D1 inhibited by LMTK2-KD. (E) Overexpression of p65 reversed the mRNA level of *CCND1* decreased by LMTK2-KD. (F) Knockdown of LMTK2 downregulated the expression of *IL-8*, *A20* and *IκBα*. Data are mean ± SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

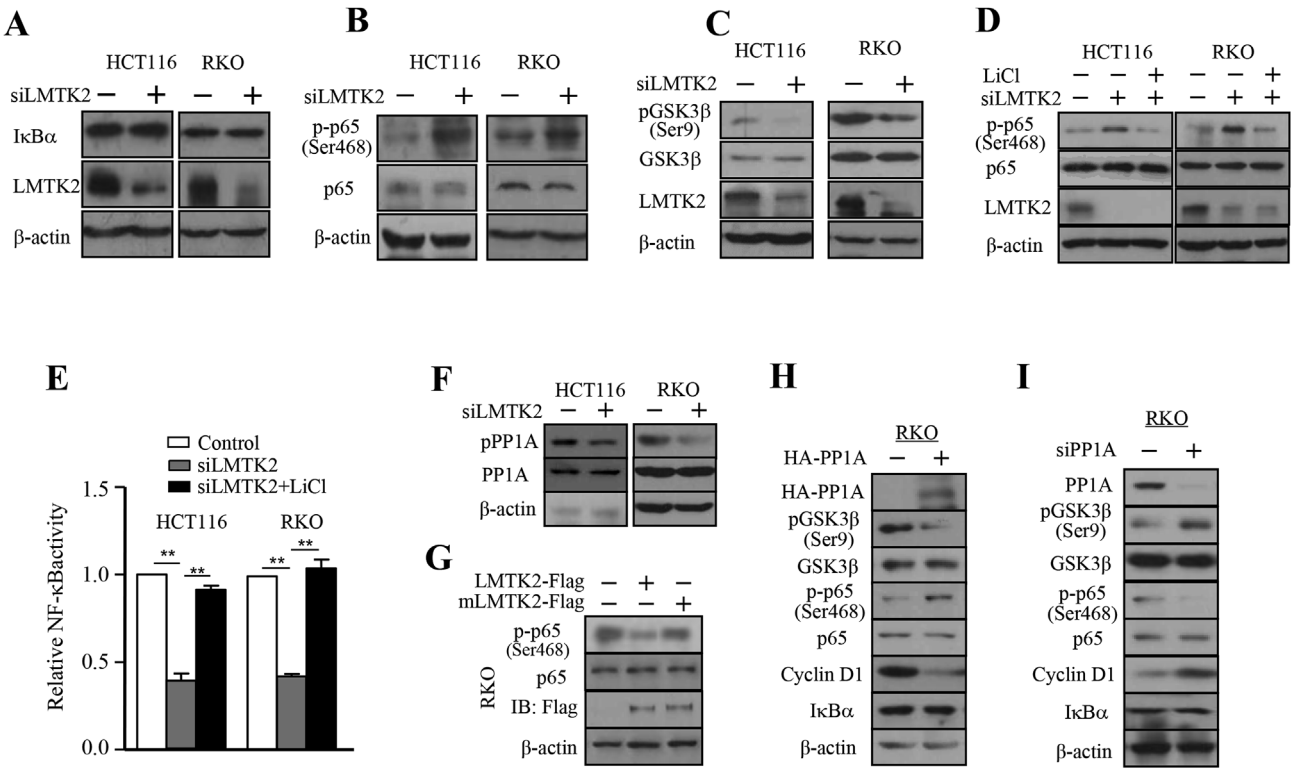

Fig. 4. LMTK2 modulates NF-κB activity via PP1A-GSK3β in unstimulated condition. (A) Knockdown of LMTK2 did not influence the expression of IκBα. The cells were transfected with control or siLMTK2 oligos as indicated. After 48 h, the cells were harvested for westernblot. (B, C) Knockdown of LMTK2 induced p-p65(Ser468) and inhibited p-GSK3β(Ser9). The cells were treated as in A. (D) LiCl prevented LMTK2-KD from inducing p-p65(Ser468). The cells were transfected with control or LMTK2 siRNA oligos. After 48 h, LiCl (40 mM) was added and the cells were incubated for another 1 h. (E) LiCl reversed the NF-κB activities blocked by LMTK2-KD. The cells were transfected with NF-κB reporter plasmid and LMTK2 siRNA oligos as indicated. After 48 h, LiCl (40 mM) was added and the cells were incubated for another 1 h. (F) Knockdown of LMTK2 inhibited p-PP1A (Thr320). The cells were treated as in A. (G) Overexpression of LMTK2, but not LMTK2(V1355AF1357A), inhibited p-p65(Ser468). RKO cells were transfected with control, LMTK2 or mutated LMTK2 vector as indicated. After 24 h, the cells were harvested for determining p-p65(Ser468). (H) RKO cells were transfected with control or PP1A vector. After 24 h, the cells were harvested for determining p-GSK3β(Ser9) and p-p65(Ser468). (I) RKO cells were transfected with control or PP1A siRNA oligos. After 48 h, the cells were harvested to determine p-GSK3β(Ser9) and p-p65(Ser468). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

