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TIFA promotes colorectal cancer cell proliferation in an RSK- and PRAS40-dependent manner

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Abstract

Previous studies have reported that TIFA plays different roles in various tumor types. However, the function of TIFA in colorectal cancer (CRC) remains unclear. Here, we showed that the expression of TIFA was markedly increased in CRC versus normal tissue, and positively correlated with CRC TNM stages. In agreement, we found that the CRC cell lines show increased TIFA expression levels versus normal control. The knockdown of TIFA inhibited cell proliferation but had no effect on cell apoptosis in vitro or in vivo. Moreover, the ectopic expression of TIFA enhanced cell proliferation ability in vitro and in vivo. In contrast, the expression of mutant TIFA (T9A, oligomerization site mutation; D6, TRAF6 binding site deletion) abolished TIFA-mediated cell proliferation enhancement. Exploration of the underlying mechanism revealed that the protein synthesis-associated kinase RSK and PRAS40 activation were responsible for TIFA-mediated CRC progression. In summary, these findings suggest that TIFA plays a role in mediating CRC progression. This could provide a promising target for CRC therapy.

KEYWORDS
cell proliferation, colorectal cancer, PRAS40, RSK, TIFA

Abbreviations: CRC, colorectal cancer; eIF, eukaryotic translation initiation factor; HCC, hepatocellular carcinoma; IHC, immunohistochemistry; IKK, IκB kinase; mTORC1, mTOR complex 1; NEAA, non-essential amino acid; NF-κB, nuclear factor-κB; PI, propidium iodide; PRAS40, proline-rich *akt* substrate of 40 kDa; RSK, ribosomal S6 protein kinase; TAK, transforming growth factor β-activated kinase; TIFA, TRAF-interacting protein with a Forkhead-associated domain; TRAF6, tumor necrosis factor receptor-associated factor 6.

1 | INTRODUCTION

As one of the common human malignancies, CRC has high morbidity and mortality and is ranked as the third most commonly diagnosed cancer in me and the second in women.[1] Globally, more than 1.4 million new cases and nearly 0.7 million deaths from CRC are reported per year.[2] With significant progress in screening and treatment strategies, the prognosis of CRC patients has steadily improved, and the relative 5-year survival rate has improved.[3] However, effective strategies targeting tumor metastasis, chemoresistance, or recurrence to prevent CRC progress remain lacking. Based on this, the development of novel prognostic biomarkers or targets to predict CRC prognosis and develop CRC targeted therapy is urgently needed.

First named T2BP,[4] TIFA was identified as a novel TRAF6-interacting protein by using yeast two-hybrid screening.[5] The structure of TIFA includes a Forkhead-associated domain that could directly bind phosphothreonine/phosphoserine, and a consensus TRAF6-binding motif that could interact with TRAF6. Oligomerization of TIFA, which induces oligomerization and polyubiquitinylation of TRAF6, in turn activates TAK and IKK,[6] finally mediates NF-κB activation in the Toll-like receptor 4/interleukin-1 signaling pathway.[7] Recent research has also offered an insight into the role of TIFA for innate immunity induced by bacterial metabolite heptose- 1, 7-bisphosphate (HBP) or NLRP3 inflammasome.[8–10] Moreover, it has also been proven that TIFA promotes cancer cell migration and cell survival in lung adenocarcinoma.[11] Our previous work indicated that TIFA expression is downregulated in HCC progression and TIFA reconstitution promotes HCC cell apoptosis, while suppressing HCC cell proliferation among surviving cells.[12,13] The above previous findings imply that TIFA plays various roles in different cancer types. However, the function of TIFA in CRC was not dissected.

Cell cycling and protein synthesis both play irreplaceable role in cancer cell proliferation.[14] Protein synthesis is also a key step that occurs in G_0/G_1 and G_2/M phase of the cell cycle. Ribosomal S6 protein kinase is a family of serine/threonine protein kinases comprises four main members (RSK1, RSK2, RSK3, and RSK4), and they could activate downstream of the MAPK pathway.[15] Ribosomal S6 protein kinase has multiple functions, including involvement in cancer cell proliferation, invasion, and migration.[16–19] Ribosomal S6 protein kinase was proved to regulate the stability of eIF to mediate protein synthesis.[20,21] It was also reported that RSK could combine with TRAF6 and they could activate each other.[22] Nevertheless, the specific function of RSK in TIFA-mediated cancer progression remains unreported.

