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NG25, a novel inhibitor of TAK1, suppresses *KRAS*-mutant colorectal cancer growth in vitro and in vivo

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Abstract

KRAS mutations are one of the most prevalent genetic alterations in colorectal cancer (CRC). Although directly targeting KRAS still is a challenge in anti-cancer therapies, alternatively inhibiting KRAS related signaling pathways has been approached effectively. Here we firstly reported that MAP kinase, transforming growth factor-β-activated kinase 1 (TAK1), commonly expressed in CRC cell lines and significantly associated with *KRAS* mutation status. Inhibition of TAK1 by the small molecular inhibitor NG25 could inhibit CRC cells proliferation in vitro and in vivo, especially in *KRAS*-mutant cells. NG25 induced caspase-dependent apoptosis in *KRAS*-mutant cells and in orthotopic CRC mouse models by regulating the B-cell lymphoma-2 (Bcl-2) family and the inhibitor of apoptosis protein (IAP) family. Besides inhibiting molecules downstream of MAPK, including ERK, JNK and p38 phosphorylation, NG25 could block NF-κB activation in *KRAS*-mutant cells. As a target gene of NF-κB, down-regulated XIAP expression may be not only involved in apoptosis induced by NG25, but also reducing the formation of TAK1-XIAP complex that can activate TAK1 downstream signaling pathways, which forms a positive feedback loop to further induce apoptosis in *KRAS*-mutant CRC cells. Together, these findings indicated that TAK1 is an important kinase for survival of CRCs harboring *KRAS* mutations, and that NG25 may be a potential therapeutic strategy for *KRAS*-mutant CRC.

Keywords Colorectal cancer · *KRAS* · NG25 · TAK1 · Bcl-2 · XIAP

Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide and the fourth most common cause of cancer-related death [1]. Although treatment of CRC has improved in the past several decades, overall survival rates have not significantly increased [2]. Several critical genes and pathways contribute to the initiation and progression of CRC. One of the most prevalent genetic alterations in CRC is the *KRAS* activating mutation, which is observed in 30–50% of CRC cases and is predictive of poor responses to drugs targeting EGFR [3, 4]. Although its high mutation frequency and roles in therapeutic resistance make KRAS an ideal target for treating CRC, directly targeting KRAS is still a challenge in clinical therapy [5, 6].

One way in which *KRAS* mutations contribute to colorectal carcinogenesis is uniquely linked to the transforming growth factor-β (TGF-β) and bone morphogenetic protein (BMP)-7 signaling pathway. *MAP3K7*, which encodes TGF-β-activated kinase (TAK1), promotes survival in KRAS-dependent cells [6]. KRAS activates the TGF-β and

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BMP-7 signaling pathway, leading to TAK1 activation [6, 7]. Activated TAK1 phosphorylates and activates mitogen-activated protein kinase kinases (MAPKKs) leading to activation of mitogen-activated protein kinases (MAPKs), such as ERK, JNK and p38, which are all well-known survival downstream pathways of KRAS [6, 8]. In addition, TAK1 activates the NF-κB pathway to regulate pro-survival genes, such as the B-cell lymphoma-2 (Bcl-2) family or the inhibitor of apoptosis protein (IAP) family [8, 9]. Taken together, TAK1 plays an important role in cell survival, proliferation, differentiation and migration [10, 11].

NG25 is a novel synthetic inhibitor of TAK1 which binds to the ATP binding pocket of the target kinase in addition to an adjacent hydrophobic pocket that is created when the activation loop, which contains the conserved DFG motif, is in an "out" conformation [12]. A recent report showed that NG25 was able to sensitize breast cancer cells to Dox-mediated cytotoxic effects by suppressing TAK1 expression [13]. However, the potential anti-tumor effects of NG25 in CRC have not been investigated.

In the current study, we tested the effects of NG25 on the sensitivity of CRC cells. Our data showed that NG25 could induce apoptosis in CRC cells, especially those harboring mutant *KRAS* in vitro and in vivo, by inhibiting the activation of the TAK1 signaling pathways.

Materials and methods

Compounds and reagents

NG25 was purchased from Sigma-Aldrich (Cat. No. SML1332, St. Louis, MO, USA). Z-VAD-fmk (FMK001) and z-DEVD (FMK004) were obtained from R&D Systems (Minneapolis, MN, USA). The indicated antibodies used in this paper were against caspase-3 (CST #9664), cleaved-PARP (CST #5625), p-38 (CST #8690), p-p38 (CST #4511), JNK (CST #9252), p-JNK (CST #9255), ERK1/2 (CST #9102), p-ERK1/2 (CST #9106), IκBα (CST #4814), p-IκBα (CST #2859), Bad (CST #9239), IAP family (XIAP, c-IAP1, c-IAP2, Survivin and Livin, CST #9770), TAK1 (SCBT sc-166562), p-TAK1 (SCBT sc-4531), Bcl-2 (SCBT sc-7382), Bcl-xl (SCBT sc-8392), Mcl-2 (SCBT sc-12756), Bax (SCBT sc-23959) and β-actin (Sigma-Aldrich A2228). HRP conjugated goat anti-rabbit IgG was purchased from Thermo Scientific (35561, Waltham, MA, USA). HRP conjugated goat anti-mouse IgG was purchased from Abcam (ab150113, Cambridge, UK).

Cell lines and cell culture

$HCT116^{KRASG13D}$, $HCT116^{KRASWT}$, $DLD1^{KRASG13D}$, $DLD1^{KRASWT}$, $SW480^{KRASG12V}$, $SW620^{KRASG12V}$ and $HCT15^{KRASG13D}$ were kindly provided by Dr. Kevin Haigis (Beth Israel Deaconess Medical Center, Boston, USA). The other CRC cell lines used in this study were purchased from Kunming Cell Bank (KCB, China). All cell lines were incubated under standard conditions.

Immunohistochemical analysis

Apoptosis was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining (11684817910, In Situ Cell Death Detection Kit, Roche Molecular Biochemicals, NJ, USA), following the manufacturer's instructions.

MTT assay of cell viability

Cell viability assays were performed using MTT (MKBH9272V, Sigma-Aldrich) as described previously [14]. $HCT116^{KRASG13D}$ and $HCT116^{KRASWT}$ cell lines in the logarithmic growth phase were digested, counted, and seeded in 96-well plates at 5000 cells/well. After different concentrations of NG25 (0.1 μM, 0.5 μM, 1 μM, 5 μM, and 10 μM) were applied to the cells for 48 h, 20 μl of MTT (5 mg/mL) was added to each well for 4 h. The culture medium was discarded, 150 μL of DMSO was added to each well, and shaken at 37 °C for 10 min at 500 rpm. The absorbance of each well was measured with a microplate reader at 540 nm. At least six replicate wells were set for each concentration. Each experiment was performed in triplicate.