work, we found that LMTK2-KD decreased the level of p-PP1A (Thr320) in HCT116 and RKO cells (Fig. 4F). Moreover, overexpression of LMTK2 decreased p-p65(Ser468) (Fig. 4G). To confirm that LMTK2 modulates NF-κB through PP1A, we constructed a vector encoding Flag-tagged LMTK2(V1355AF1357A). The residues V1355 and F1357 are critical for LMTK2 binding PP1A and mutation at these sites disabled LMTK2 to bind and phosphorylate PP1A [1]. We found that overexpression of LMTK2(V1355AF1357A) had little effect on phosphorylation of p65 (Fig. 4G), suggesting that LMTK2 regulates p-p65(Ser468) through PP1A. Together, these results suggest that LMTK2 modulates phosphorylation of p65 through PP1 − GSK3β pathway.

We also determined the effect of PP1A in the regulation of p65 phosphorylation and cyclin D1 expression in colon cancer cells. We found that overexpression of PP1A in RKO cells decreased p-GSK3β(Ser9) and increased p-p65(Ser468) (Fig. 4H). As expected, overexpression of PP1A attenuated the expression of cyclin D1. In agreement with the results, knockdown of PP1A increased p-GSK3β(Ser9), decreased p-p65(Ser468) and increased cyclin D1 (Fig. 4I). Together, these results suggest LMTK2 signals via PP1A to increase p-GSK3β(Ser9), leading to p-p65(Ser468) reduction and thereafter cyclin D1 induction. It is noted that neither PP1A overexpression nor PP1A knockdown had effect on the protein level of IκBα.

3.4. LMTK2 modulates TNFα-stimulated NF-κB activity

We were also interested in whether LMTK2 modulated the stimulus-induced NF-κB activity. To this end, we determined the effect of LMTK2-KD on TNFα-induced activation of NF-κB. We found that LMTK2-KD inhibited NF-κB activity induced by TNFα (Fig. 5A). In agreement with this, LMTK2-KD attenuated TNFα-induced expression of NF-κB downstream genes including *CCND1* (Fig. 5B), *IL-8*, *IκBα* and *A20* (Fig. 5C). It is known that PP1 inhibited TNFα-induced IKKβ phosphorylation and NF-κB activation [27,28]. As LMTK2 is an inhibitor of PP1A, we therefore presumed that LMTK2 regulated TNFα-stimulated NF-κB through PP1/IKK pathway. We determined the phosphorylation of IKKβ and found that LMTK2-KD inhibited TNFα-induced p-IKKβ (Fig. 5D). We checked IKK substrates IκBα and p65 and found that LMTK2-KD suppressed TNFα-induced p-IκBα(Ser32) and p-p65(Ser536) (Fig. 5E). Though LMTK2-KD had no effect on protein level of IκBα in unstimulated condition, it inhibited TNFα-induced degradation of IκBα (Fig. 5E). We determined the effect of LMTK2-KD on protein levels of A20 and IL-8. Consistent with the mRNA results (Figs. 3F and 5C), knockdown of LMTK2 decreased the protein levels of A20 and IL-8 in both unstimulated and TNFα-stimulated conditions (Fig. 5F).

To know whether PP1A was involved in LMTK2-regulated NF-κB activation by TNFα, we performed NF-κB reporter assay. We found that knockdown of PP1A prevented LMTK2-KD from inhibiting TNFα-induced NF-κB activities (Fig. 5G). These results suggest that LMTK2 suppresses TNFα-stimulated NF-κB activation through PP1/IKK pathway. We determined phosphorylation of GSK3β at Ser9. Unlike in unstimulated condition, knockdown of LMTK2 had little effect on pGSK3β in the presence of TNFα (Fig. 5H).