Also called 14-3-3 binding protein, PRAS40 was first identified in insulin-treated hepatoma cells lysates.[23] Further studies of PRAS40 found that it was a component and substrate of mTORC1.[24,25] PRAS40 phosphorylated by the kinases like AKT or by mTORC1 itself could induce the dissociation of PRAS40 from mTORC1 and relieve an inhibitory constraint on mTORC1 activity.[26] But PRAS40 is also indispensable for the activity of the mTORC1 complex.[27] PRAS40 was reported to be highly expressed in cancer and mediated cancer progression by activating mTOR signaling.[28–30] But the role of PRAS40 in TIFA-mediated cancer progression remains unknown.

In the present study, we investigated the specific function and mechanism of TIFA in promoting CRC progression in vitro and in vivo. We found that the expression of TIFA was markedly increased in CRC tissue and cell lines versus normal control. The knockdown of TIFA inhibited CRC cell proliferation but had no effect on cell apoptosis in vitro or in vivo. Moreover, the ectopic expression of TIFA enhanced cell proliferation ability in vitro and in vivo. In contrast, the expression of mutant TIFA (T9A, oligomerization site mutation; D6, TRAF6 binding site deletion) abolished TIFA mediated cell proliferation enhancement. Exploration of the underlying mechanism indicated that the protein synthesis-associated kinase RSK and PRAS40 activation were all responsible for TIFA-mediated CRC progression. In summary, these findings suggest that TIFA plays a role in mediating CRC progression. This could provide a promising target for CRC therapy.

2 | MATERIALS AND METHODS

2.1 | Tumor tissues

The CRC tissues we used were obtained from patients who underwent surgical treatment at the Affiliated Hospital of Jining Medical University from 2015 to 2019; more information is listed in Table S1. All tissues from the patients were staged based on the American Joint Committee on Cancer TNM staging system. This research was approved by the institutional ethics committees at Jining Medical University.

2.2 | Cell culture

SW620 colon cancer cells were cultured in L15 medium (Gibco) supplemented with 10% FBS (Gibco). RKO cancer cells were cultured in DMEM medium (HyClone) supplemented with 10% FBS, 1% nonessential amino acid (Gibco), 1% sodium pyruvate (Gibco), and 1.5 g/L $NaHCO_3$ (Gibco). SW480 cancer cells were cultured in DMEM (HyClone) supplemented with 10% FBS and 2% L-glutamine (Gibco). RKO and SW480 cells were grown at 37°C in 5% CO_2 incubators and SW620 cells were grown at 37°C in 0% CO_2 incubators. All cells were passaged for less than 3 months before renewal from frozen, early-passage stocks. All cells were purchased from ATCC and all were tested to ensure that they were *Mycoplasma* negative.

2.3 | Vector construction

Short hairpin RNA targeting human TIFA and human TIFA or mutant TIFA overexpression plasmid were constructed following the published protocol.[12,13] The primers used in this assay are listed in Table S2.

2.4 | Cell proliferation assay

The CCK-8 assay was carried out on colon cancer cells following the previous protocols.[31] Cells were seeded in a 96-well plate at 3×10^3 cells per well (day 0) and incubated for 4–5 days. CCK-8 reaction agent (10 μl) was added to each well at the time of cell collection, then the optical density was measured at 450 nm by multiscanner autoreader.

2.5 | Cell colony formation

Colon cancer cells were seeded at a density of 500 cells/ml in a 6-well plate, then cultured with complete medium for 2 weeks. The cell clones were treated with 4% paraformaldehyde fixation and stained with Giemsa (containing 1% Trion X-100) for 4-5 hours. Clones were photographed and the colony numbers were calculated using ImageJ software.[32]

2.6 | Flow cytometry analysis of apoptosis and cell cycle

For the cell cycle assay, colon cancer cells were stained with PI (BD Biosciences). For the cell apoptosis assay, apoptotic cells were stained with PI and annexin-V-FITC (BD Biosciences). The details of these assays followed previously published protocols.[12,13]

2.7 | Western blot analysis

Western blot analysis was carried out according to established protocols described previously.[12,13] Primary Abs used in this assay are listed in Table S3. All western blot results are provided as representative images from three independent experiments.