Colony formation assay

The colony formation assay was performed as described previously [13]. Briefly, $HCT116^{KRASG13D}$ and $HCT116^{KRASWT}$ cell lines were treated with NG25 at the indicated concentrations (2.5 μM and 5 μM) for 48 h, and then replaced with fresh medium. One week later, the cells were fixed and stained with methanol/crystal violet dye and photographed. Then, 33% acetic acid was added to dissolve the dye and incubated for 10 min. The absorbance of each well was measured with a microplate reader at 590 nm. Each experiment was performed in triplicate.

Apoptosis assay

Apoptosis assays were performed using propidium iodide (PI 81845, Sigma-Aldrich) and a FITC Annexin V Apoptosis Detection Kit (11988549001, Roche). Briefly, after $HCT116^{KRASG13D}$ and $HCT116^{KRASWT}$ cell lines were treated with NG25 (2.5 μM and 5 μM for PI Apoptosis Detection, 5 μM for FITC Annexin V Apoptosis Detection) for 48 h, the cells were stained according to the manufacturer's

instructions, and analyzed by flow cytometry (FACSort, Becton Dickinson, San Jose, CA, USA).

Immunoblotting assay

Immunoblotting assays were performed as described previously [15]. The membranes were visualized with the ECL Select™ Western Blotting Detection Reagent (RPN2235, GE Healthcare, Buckinghamshire, UK). The images were captured with ImageQuant™ LAS 4000 (GE Healthcare) and were quantified with ImageJ software. Anti-β-actin antibodies were used as the control for whole-cell extracts in all samples.

Immunoprecipitation assay

Immunoprecipitation assays were performed using Pierce™ Protein G Magnetic Beads (#88847, Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, treated cells were collected and lysed following the procedures of the immunoblotting assay. Equal amounts of total proteins were immunoprecipitated with the appropriate antibodies overnight at 4 °C with agitation, followed by incubation with pre-washed magnetic beads for 1 h with mixing. Another portion of the cell lysate was prepared for the input assay. The immunoprecipitation proteins and input proteins were analyzed by SDS-PAGE and immunoblotting to establish their interactions with other proteins.

Active RAS determination by RBD pulldown assay

Cells (1×10^7) were treated with NG25 for 48 h. RAS activity was determined by the RAS activation kit from Cell Signaling Technology according to the manufacturer's instructions. In brief, cells were lysed with 1 mL of lysis buffer. Cells were scraped off and lysate was centrifuged at 14,000 rpm for 5 min at 4 °C. Precleared lysates were subsequently added to prewashed glutathione agarose beads for 1 h at 4 °C under constant rocking. The beads were subsequently pelleted and washed 3 times and eluted for Western blotting with SDS-PAGE sample buffer. KRAS protein was detected using Western blot.

RNA extraction and RT-PCR assay

Total RNA was extracted using the TRIzol reagent, following the manufacturer's instructions. RNA (1 μg) was reverse-transcribed to cDNA using RevertAid First Strand cDNA Synthesis Kit (#1622, Thermo Scientific). The cDNA was amplified with the 7500 Real Time PCR System with QuantiTect™ SYBR Green PCR kits (19317900, Roche). The primer sequences of TAK1 is forward 5′-GATGCGGTA CTTTCCAGGAG-3′, reverse 5′-CATGAATGAGCCTGT ACTGGTG-3′. Relative expression levels of genes were calculated using the $2^{-\Delta\Delta Ct}$ method, with values normalized to the reference gene GAPDH.

Transient transfection and luciferase reporter assay

The activity of NF-κB was detected using a luciferase reporter assay as described previously [16]. Briefly, the cells were seeded and treated with NG25 for 48 h. Luciferase activity was measured using the Dual-Luciferase® Reporter Assay System (E1910, Promega Corporation, Madison, WI, USA). The luminescence was measured using the BioTek Synergy HT (Com3) and normalized to *Renilla* luciferase gene expression. Each experiment was performed in triplicate.

Immunofluorescence staining

The interaction between XIAP and TAK1 was examined by immunofluorescence staining, as described previously [17]. Briefly, cell slides were sequentially incubated with anti-XIAP (RPS3331, Sigma-Aldrich) and anti-TAK1 (sc-166562, Santa Cruz) primary antibodies, DyLight 594 Goat anti-Rabbit IgG secondary antibodies (#35561, ThermoFisher Scientific), Alexa Fluor 488 Goat anti-Mouse IgG secondary antibodies (A11001, Life Technologies, CA, USA), and 4′,6-diamidino-2-phenylindole (DAPI; H-1200, Vector Laboratories, CA, USA), and photographed using a confocal microscope.

Orthotopic mouse model and anti-tumor activity assay

A surgical orthotopic implantation mouse model of CRC was generated as described previously [18]. Briefly, mice were housed under standard conditions. Male BALB/c mice (6–8 weeks of age, 20–22 g) were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine via intraperitoneal injection. $CT26^{KRASG12D}$ cells (2.5×10^6 per mouse) were injected into the triangular region enclosed by the intestinal wall comprising the attachment of the cecum mesentery and two layers of the peritoneum (referred to as the cecum mesentery triangle). All mice were raised under specific pathogen-free conditions. After 1 week of tumor growth, the mice were randomly divided into 3 groups (4 mice per group): control (N.S. treatment), 5-fluorouracil (20 mg/kg/day, once every 2 days, intraperitoneal administration) and NG25 (30 μM/kg/day, once a day, intraperitoneal administration). One week later, the mice were sacrificed, and the tumor was separated, photographed and weighed. The tumor volume (V) was measured and calculated as the formula: $V = (\text{length} \times \text{width} \times \text{height} \times \pi)/6$ (mm^3). All animal procedures were approved by the Institutional Animal Care and Use Committee at the

Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences (No. SLXD-15-10-15).

Statistical analysis

Results are expressed as mean ± standard deviation (SD). Student's *t* test and ANOVA were used to determine the statistical significance of differences between drug treatment groups and the control group. $p < 0.05$ was considered to be statistically significant.

Results

TAK1 high-expression in *KRAS*-mutant CRC cell lines

We detected the KRAS and TAK1 expression in a panel of CRC cell lines using western blot. The result showed that KRAS protein and TAK1 protein are commonly expressed in CRC cell lines, but the expression level is different (Fig. 1a). Consistent with *KRAS* mutation state, *KRAS*-mutant CRC cell lines expressed higher KRAS GTP bound protein but not KRAS wildtype CRC cell lines (Fig. 1a). Then we analyzed the association between TAK1 expression and *KRAS* mutation. The result showed that the expression of TAK1 protein in the *KRAS*-mutant CRC cell lines was higher than that of the *KRAS* wildtype CRC cell lines ($p < 0.05$, Fig. 1b).

NG25, an inhibitor of TAK1, inhibited the proliferation and induced the apoptosis of CRC cells, especially in *KRAS*-mutant cells

NG25 has been reported to be a novel synthetic inhibitor of TAK1. The inhibitory activity against TAK1 was confirmed in paired isogenic cells, $HCT116^{KRASG13D}$ and $HCT116^{KRASWT}$. As shown in Fig. 2a, the mRNA levels of TAK1 were decreased after treatment with NG25 in both cell lines, as were the protein levels of TAK1 in both cell lines (Fig. 2b), and TAK1 inhibition by NG25 was more significant in *KRAS*-mutant CRC cell lines, which may be related to the higher expression of TAK1 in *KRAS*-mutant CRC cell lines.