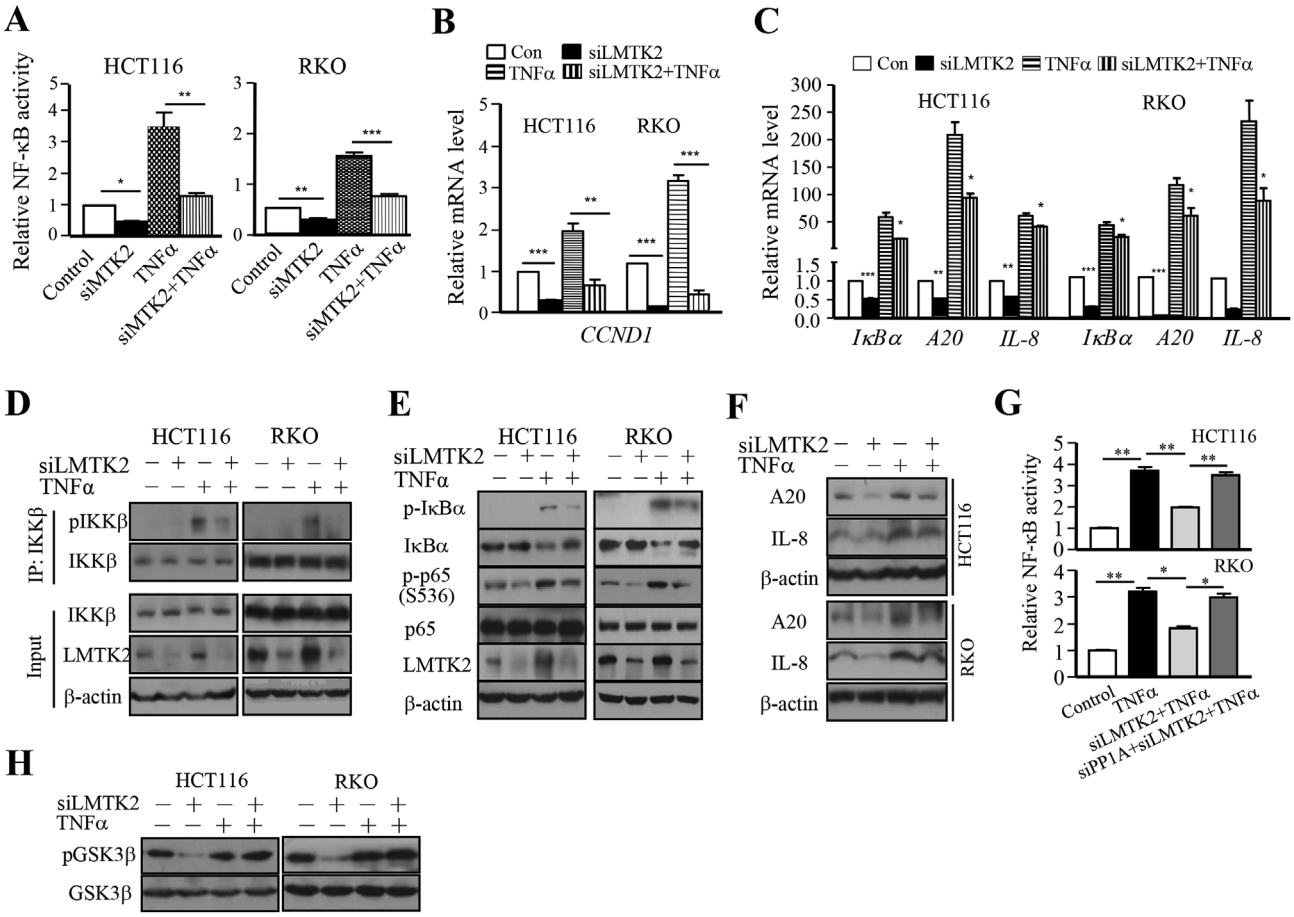

Fig. 5. LMTK2 regulates TNFα-induced NF-κB activity through PP1-IKK pathway. (A) LMTK2-KD prevented TNF from activating NF-κB. The cells were transfected with NF-κB reporter plasmid and LMTK2 siRNA oligos as indicated. After 48 h, the cells were treated with or without TNFα (20 ng/ml) for another 8 h. (B, C) LMTK2-KD prevented TNFα from inducing the expression of downstream target genes. The cells were transfected with control or LMTK2 siRNA oligos as indicated. After 48 h, the cells were treated with or without TNFα (20 ng/ml) for 2 h. The expression of *CCND1* (B) and *IL-8*, *IκBα* and *A20* (C) was determined by qPCR. (D) LMTK2-KD prevented TNFα from inducing p-IKKβ. The cells were transfected as indicated. Forty-eight hours post-transfection, TNFα (20 ng/ml) was added and the cells were incubated for 5 min, followed by immunoprecipitation and westernblot. (E) LMTK2-KD prevented TNFα from inducing the degradation of IκBα. The cells were transfected as indicated. 48 h post-transfection, TNFα (20 ng/ml) was added and the cells were incubated for 20 min. (F) Knockdown of LMTK2 inhibited the expression of A20 and IL-8. The cells were transfected as indicated. After 48 h, the cells were treated with or without TNFα (20 ng/ml) for 30 min. (G) Knockdown of PP1A reversed LMTK2-KD's inhibitory effect on TNFα-induced NF-κB activities. The cells were transfected as indicated. After 48 h, TNFα (20 ng/ml) was added and the cells were incubated for another 8 h. **(H)** LMTK2-KD had little effect on pGSK3β in the presence of TNFα. The cells were treated as in Fig. 5E. Phosphorylation of GSK3β at Ser9 was determined. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