2.8 | Immunohistochemistry

Immunohistochemistry assay was undertaken on human CRC tissue microarrays (cat. # col11042; Avilabio Company) or paraffin-embedded specimens from in vivo tumors. The specific Abs used for targeting corresponding proteins (listed in Table S2) were scored based on the percentage of protein-positive cells in each tissue. The photographs were taken using a panoramic viewer under a×10 or ×40 objective (3DHistech).[33]

2.9 | Immunoprecipitation

Immunoprecipitation was carried out according to the established protocols described previously.[33,34] Antibodies used in this assay are listed in Table S2.

2.10 | Human phosphokinase array

The phosphokinase array was undertaken using the Human Phosphokinase Array Kit (Cat. # ARY003B; R&D Systems) following the manufacturer's instructions.

2.11 | Small molecular inhibitors

The RSK-specific inhibitor BRD7389 was purchased from MCE and the effective concentration in our assay was 4 μM. The IKK-specific inhibitor IKK-16 was purchased from MCE and the effective concentration in our assay was 5 μM.

2.12 | Xenograft model study

The in vivo xenograft model studies were first approved by Jining Medical University Ethics Committee. Nude mice, 6–8 weeks of age, were randomly assigned to each group ($n \geq 6$). The stable cell lines (1×10^6 cells) were injected into each mouse subcutaneously. When palpable, the tumors were calculated and recorded. The tumor volume was calculated by the following formula: volume (mm^3) = (width^2 (mm^2) × length (mm))/2. Finally, the mice were humanely killed using avertin (Sigma-Aldrich) (dissolved by tert-pentanol and 0.9% saline, 0.2–0.4 ml/10 g mice weight, i.p. injection).[35] The tumors were excised, fixed with formalin, paraffin-embedded, and sectioned for further analysis.[33]