In order to assess the inhibitory effect of NG25 on the proliferation of CRC cell lines, the $HCT116^{KRASG13D}$ cell line and $HCT116^{KRASWT}$ cell line were treated with NG25 at the indicated concentrations (0.1–10 μM) for 48 h. The MTT assay showed that NG25 treatment reduced cell viability in a dose-dependent manner. NG25 at 5 μM reduced the viability of $HCT116^{KRASG13D}$ and $HCT116^{KRASWT}$ by 75.1% and 43.1%, respectively (Fig. 2c). Consistently, NG25 also

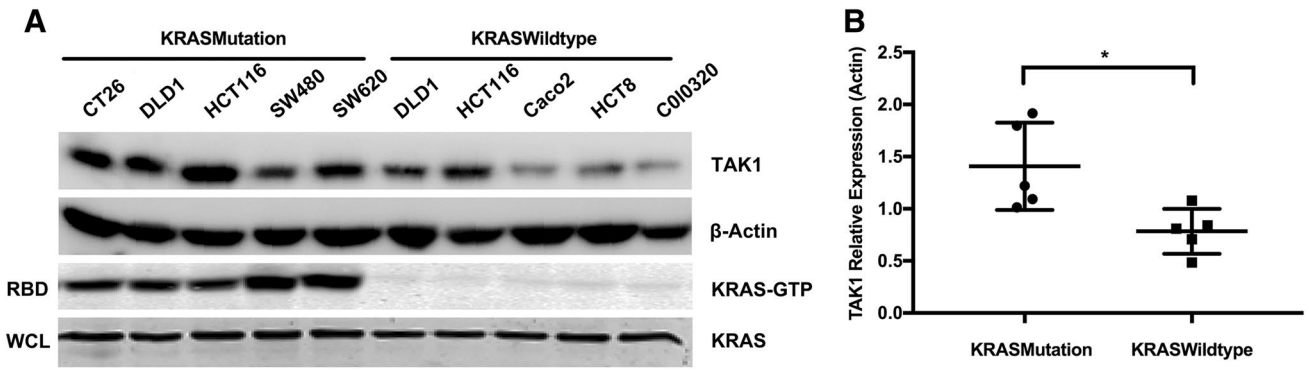
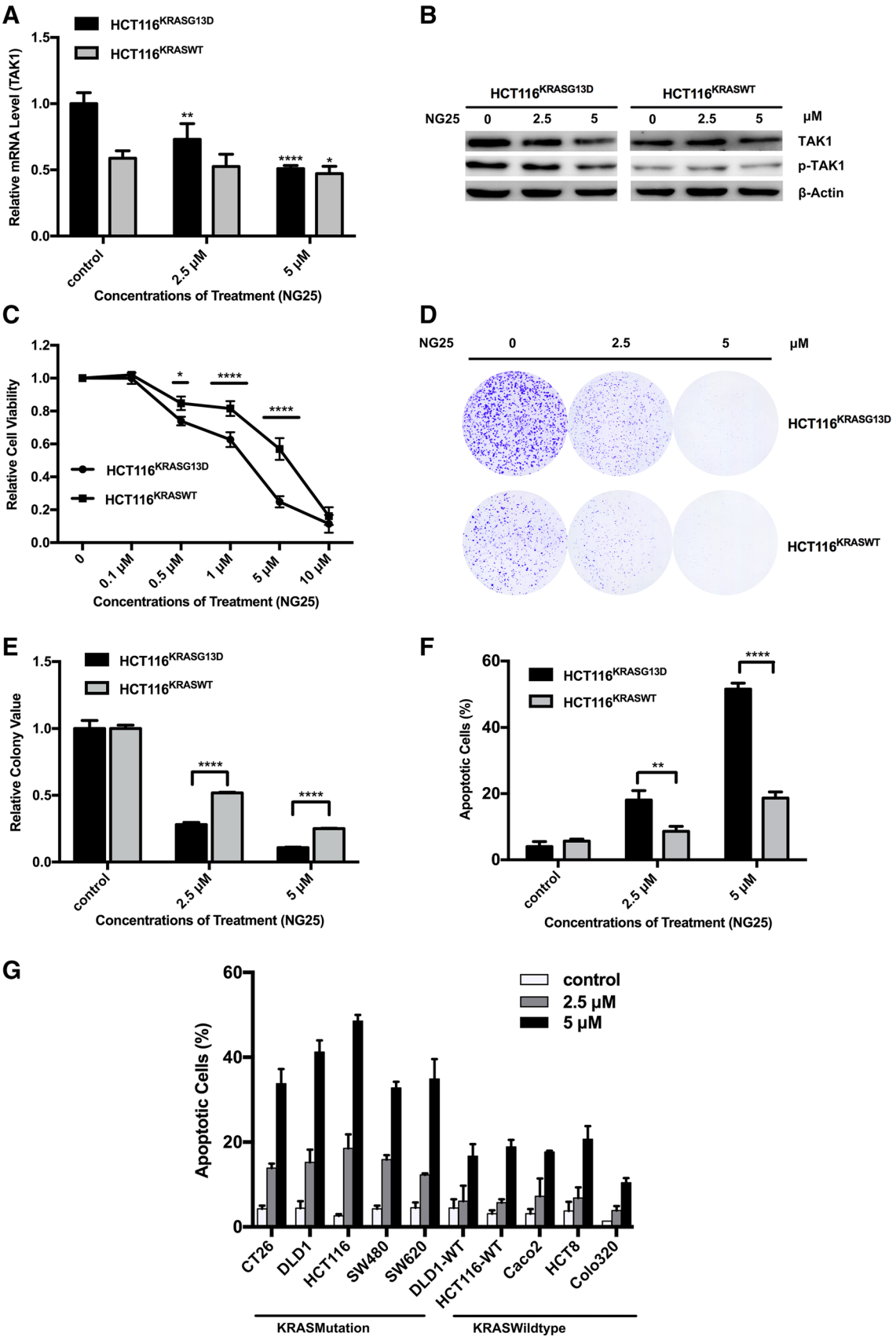


Fig. 1 TAK1 high-expression in *KRAS*-mutant CRC cell lines. **a** The expressions of TAK1 protein in CRC cell lines were analyzed using western blot. Detection of KRAS activation in KRAS-mutant and -wildtype CRC cell lines by RAS-binding domain (RBD) pull-downs assay. WCL: whole cell lysates. **b** The expressions of TAK1 protein were quantified using ImageJ software, and the association between TAK1 expression and *KRAS* mutation in CRC cell lines was analyzed. The data are from three independent experiments. *p* values < 0.05 (*) is indicated