3.5. Knockdown of LMTK2 retards xenograft growth

Finally, we determined the effect of LMTK2 on xenograft tumor growth. HCT116 cells stably expressing short hairpin RNA of LMTK2 (shLMTK2) were selected and employed in our work. We found that knockdown of LMTK2 restrained the xenograft growth, as evidenced by decreased tumor size (Fig. 6A and B) and tumor weight (Fig. 6C). The Ki67 protein is strictly associated with cell proliferation and is used as a marker for cell proliferation [29]. We stained the tumor tissues with Ki67 antibody and found that knockdown of LMTK2 reduced the amount of Ki67-positive cells (Fig. 6D). Western-blotting shows that knockdown of LMTK2 decreased cyclin D1 and increased p-p65(Ser468) (Fig. 6E). As expected, knockdown of LMTK2 decreased p-GSK3β(Ser9) and p-PP1A (Thr320). Quantitative PCR results indicate that LMTK2-KD decreased the transcription of NF-κB downstream genes *CCND1*, *IL-8*, *IκBα* and *A20* (Fig. 6F). A working model for LMTK2 is proposed (Fig. 6G).

4. Discussion

Thus far, LMTK2 is less studied and little is known about its biological function. These drove us to determine the possible role of LMTK2 in colon cancer cells. In this manuscript, we have demonstrated that LMTK2 modulates positively the activity of NF-κB and proliferation of colon cancer cells in vitro and xenograft growth in vivo. We have shown that LMTK2 regulates the NF-κB activity through PP1 − GSK3β pathway in unstimulated condition and TNF-induced NF-κB activity through PP1 − IKK pathway.

We were interested in whether LMTK2 played a role in proliferation of colon cancer cells. We firstly determined the expression of LMTK2 in a few colon cancer cells and normal colon epithelial CCD841 cells. We found that all the cells produced LMTK2 and the cancer cells produced more LMTK2 than normal CCD841 cells did (Fig. 1A), suggesting that LMTK2 might function to promote colon cancer cells. As expected, our results show that LMTK2 regulated positively the proliferation of colon cancer cells (Fig. 1B–F). Further investigation indicates that LMTK2 regulates cell proliferation through modulating the activation of NF-κB (Figs. 2 and 3).

In unstimulated condition, knockdown of LMTK2 decreased the activity of NF-κB (Fig. 3B) with little effect on protein levels of IκBα (Fig. 4A), suggesting that LMTK2 modulates NF-κB in a manner rather than influencing IκBα expression in the unstimulated condition. It is