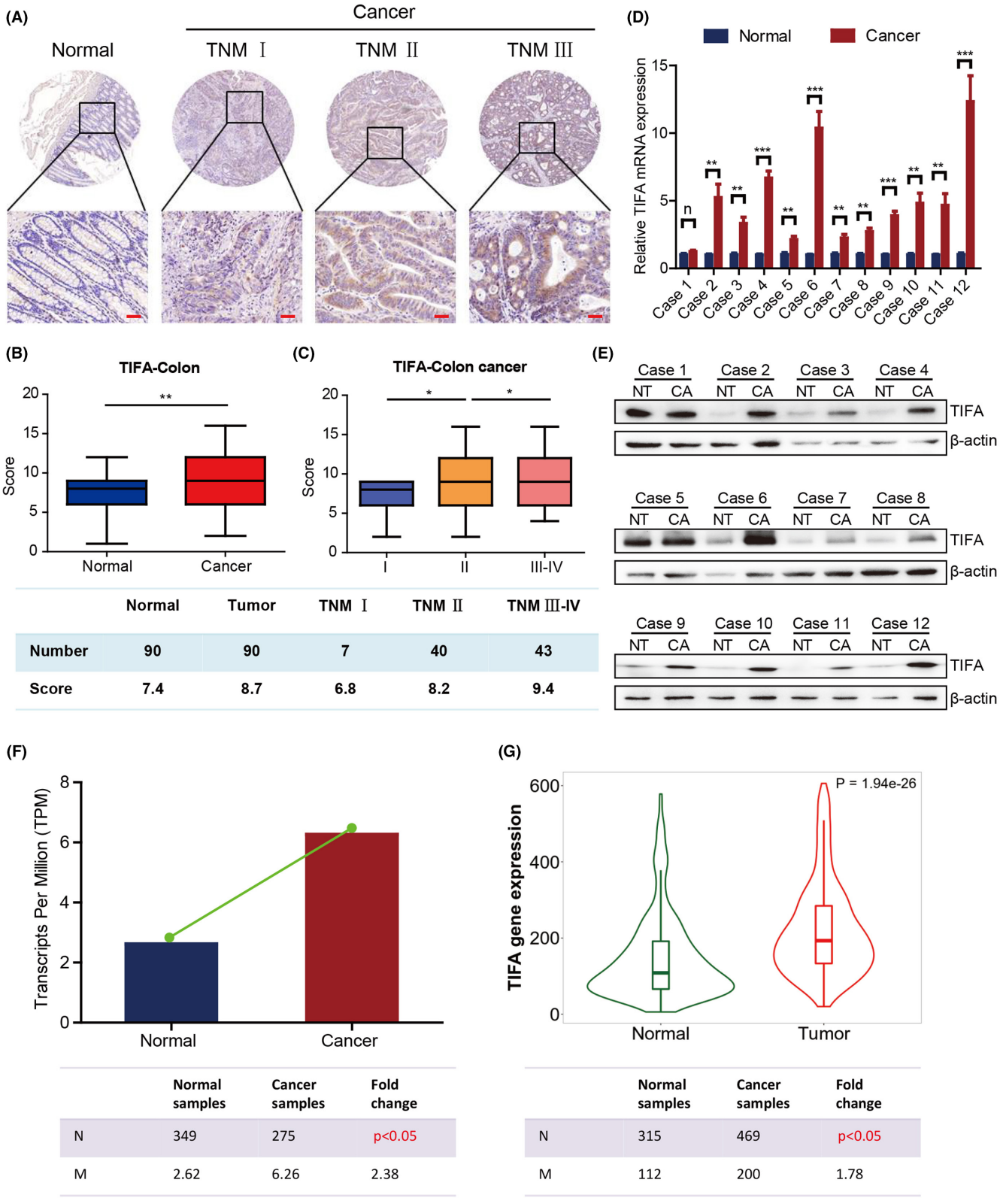
2.13 | Statistical analysis

All data were analyzed using GraphPad Prism5 software. Values were expressed as means ± SEM. The p values were calculated using a two-tailed Student's t-test (two groups) or one-way ANOVA (more than two groups) unless otherwise noted. A value of $p<0.05$ was used as the criterion for statistical significance. *Significant difference with $p<0.05$, **significant difference with $p<0.01$, and ***significant difference with $p<0.001$.[33,36,37]

3 | RESULTS

3.1 | TIFA upregulated in human CRC tissues

To investigate the expression pattern of TIFA in human CRC, we undertook IHC analyses with a TIFA-specific Ab in human CRC tissue microarrays containing 180 samples (90 normal colon tissues and 90 CRC tissues). The results showed that expression levels of TIFA were increased in tumor biopsies versus comparable normal tissues (Figure 1A,B). We also explored the correlation between the expression levels of TIFA and the TNM stages of the tumor biopsies. We found a positive correlation between elevated



	Normal	Tumor	TNM Ⅰ	TNM Ⅱ	TNM Ⅲ-Ⅳ
Number	90	90	7	40	43
Score	7.4	8.7	6.8	8.2	9.4

	Normal samples	Cancer samples	Fold change
N	349	275	p<0.05
M	2.62	6.26	2.38

	Normal samples	Cancer samples	Fold change
N	315	469	p<0.05
M	112	200	1.78

FIGURE 1 TIFA was upregulated in human colorectal cancer (CRC) tissues. (A) Representative immunohistochemistry of TIFA in a human CRC tissue array. Scale bar, 50 μm. (B) Quantification of TIFA immunohistochemistry in a CRC tissue array. (C) Quantification of TIFA immunostaining and TNM stages. (D, E) Representative quantitative RT-PCR and western blot analyses of 12 pairs of fresh colon cancer tissue (CA) and normal adjacent tissue (NT). (F, G) Transcriptional expression of TIFA in CRC cancers (cancer vs. corresponding normal tissue) were evaluated using the GEPIA and TNMplot online database number (N) and mean (M). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. n, not significant

expression levels of TIFA and high TNM stage tumor biopsies (Figure 1C). To further confirm this, we also checked the TIFA expression in 12 pairs of fresh CRC and the adjacent nontumor tissues by quantitative RT-PCR (Figure 1D) and western blot analysis (Figure 1E). The results showed that TIFA was overexpressed in CRC tissues and rarely in the adjacent nontumor tissues. To validate our results, we also analyzed the expression level of TIFA in patients with CRC in GEPIA and TNMplot online databases. We found that TIFA was overexpressed in CRC tissues versus comparable normal tissues in both databases ($p < 0.05$; Figure 1F,G). Together, the above-mentioned findings suggest that TIFA acts as a potential cancer-related mediator that is upregulated in human CRC tissues.

3.2 | Silencing of TIFA inhibits CRC cell proliferation in vitro

We investigated whether the observations in clinical samples were represented in tumor cell lines. The mRNA level of TIFA was found to be upregulated in CRC cell lines relative to a normal colon cell line. Immunoblot analysis of TIFA protein levels supported the quantitative PCR results, suggesting that TIFA was increased in CRC cell lines, especially in RKO and SW620 cells (Figure 2A).