Fig. 2 NG25 inhibited the proliferation and induced the apoptosis of CRC cells, especially in KRAS-mutant cells. **a, b** Cells were treated with NG25 at 2.5 and 5 μM, respectively, for 48 h. The expression levels of TAK1 were detected by qRT-PCR (**a**) and western blot (**b**). *p* values < 0.05 (*), < 0.01 (**), or < 0.0001 (****) are presented versus the respective control. **c** Cells were treated with NG25 at 0–10 μM for 48 h, then subjected to an MTT assay. *p* values < 0.05 (*) or < 0.0001 (****) represent the $HCT116^{KRASG13D}$ cell line versus the $HCT116^{KRASWT}$ cell line at 0.5 μM, 1 μM or 5 μM, respectively. **d** Cells were seeded in 12-well plates at 2×10^3 per well, and then cultured with 2.5 μM and 5 μM NG25, respectively, for 48 h, and then in fresh medium without the drug for one week. Then, cell colonies were fixed, stained with methanol/crystal violet dye and photographed. **e** Cells were treated with 33% acetic acid following **d**, and absorbance of each well was measured at 590 nm and plotted for the cell viability curve. **f** Cells were treated with 2.5 μM and 5 μM NG25, respectively, for 48 h, and were analyzed by Annexin-PI staining. *p* values < 0.01 (**) or < 0.0001 (****) are indicated. **g** Flow cytometry analysis of PI staining in a panel of CRC cells treated with 2.5 μM and 5 μM NG25, respectively, for 48 h. The data are presented as mean ± SD from three independent experiments


A
HCT116KRASG13D
HCT116KRASWT
Relative mRNA Level (TAK1)
1.5
1.0
0.5
0.0
**
****
*
control
2.5 μM
5 μM
Concentrations of Treatment (NG25)
B
HCT116KRASG13D
HCT116KRASWT
NG25
0
2.5
5
0
2.5
5
μM
TAK1
p-TAK1
β-Actin
C
Relative Cell Viability
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
****
****
HCT116KRASG13D
HCT116KRASWT
0
0.1 μM
0.5 μM
1 μM
5 μM
10 μM
Concentrations of Treatment (NG25)
D
NG25
0
2.5
5
μM
HCT116KRASG13D
HCT116KRASWT
E
Relative Colony Value
1.5
1.0
0.5
0.0
HCT116KRASG13D
HCT116KRASWT
****
****
control
2.5 μM
5 μM
Concentrations of Treatment (NG25)
F
Apoptotic Cells (%)
60
40
20
0
HCT116KRASG13D
HCT116KRASWT
**
****
control
2.5 μM
5 μM
Concentrations of Treatment (NG25)
G
Apoptotic Cells (%)
60
40
20
0
control
2.5 μM
5 μM
CT26
DLD1
HCT116
SW480
SW620
DLD1-WT
HCT116-WT
Caco2
HCT8
Colo320
KRASMutation
KRASWildtype

reduced *KRAS*-mutant cells colony formation to a greater extent than in *KRAS*-wildtype cells, as shown in Fig. 2d, e.

The inhibition of CRC cell viability by NG25 appeared to be largely due to the induction of apoptosis, as shown in Fig. 2f. NG25 induced apoptosis in a dose-dependent manner (Fig. 2f). Under treatment with NG25 (5 μM), there was 51.5% apoptosis in the HCT116^{KRASG13D} cell line, but only 18.7% in HCT116^{KRASWT} cell line. The induction of apoptosis by NG25 was confirmed in a panel of CRC cell lines (Fig. 2g). Consistently, *KRAS*-mutant CRC cell lines were more sensitive to NG25 than *KRAS*-wildtype CRC cell lines.

NG25 induced caspase-dependent apoptosis in *KRAS*-mutant CRC cells

Next, we determined whether NG25-induced apoptosis occurred in a caspase-dependent manner. Western blot analyses revealed that NG25 activated caspase-3 in HCT116^{KRASG13D} cell lines in a dose-dependent manner (Fig. 3a). NG25 also induced the cleavage of PARP, which is a substrate of activated caspase-3, in the HCT116^{KRASG13D} cell line, but not in the HCT116^{KRASWT} cell line.

To further explore the role of caspase in NG25-induced apoptosis, the cells were pretreated with apoptosis inhibitors, including the pan-caspase inhibitor z-VAD-fmk and the caspase 3 inhibitor z-DEVD-fmk, for 2 h, and then exposed to 5 μM NG25 for another 48 h. As shown in Fig. 3b, c, z-VAD-fmk and z-DEVD-fmk significantly inhibited NG25-induced apoptosis. This suggested that the apoptosis induced by NG25 was caspase-3-dependent.

Since the Bcl-2 family and the IAP family play key roles in regulating caspase-dependent apoptosis, we investigated the expression of the Bcl-2 family and the IAP family proteins after NG25-treatment. As shown in Fig. 3d, NG25 down-regulated the expression of the pro-survival proteins Bcl-2, Bcl-xl and Mcl-1, and up-regulated the expression of the pro-apoptotic proteins Bax and Bad in a dose-dependent manner in the HCT116^{KRASG13D} cell line, but not in the HCT116^{KRASWT} cell line. Of the IAP family members, XIAP, c-IAP1, c-IAP2, Survivin and Livin were markedly down-regulated after treatment with different concentrations of NG25 in the HCT116^{KRASG13D} cell line, but not in the HCT116^{KRASWT} cell line (Fig. 3e).

NG25 regulated the downstream signaling pathways of TAK1

As shown in Fig. 1a, b and Supplementary Fig. 2a, b, NG25 down-regulated the expression of TAK1 significantly in CRC cell lines, at both the transcriptional and translational levels. We further studied how the downstream signaling pathways of TAK1 were affected by NG25 treatment in CRC cells.

We investigated the protein expression of TAK1 related downstream molecules, ERK, JNK and p38, in the HCT116^{KRASG13D} cell line and the HCT116^{KRASWT} cell line. Figure 4a showed that NG25 treatment suppressed the expression of ERK, JNK and p38 in a dose-dependent manner in both cell lines.

TAK1 is a well-known upstream kinase for NF-κB activation. To clarify whether NF-κB activation is inhibited by NG25 treatment in *KRAS*-mutant CRC cells, we detected the activation of NF-κB in CRC cells after NG25 treatment for 48 h. The NF-κB activity reporter assay demonstrated that NF-κB activity was suppressed in a dose-dependent manner in the HCT116^{KRASG13D} cell line, but not in the HCT116^{KRASWT} cell line (Fig. 4b). Meanwhile, we tested the expression levels of phosphorylated IκBα (p-IκBα) in both cell lines. Consistently, NG25 reduced the phosphorylation of IκBα in the HCT116^{KRASG13D} cell line, but not in the HCT116^{KRASWT} cell line (Fig. 4c, d).