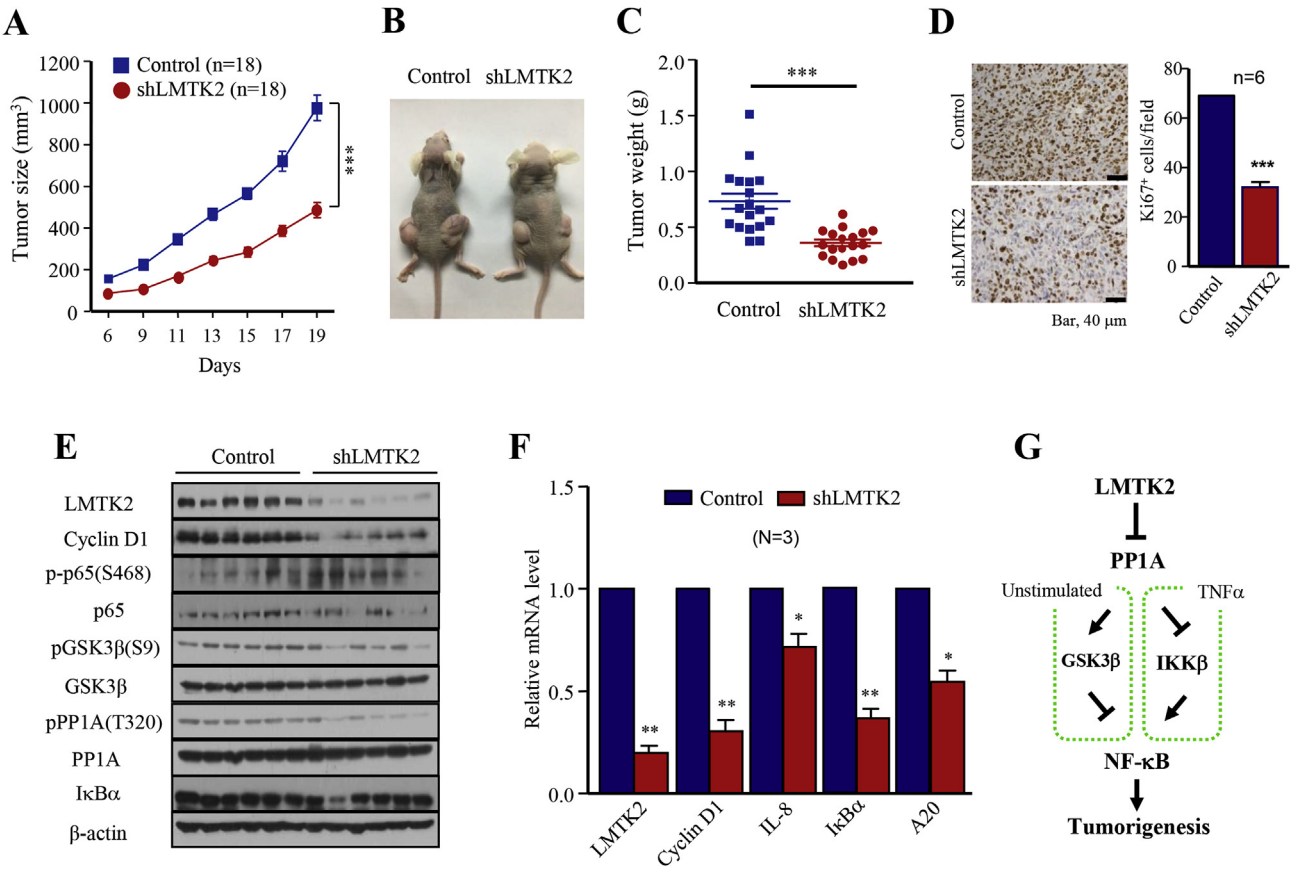

Fig. 6. Knockdown of LMTK2 suppresses HCT116 xenograft growth. (A) HCT116 cells were implanted in nude mice as described in Methods. Tumor sizes were measured every two or three days. Statistical analysis was conducted using ANOVA. (B) Representative of mice with tumors on 19th day. (C) Tumor weight. (D) Immunohistochemical stain of xenografts with Ki67 antibody. (E) Determination of the levels of interested proteins and phosphorylated proteins in xenografts by western-blot. (F) Determination of the mRNA levels of *CCND1*, *IL-8*, *IκBα* and *A20* in xenografts by quantitative PCR. (G) A proposed working model for LMTK2. Data are mean ± SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

known that GSK3β controls the basal NF-κB activity via phosphorylating p65 at Ser468 [24]. So, we examined p65 and found that LMTK2 modulated p-p65(S468) through GSK3β (Fig. 4). LMTK2 induced the inhibitory phosphorylation of GSK3β through phosphorylating and inactivating PP1A. These data imply that, in unstimulated condition, LMTK2 signals via PP1A-GSK3β to activate NF-κB. We noted that, in unstimulated condition, LMTK2-KD reduced the mRNA level of *IκBα* (Fig. 3F) with little influence on IκBα protein level (Fig. 4A). One possible reason is that the siLMTK2-mdeiated the change of IκBα mRNA level did not result in a change of IκBα protein level in the experimental conditions. Our results show that LMTK2-KD also reduced the mRNA levels of *A20* and *IL-8* (Fig. 3F). So, we determined the protein levels of A20 and IL-8 and found that siLMTK2 decreased the levels of these proteins (Fig. 5F).

We found that LMTK2 also regulated TNF-induced activation of NF-κB (Fig. 5). It is well-known that the stimuli activate the IKK complex and the activated IKK phosphorylates IκBα, leading to IκBα degradation. This allows NF-κB to enter the nucleus where it initiates gene expression. It was reported that PP1 formed a complex with CUEDC2 [27] or hCINAP [28] to dephosphorylate TNFα-induced p-IKK, resulting in IκBα stabilization and thereafter NF-κB inactivation. As LMTK2 is a negative regulator of PP1A [1], we presumed that LMTK2 regulated NF-κB through PP1A. As expected, knockdown of LMTK2 inhibited TNFα-induced phosphorylation of IKKβ, IκBα and p65 (Fig. 5D and E). Knockdown of PP1A prevented LMTK2-KD from inhibiting TNFα-induced NF-κB activation (Fig. 5G), implying that LMTK2 regulates TNFα-activated NF-κB through PP1-IKK pathway. In the presence of TNFα, knockdown of LMTK2 did not influence the phosphorylation of GSK3β (Fig. 5H). Thus, LMTK2 may not modulate TNF-activated NF-κB through GSK3β. In unstimulated condition, the CUE domain-containing 2 (CUEDC2) recruits IKK and PP1 to form a complex and maintains IKK in an inactive state [27]. In this condition, LMTK2 may modulate the basal NF-κB activity through PP1-GSK3β axis. When stimulated with TNFα, IKK is released from the IKK-CUEDC2-PP1 complex and is phosphorylated [27]. The activated IKK is re-recruited by CUEDC2 and is inactivated by PP1 [27]. Under stimulated condition, phosphorylation of GSK3β is not influenced by LMTK2 (Fig. 5H). So, GSK3β may not be involved in LMTK2-regulated NF-κB activated by TNFα. Together, these results suggest that LMTK2 modulates stimulated and unstimulated NF-κB activities in different mechanisms.