To investigate the functional role of TIFA in tumor progression, we knocked down TIFA expression in RKO and SW620 cell lines As shown in Figure 2B, the protein level was decreased by using TIFA-specific shRNAs. We used the CCK-8 assay to detect the cell viability and the results showed that knockdown of TIFA expression inhibited the speed of cell growth at the indicated time points (Figure 2C). We also undertook the cell clone formation assay to test the cell proliferation ability (Figure 2D). We found that knockdown of TIFA expression decreased the cell clone formation ability (Figure 2E). Moreover, the PI single staining assay was used to analyze cell cycle progression. The results revealed that TIFA knockdown reduced the number of cells in S phase, but increased the number of cells in G_2/M phase, which further suppressed the cell proliferation ability (Figure 2F,G). In addition, PI–annexin V double-staining assay was used to detect the function of TIFA interference in cell apoptosis. As shown in Figure 2H, knockdown of TIFA expression in RKO and SW620 cell lines made no difference to the cell apoptosis ability of either cell line (Figure 2I). Thus, these results indicated that silencing TIFA inhibits CRC cell proliferation but has no effect on cell apoptosis in vitro.

3.3 | TIFA deficiency suppresses CRC cell proliferation in vivo

As TIFA contributes to CRC tumor progression by promoting cell proliferation in vitro, we next tested the results in the xenograft animal model. To this end, stable RKO-shCtrl, RKO-sh1, or RKO-sh2 cells were injected into the fourth fat pad of nude mice. As shown in Figure 3A,B, the tumor growth and tumor volume in the RKO-sh1 and RKO-sh2 groups showed a marked decrease versus the RKO-shCtrl control group. Moreover, consistent with the in vitro findings, the IHC staining results also revealed that knockdown of TIFA expression reduced the expression of Ki-67 while the expression of apoptosis marker cleaved caspase 3 did not change (Figure 3C,D). These findings collectively suggested that TIFA deficiency suppresses CRC tumor progression in vivo.

3.4 | TIFA mediates RSK and PRAS40 signaling activation in vitro

To investigate the underlying mechanism of TIFA-mediated CRC cell proliferation, we used a human phosphokinase array that contains 39 kinase phosphorylation sites and two related total proteins in RKO-shTIFA and RKO-shCtrl cells. Our results showed that TIFA knockdown reduced the expression of many phosphokinases, especially p-RSK and p-PRAS40 (Figure 4A,B). Based on this finding, we focused our attention on p-RSK and p-PRAS40 using a variety of methods. Using western blot analysis, we observed that TIFA knockdown consistently decreased p-RSK and p-PRAS40 expression levels in RKO and SW620 cells (Figure 4C). Furthermore, using tissue microarray and IHC, we checked coexpression levels of TIFA and p-RSK/p-PRAS40 in the tumor tissue microarray. The results showed that TIFA has good coexpression with p-RSK and p-PRAS40 (Figure 4D–G). In addition, we selected TIFA high expression tumor tissue pairs and checked the expression of p-RSK/p-PRAS40. Consistently, we found that p-RSK and p-PRAS40 also had higher expression in tumor tissues compared to the normal control (Figure 4H). Collectively, these results suggested that TIFA mediates RSK and PRAS40 signaling activation in vitro, which could further regulate CRC cell proliferation.

3.5 | Ectopic expression of TIFA facilitates CRC cell proliferation rely on its oligomerization site and TRAF6 binding site in vitro

To further identify the function of TIFA, we ectopically expressed either WT TIFA (TIFA), an oligomerization site mutant TIFA (T9A), or TRAF6 binding site deletion TIFA (D6) (Figure 5A) in SW480 cells. Western blot analysis results showed that the three TIFA constructs were all reconstituted in SW480 cells (Figure 5B). The CCK-8 results noted that the cell proliferation ability was increased following the expression of TIFA and this effect was abolished by the expression of TIFA-T9A and TIFA-D6 (Figure 5C). Moreover, cell clone formation results showed that ectopic expression of TIFA accelerated cell growth at the indicated time points; however, the TIFA-T9A and TIFA-D6 showed no effects (Figure 5D,E). In addition, the PI single staining results revealed that ectopic expression of TIFA increased the number of cells in S phase and reduced the number of cells in G_2/M phase, whereas TIFA-T9A