NG25 further inhibited NF-κB activation by interfering with the formation of the TAK1-XIAP complex

c-IAP1, c-IAP2 and XIAP, members of the IAP family that were down-regulated by NG25 treatment in *KRAS*-mutant cell lines, harbor E3 ligase activity. Beyond the inhibition of apoptosis, they affect TAK1 activity by directly or indirectly interacting with TAK1 [19]. We investigated that IAP proteins interacted with TAK1 using co-immunoprecipitation experiments. As shown in Fig. 5a, XIAP associated with TAK1 under untreated conditions and complex formation decreased after NG25(5 μM) treatment for 48 h in the HCT116^{KRASG13D} cell line. There was no binding between c-IAP1, c-IAP2 and TAK1 in the NG25-treated HCT116^{KRASG13D} cell line (Fig. 5b).

Immunofluorescence microscopy showed that cells clearly expressed both TAK1 and XIAP proteins, and there was a significant amount of co-localisation in the cytoplasm. After NG25 treatment, both TAK1 (green) and XIAP (red) fluorescence intensity were decreased but the merged fluorescent (yellow) almost was invisible (Fig. 5c). These results suggested that NG25 interfered with the binding of XIAP and TAK1. XIAP may be involve in the inhibition of TAK1 by forming the TAK1-XIAP complex in NG25-treated *KRAS*-mutant CRC cell lines.

NG25 suppressed tumor growth in the orthotopic CRC mouse model

The orthotopic-implantation tumor model was generated to validate the anti-tumor activity of NG25 in vivo.

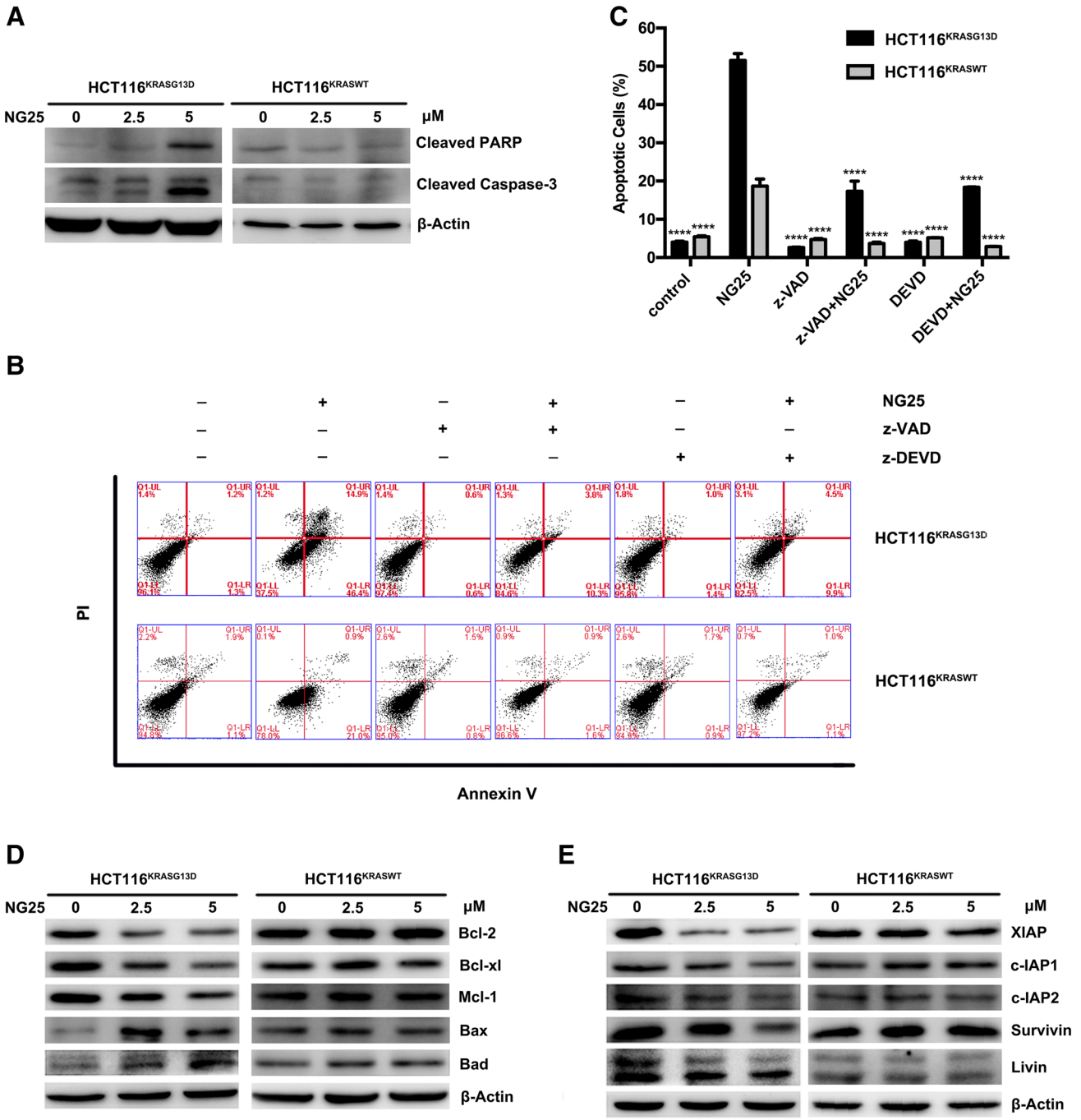


Fig. 3 NG25-induced caspase-dependent apoptosis in KRAS-mutant CRC cells. **a** Cells were treated with 2.5 μM and 5 μM NG25, respectively, for 48 h, and the protein expression was analyzed by western blot. **b, c** Cells were pretreated with caspase inhibitors z-VAD-fmk (20 μM) or z-DEVD-fmk (20 μM), respectively, for 2 h, and subsequently exposed to 5 μM NG25 for another 48 h. Apoptosis was analyzed by Annexin-PI staining and quantified. *p* values < 0.0001 (****) is presented versus the respective NG25-treated group. **d, e** Cells were treated with 2.5 μM and 5 μM NG25, respectively, for 48 h. The protein expression of the Bcl-2 family and the IAP family was analyzed by western blot (**d, e**). The data are presented as mean ± SD from three independent experiments

$CT26^{KRASG12D}$ cells were injected into the cecum mesentery triangle of BALB/c mice. 5-FU, which is a traditional chemotherapeutic drug for CRC in the clinic, was used as the treatment control in the experiment.