We noted that LMTK2-KD also had a minor effect on p-p65(S536) in unstimulated condition (Fig. 5E). As LMTK2-KD had no effect on IκBα (Fig. 4A), it might not influence p-p65(S536) via IKK. It is known that, besides IKK, other kinases such as RSK1 [30] and TKB1 [31] can also phosphorylate p65 at Ser536. And p-p65(S536) is dephosphorylated by PP2A [32] and Wip1 [33]. So the change of p-p65(S536) might be due to other mechanism and this needs investigation in future.

The aberrant constitutive activation of NF-κB is often associated with inflammatory diseases [34]. NF-κB is also constitutively active in many types of human tumors including colon cancer [35], due to the inflammatory microenvironment and/or oncogenic mutations. The constitutively activated NF-κB is associated with several aspects of tumorigenesis, including promoting cell proliferation, suppressing apoptosis, inducing angiogenesis and facilitating metastasis. For instance, NF-κB promotes cell proliferation through transcriptional activation of *CCND1* [36,37]. We have demonstrated in this manuscript that

inhibition of expression of LMTK2 inactivated NF-κB, attenuated expression of cyclin D1 and inhibited proliferation of colon cancer cells. Our findings highlight a role of LMTK2 in regulating the activity of NF-κB, thereby contributing to proliferation of colon cancer cells. These results also suggest that LMTK2-targeting might be a strategy for chemotherapy of colon cancer.

Author contribution

RZ, XL, LW, data acquisition; YQ, technique support; JF, work design, supervision and paper writing.

Conflicts of interest

The authors declare that there is no conflict of interest.

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References

[1] H. Wang, D.L. Brautigan, A novel transmembrane Ser/Thr kinase complexes with protein phosphatase-1 and inhibitor-2, J. Biol. Chem. 277 (2002) 49605–49612.
[2] C. Manser, A. Vagnoni, F. Guillot, J. Davies, C.C. Miller, Cdk5/p35 phosphorylates lemur tyrosine kinase-2 to regulate protein phosphatase-1C phosphorylation and activity, J. Neurochem. 121 (2012) 343–348.
[3] C. Manser, F. Guillot, A. Vagnoni, J. Davies, K.F. Lau, D.M. McLoughlin, K.J. De Vos, C.C. Miller, Lemur tyrosine kinase-2 signalling regulates kinesin-1 light chain-2 phosphorylation and binding of Smad2 cargo, Oncogene 31 (2012) 2773–2782.
[4] S. Kawa, J. Fujimoto, T. Tezuka, T. Nakazawa, T. Yamamoto, Involvement of BREK, a serine/threonine kinase enriched in brain, in NGF signalling, Genes Cells 9 (2004) 219–232.
[5] M.V. Chibalina, M.N. Seaman, C.C. Miller, J. Kendrick-Jones, F. Buss, Myosin VI and its interacting protein LMTK2 regulate tubule formation and transport to the endocytic recycling compartment, J. Cell Sci. 120 (2007) 4278–4288.
[6] T. Inoue, T. Kon, R. Ohkura, H. Yamakawa, O. Ohara, J. Yokota, K. Sutoh, BREK/LMTK2 is a myosin VI-binding protein involved in endosomal membrane trafficking, Genes Cells 13 (2008) 483–495.
[7] S. Kawa, C. Ito, Y. Toyama, M. Maekawa, T. Tezuka, T. Nakamura, T. Nakazawa, K. Yokoyama, N. Yoshida, K. Toshimori, T. Yamamoto, Azoospermia in mice with targeted disruption of the Brek/Lmtk2 (brain-enriched kinase/lemur tyrosine kinase 2) gene, Proc. Natl. Acad. Sci. U.S.A. 103 (2006) 19344–193499.
[8] X. Liu, Y. Jia, M.B. Stoopler, Y. Shen, H. Cheng, J. Chen, M. Mansukhani, S. Koul, B. Halmos, A.C. Borczuk, Next-generation sequencing of pulmonary sarcomatoid carcinoma reveals high frequency of actionable MET gene mutations, J. Clin. Oncol. 34 (2016) 794–802.
[9] J.S. Seo, Y.S. Ju, W.C. Lee, J.Y. Shin, J.K. Lee, T. Bleazard, J. Lee, Y.J. Jung, J.O. Kim, J.Y. Shin, S.B. Yu, J. Kim, E.R. Lee, C.H. Kang, I.K. Park, H. Rhee, S.H. Lee, J.I. Kim, J.H. Kang, Y.T. Kim, The transcriptional landscape and mutational profile of lung adenocarcinoma, Genome Res. 22 (2012) 2109–2119.
[10] R.A. Eeles, Z. Kote-Jarai, G.G. Giles, A.A. Olama, M. Guy, S.K. Jugurnauth, S. Mulholland, D.A. Leongamornlert, S.M. Edwards, J. Morrison, H.I. Field, M.C. Southey, G. Severi, J.L. Donovan, F.C. Hamdy, D.P. Dearnaley, K.R. Muir, C. Smith, M. Bagnato, A.T. Ardern-Jones, A.L. Hall, L.T. O'Brien, B.N. Gehr-Swain, R.A. Wilkinson, A. Cox, S. Lewis, P.M. Brown, S.G. Jhavar, M. Tymrakiewicz, A. Lophatananon, S.L. Bryant, UK Genetic Prostate Cancer Study Collaborators; British Association of Urological Surgeons' Section of Oncology; UK ProtecT Study Collaborators, A. Horwich, R.A. Huddart, V.S. Khoo, C.C. Parker, C.J. Woodhouse, A. Thompson, T. Christmas, C. Ogden, C. Fisher, C. Jamieson, C.S. Cooper, D.R. English, J.L. Hopper, D.E. Neal, D.F. Easton, Multiple newly identified loci associated with prostate cancer susceptibility, Nat. Genet. vol. 40, (2008) 316–321.
[11] L.W. Harries, J.R. Perry, P. McCullagh, M. Crundwell, Alterations in LMTK2, MSMB and HNF1B gene expression are associated with the development of prostate cancer, BMC Canc. 22 (2010) 10:315.
[12] K. Shah, N.A. Bradbury, Lemur Tyrosine Kinase 2, a novel target in prostate cancer therapy, Oncotarget 6 (2015) 14233–14246.
[13] A. Conti, M.T. Majorini, E. Fontanella, A. Bardelli, M. Giacca, D. Delia, M. Mano, D. Lecis, Lemur tyrosine kinase 2 (LMTK2) is a determinant of cell sensitivity to apoptosis by regulating the levels of the BCL2 family members, Cancer Lett. 389 (2017) 59–69.
[14] J. Xue, X. Li, S. Jiao, Y. Wei, G. Wu, J. Fang, Prolyl hydroxylase-3 is down-regulated in colorectal cancer cells and inhibits IKKβ independent of hydroxylase activity, Gastroenterology 138 (2010) 606–615.
[15] H.S. Zhang, B. Yan, X.B. Li, L. Fan, Y.F. Zhang, G.H. Wu, M. Li, J. Fang, PAX2 protein induces expression of cyclin D1 through activating AP-1 protein and promotes proliferation of colon cancer cells, J. Biol. Chem. 287 (2012) 44164–44172.
[16] C.Y. Liu, X. Lv, T. Li, Y. Xu, X. Zhou, S. Zhao, Y. Xiong, Q.Y. Lei, K.L. Guan, PP1 cooperates with ASPP2 to dephosphorylate and activate TAZ, J. Biol. Chem. 286 (2011) 5558–5566.