Compared with the N.S.-treated group, tumor sizes were significantly decreased in the NG25 group, but varied widely among mice in the 5-FU-treated group (Fig. 6a), which was in accordance with the poor impact of 5-FU administration

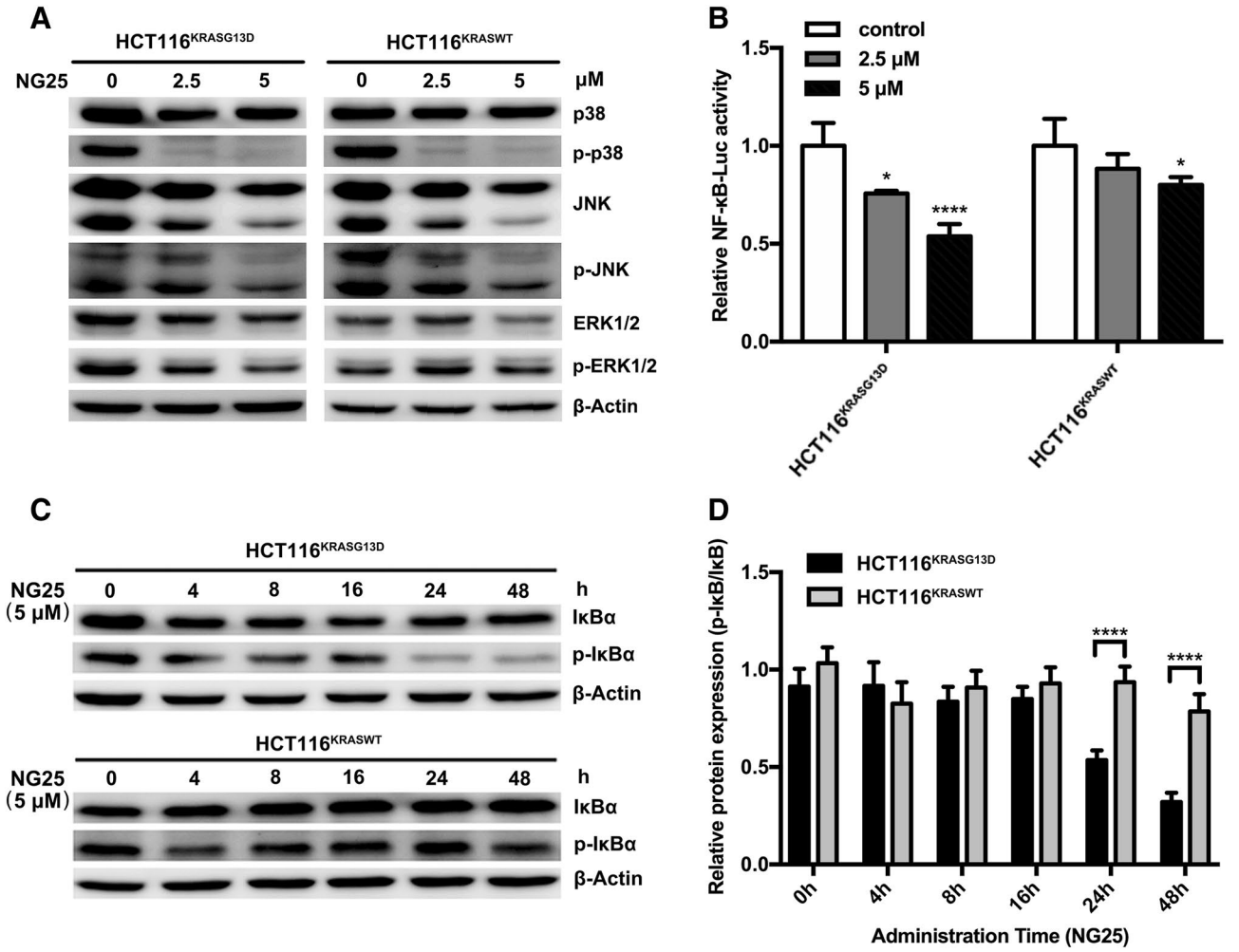


Fig. 4 NG25 regulated downstream signaling pathways of TAK1. Cells were treated with NG25 at 2.5 μM and 5 μM, respectively, for 48 h. The protein expression of p38/p-p38, JNK/p-JNK and ERK1/2/p-ERK1/2 was analyzed using western blot (**a**); NF-κB activity was analyzed using a luciferase reporter system (**b**). *p* values < 0.05 (*) or < 0.0001 (****) are presented versus the respective control. **c, d** Cells were treated with NG25 (5 μM) for the indicated time. The expression of IκB was analyzed by western blot and quantified with Image J software (**d**). *p* values < 0.0001 (****) is indicated. The data are presented as mean ± SD from three independent experiments

in *KRAS*-mutant CRC patients in the clinic. As shown in Fig. 6b, c, tumor volumes and tumor weights in the NG25 treatment group were significantly decreased, but not in the 5-FU-treated group. Consistently, TUNEL staining in tumor tissue sections showed that NG25 induced significant apoptosis (Fig. 6d).

We detected the protein expression in tumor tissues using western blot. The data showed that NG25 inhibited the expression levels of TAK1, and the phosphorylation of IκBα, as expected (Fig. 6e), but 5-FU treatment did not have a significant effect on the expression of these proteins (Fig. 6e). Meanwhile, the protein expression of IAP family members was down-regulated by NG25 treatment, which was consistent with the decreased IAP expression of NG25-treated HCT116^{KRASG13D} observed in vitro (Fig. 6f).

Discussion

Although KRAS is a downstream effector of EGFR, *KRAS* mutations were predictive factors of lack of response to anti-EGFR-targeted therapy [3, 4]. *KRAS* mutations have been increasingly accepted as a poor prognostic factor, as shown in several studies [20, 21]. As downstream molecules of KRAS, TAK1 plays a pivotal role in tumorigenesis, development, invasion and metastasis [6, 22]. In addition, RNAi-mediated silencing of TAK1 and TAK1 inhibitors can induce apoptosis in *KRAS*-mutant CRC cells [6]. However, the association between TAK1 and *KRAS* mutation, in CRC cells, have not been reported.

Herein, we found that the expression of TAK1 protein is associated with *KRAS* mutation in CRC cell lines. The

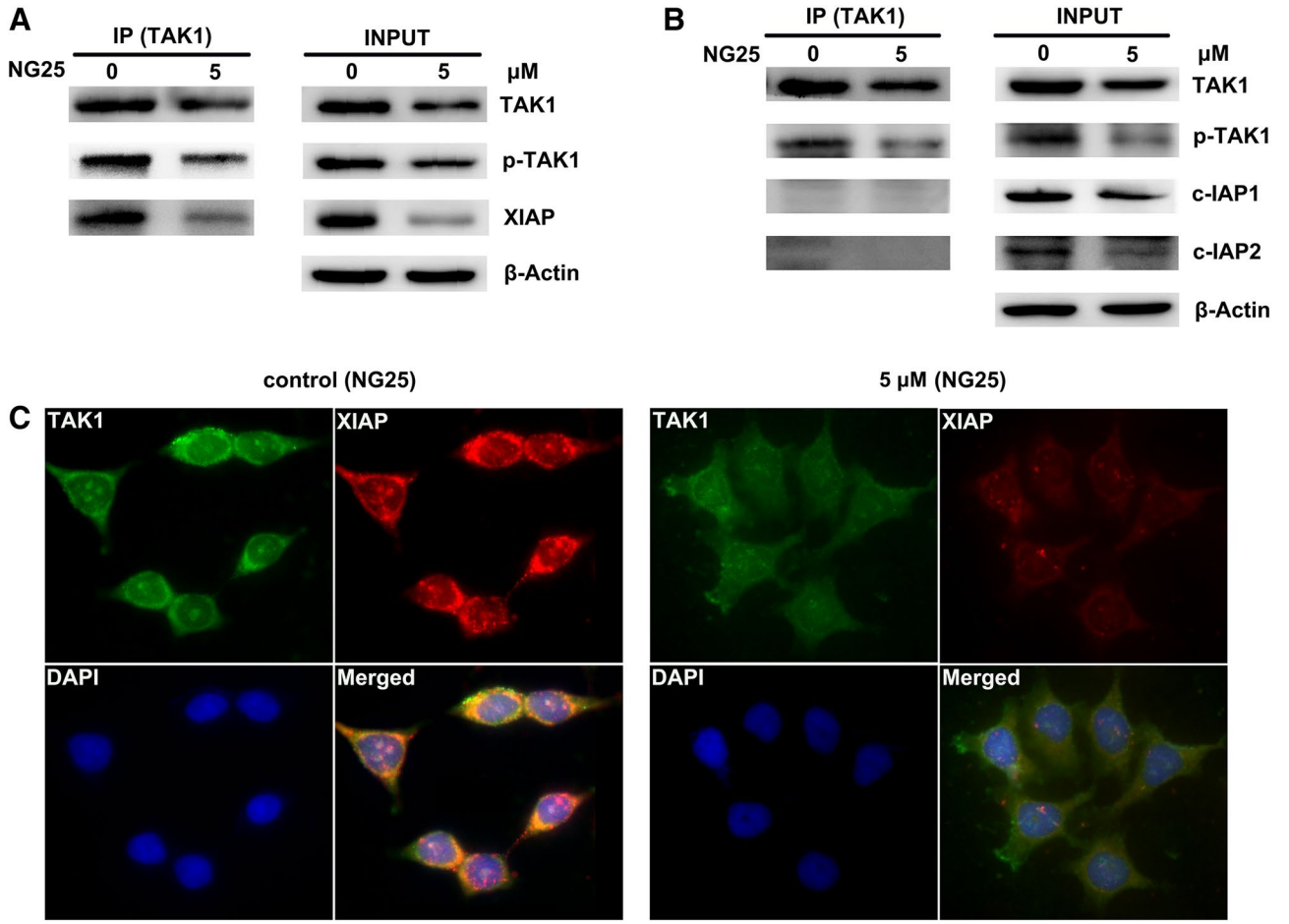


Fig. 5 NG25 further inhibited NF-κB activation by interfering with the formation of the TAK1-XIAP complex. Cells were treated with NG25 at 5 μM for 48 h. **a, b** The binding activity of XIAP, c-IAP1/2 and TAK1 was analyzed using a co-immunoprecipitation assay in the HCT116^{KRASG13D} cell line. **c** Representative photomicrographs showed the immunofluorescence staining for XIAP (red) and TAK1 (green) after NG25-treatment for 48 h in the HCT116^{KRASG13D} cell line. Nuclei (blue) were stained with DAPI

expression of KRAS protein and TAK1 protein was higher in *KRAS*-mutant CRC cell lines than in *KRAS*-wildtype cell lines. These data indicate that inhibiting TAK1 may be a viable therapeutic strategy in *KRAS*-mutant CRC.

In this study, NG25, a potent inhibitor of TAK1, inhibited CRC cell proliferation in vitro and in vivo, and the inhibition effect was more significant in *KRAS*-mutant CRC cell lines. As a MAPK inhibitor, NG25 inhibited the downstream signaling pathways of MAPK. The phosphorylation of ERK1/2, JNK and p38 MAPK was down-regulated in CRC cells. This kinase-inhibitory effect of NG25 is consistent with the results of previously reported studies [13, 23, 24]. Since the kinase-inhibitory effect of NG25 was detected in both *KRAS*-mutant and wildtype cells, this can explain why the proliferation of CRC cells was inhibited, but cannot be the main reason why *KRAS*-mutant cells are more sensitive to NG25 treatment.

Our further experiments showed that NG25 can induce caspase-dependent apoptosis in *KRAS*-mutant CRC cells. The Bcl-2 family and the IAP family contributed to the apoptosis induced by NG25 in *KRAS*-mutant cells, including down-regulation of the pro-survival proteins Bcl-2, Bcl-xl, Mcl-1, XIAP, c-IAP1/2, Survivin and Livin, and up-regulation of the pro-apoptosis proteins Bax and Bad.

The Bcl-2 family and the IAP family members are target genes regulated by activated NF-κB. Previous reports have shown that TAK1 can activate the NF-κB signaling pathway [9]. Consistently, down-regulation of the phosphorylation of IκBα and the luciferase assay demonstrated that NG25 suppressed NF-κB activity in *KRAS*-mutant cells. Therefore, the reduction in the activity of NF-κB and subsequent regulation of its target genes appear to be involved in the sensitization of *KRAS*-mutant CRC cells to NG25-induced apoptosis.

As shown above, NG25 down-regulated the expression of IAPs at both the transcriptional and translational levels. Beyond the well-characterized functions of IAPs in caspase and cell death inhibition, the members containing a