[17] J. Fang, Q. Zhou, X.L. Shi, B.H. Jiang, Luteolin inhibits insulin-like growth factor 1 receptor signaling in prostate cancer cells, Carcinogenesis 28 (2007) 713–723.
[18] V. Baldin, J. Lukas, M.J. Marcote, M. Pagano, G. Draetta, Cyclin D1 is a nuclear protein required for cell cycle progression in G1, Genes Dev. 7 (1993) 812–821.
[19] C.J. Sherr, Cancer cell cycles, Science 274 (1996) 1672–1677.
[20] D.L. Burkhart, J. Sage, Cellular mechanisms of tumour suppression by the retinoblastoma gene, Nat. Rev. Canc. 8 (2008) 671–682.
[21] L. Bakiri, D. Lallemand, E. Bossy-Wetzel, M. Yaniv, Cell cycle-dependent variations in c-Jun and JunB phosphorylation: a role in the control of cyclin D1 expression, EMBO J. 19 (2000) 2056–2068.
[22] M. Hinz, D. Krappmann, A. Eichten, A. Heder, C. Scheidereit, M. Strauss, NF-κB function in growth control: regulation of cyclin D1 expression and G0/G1-to-S-phase transition, Mol. Cell Biol. 19 (1999) 2690–2698.
[23] M.D. Jacobs, S.C. Harrison, Structure of an IkappaBalpha/NF-kappaB complex, Cell 95 (1998) 749–758.
[24] H. Buss, A. Dörrie, M.L. Schmitz, R. Frank, M. Livingstone, K. Resch, M. Kracht, Phosphorylation of serine 468 by GSK-3beta negatively regulates basal p65 NF-kappaB activity, J. Biol. Chem. 279 (2004) 49571–49574.
[25] C. Sutherland, I.A. Leighton, P. Cohen, Inactivation of glycogen synthase kinase-3b by phosphorylation: new kinase connections in insulin and growth-factor signalling, Biochem. J. 296 (1993) 15–19.
[26] G. Morfini, G. Szebenyi, H. Brown, H.C. Pant, G. Pigino, S. DeBoer, U. Beffert, S.T. Brady, A novel CDK5-dependent pathway for regulating GSK3 activity and kinesin-driven motility in neurons, EMBO J. 23 (2004) 2235–2245.
[27] H.Y. Li, H. Liu, C.H. Wang, J.Y. Zhang, J.H. Man, Y.F. Gao, P.J. Zhang, W.H. Li, J. Zhao, X. Pan, T. Zhou, W.L. Gong, A.L. Li, X.M. Zhang, Deactivation of the kinase IKK by CUEDC2 through recruitment of the phosphatase PP1, Nat. Immunol. 9 (2008) 533–541.
[28] L. Qu, Y. Ji, X. Zhu, X. Zheng, hCINAP negatively regulates NF-κB signaling by recruiting the phosphatase PP1 to deactivate IKK complex, J. Mol. Cell Biol. 7 (2015) 529–542.
[29] T. Scholzen, J. Gerdes, The Ki-67 protein: from the known and the unknown, J. Cell. Physiol. 182 (2000) 311–322.
[30] J. Bohuslav, L.F. Chen, H. Kwon, Y. Mu, W.C. Greene, p53 induces NF-kappaB activation by an IkappaB kinase-independent mechanism involving phosphorylation of p65 by ribosomal S6 kinase 1, J. Biol. Chem. 279 (2004) 26115–26125.
[31] H. Buss, A. Dörrie, M.L. Schmitz, E. Hoffmann, K. Resch, M. Kracht, Constitutive and interleukin-1-inducible phosphorylation of p65 NF-{kappa}B at serine 536 is mediated by multiple protein kinases including I{kappa}B kinase (IKK)-{alpha}, IKK{beta}, IKK{epsilon}, TRAF family member-associated (TANK)-binding kinase 1 (TBK1), and an unknown kinase and couples p65 to TATA-binding protein-associated factor II31-mediated interleukin-8 transcription, J. Biol. Chem. 279 (2004) 55633–55643.
[32] J. Yang, G.H. Fan, B.E. Wadzinski, H. Sakurai, A. Richmond, Protein phosphatase 2A interacts with and directly dephosphorylates RelA, J. Biol. Chem. 276 (2001) 47828–47833.
[33] J. Chew, S. Biswas, S. Shreeram, M. Humaidi, E.T. Wong, M.K. Dhillion, H. Teo, A. Hazra, C.C. Fang, E. López-Collazo, D.V. Bulavin, V. Tergaonkar, WIP1 phosphatase is a negative regulator of NF-kappaB signalling, Nat. Cell Biol. 11 (2009) 659–666.
[34] Q. Li, I.M. Verma, NF-kappaB regulation in the immune system, Nat. Rev. Immunol. 2 (2002) 725–734.
[35] M. Karin, Y. Cao, F.R. Greten, Z.W. Li, NF-kappaB in cancer: from innocent bystander to major culprit, Nat. Rev. Canc. 2 (2002) 301–310.
[36] D.C. Guttridge, C. Albanese, J.Y. Reuther, R.G. Pestell, A.S. Baldwin, NF-kappa B controls cell growth and differentiation through transcriptional regulation of cyclin D1, Mol. Cell Biol. 19 (1999) 5785–5799.
[37] M. Hinz, D. Krappmann, A. Eichten, A. Heder, C. Scheidereit, M. Strauss, NF-kappa B function in growth control: regulation of cyclin D1 expression and G(0)G(1)-to-S-Phase transition, Mol. Cell Biol. 19 (1999) 2690–2698.