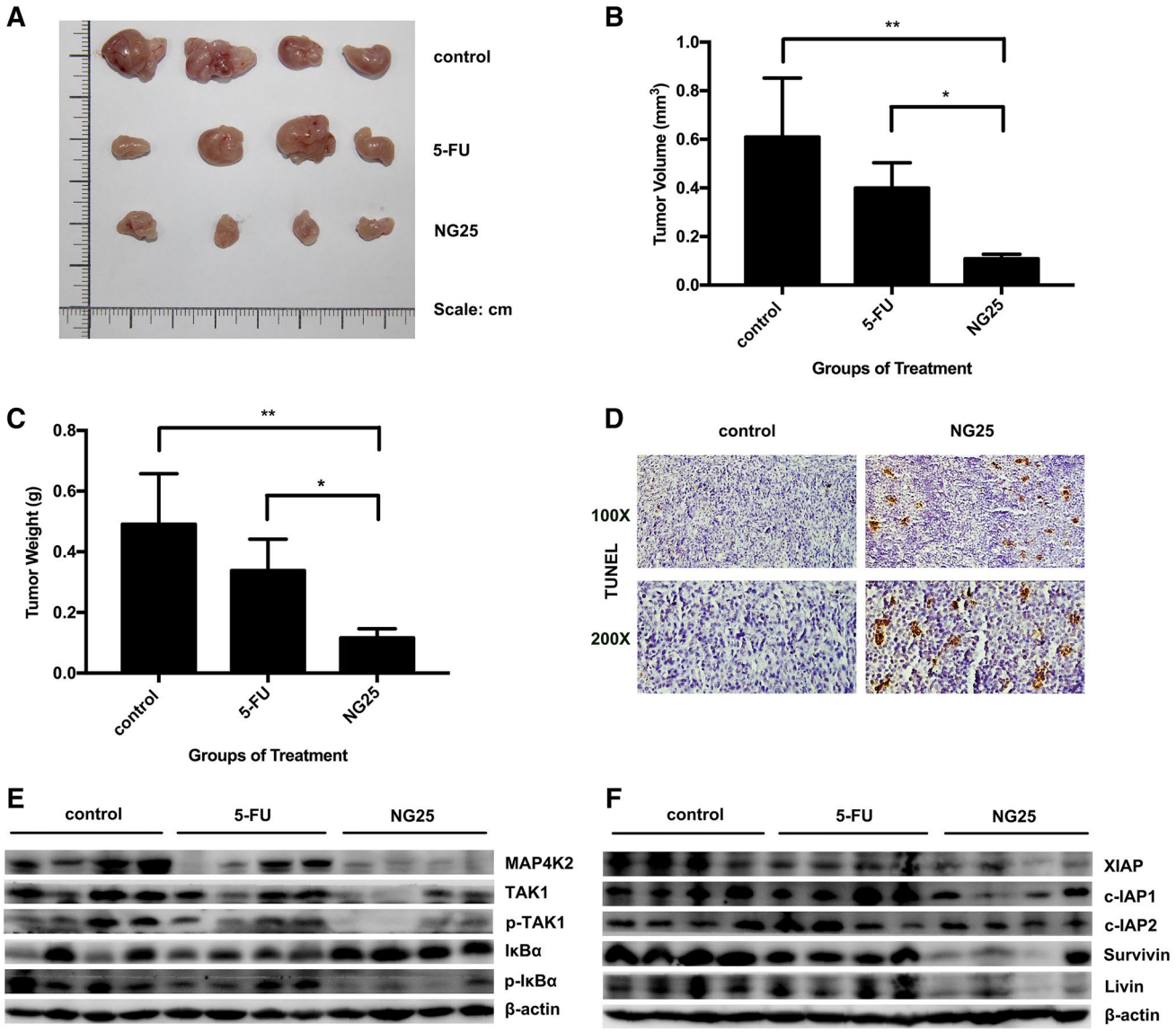


Fig. 6 NG25 suppressed tumor growth in the orthotopic colorectal cancer mouse model. **a** Photographs of orthotopic CRC tumors following treatment with N.S., 5-fluorouracil (20 mg/kg/day, once every 2 days), NG25 (30 μM/kg/day, once a day) by intraperitoneal administration. n = 4. **b, c** Tumor volumes and weights were measured on the day of harvesting. The data are presented as mean ± SD. *p* values < 0.05 (*) or < 0.01 (**) are indicated. **d** Representative photomicrographs of TUNEL staining in tumor tissues. **e, f** Protein expressions of TAK1/p-TAK1, IκBα/p-IκBα and IAP family proteins in tumors from different groups (n = 4) were detected by western blot

RING domain, which confers E3 ligase activity, can regulate the signaling pathway by interacting with other molecules [25, 26]. Our results showed that NG25 interfered with the formation of the TAK1-XIAP complex, and further inhibited the downstream signaling pathways of TAK1. Several reports showed that c-IAP1 and c-IAP2 activate both the classical and alternative arms of the NF-κB signaling pathway, by interacting with TAK1 [27–29]. However, our data showed that there was no binding activity between c-IAP1, c-IAP2 and TAK1 in CRC cells. Taken together, these results show that IAPs play potential roles in the NG25-induced apoptosis of *KRAS*-mutant CRC cells. Importantly, the down-regulation of XIAP by NF-κB further maybe inhibit the activation of TAK1 by reducing the formation of the TAK1-XIAP complex in *KRAS*-mutant CRC cells.

In conclusion, we demonstrated that NG25 induces *KRAS*-mutant CRC cell apoptosis in vitro and in vivo by blocking the TAK1 signaling pathways. NF-κB inactivation by inhibiting TAK1 further regulated target genes, including the Bcl-2 family and the IAP family, which are closed related to the apoptosis induced by NG25 in *KRAS*-mutant cells. Importantly, down-regulation of XIAP, in turn, maybe inhibit the activation of TAK1 by reducing the formation of the TAK1-XIAP complex, which forms a positive feedback

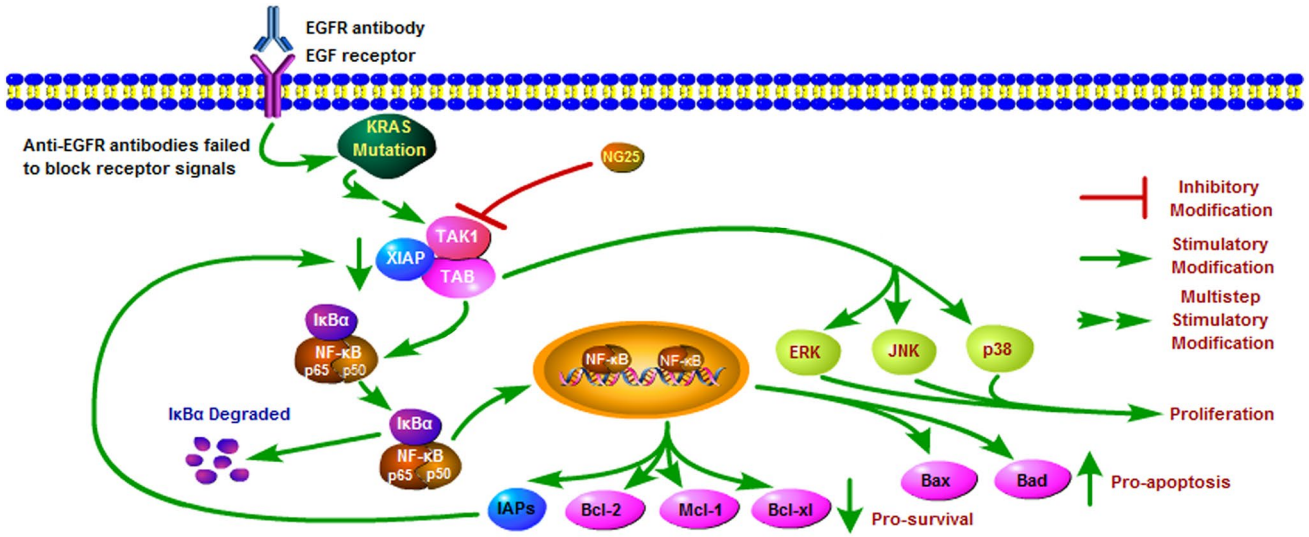


Fig. 7 A diagram of the mechanisms by which NG25 suppresses KRAS-mutant colorectal cancer growth

loop to further induce apoptosis in *KRAS*-mutant CRC cell lines (Fig. 7).

Our study illustrates that TAK1 is the therapeutically relevant kinases in CRCs harboring *KRAS* mutations. NG25, as an inhibitor of TAK1, could be a novel approach against *KRAS*-mutant CRC in clinical therapy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval All animal experiments are in accordance with International Guidelines and Protocols and approved by the Institutional Animal Care and Use Committee at the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences.

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