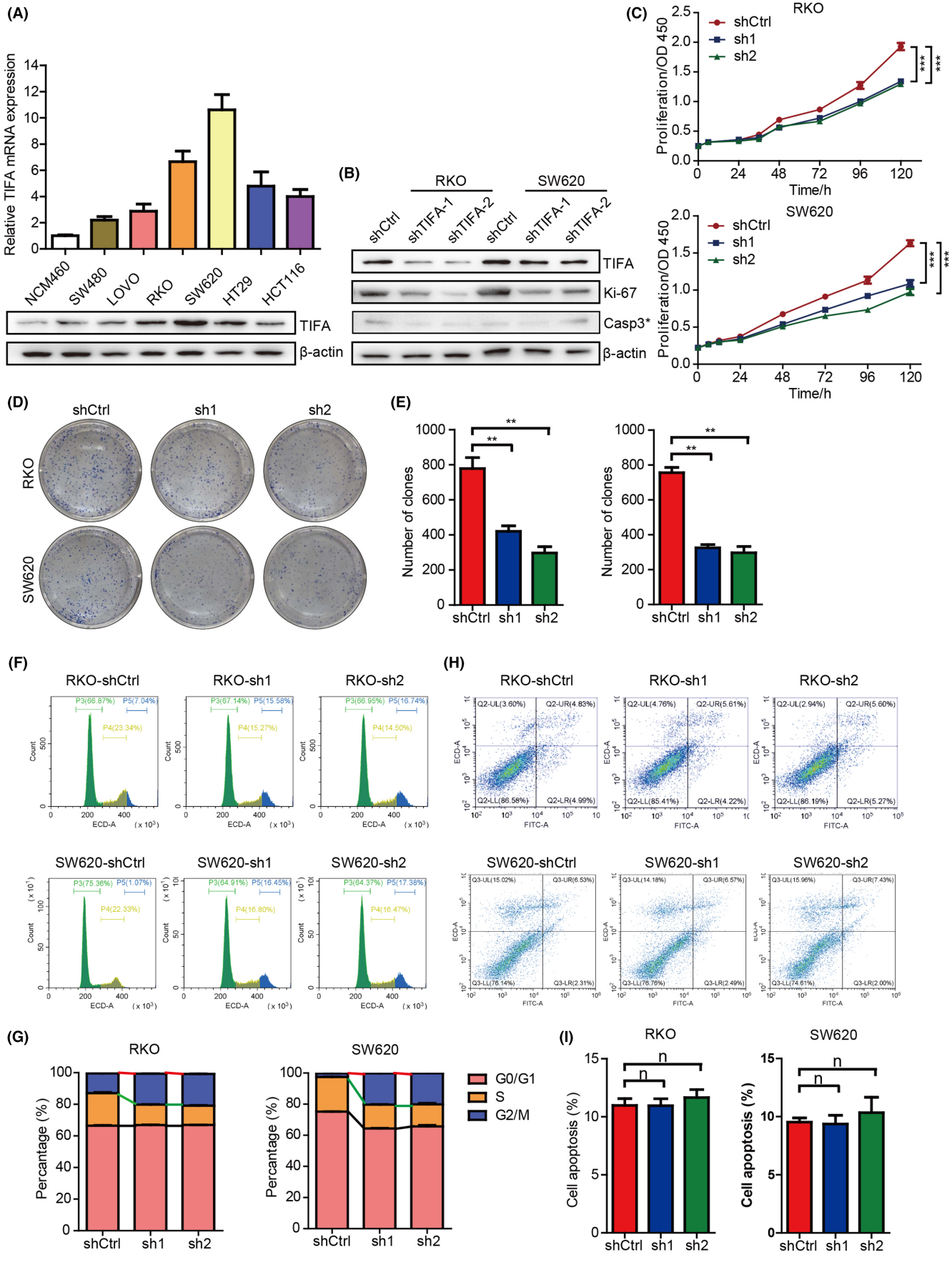

(A)
Relative TIFA mRNA expression
NCM460
SW480
LOVO
RKO
SW620
HT29
HCT116
TIFA
β-actin
(B)
RKO
SW620
shCtrl
shTIFA-1
shTIFA-2
TIFA
Ki-67
Casp3*
β-actin
(C)
RKO
SW620
shCtrl
sh1
sh2
Proliferation/OD 450
Time/h
(D)
shCtrl
sh1
sh2
RKO
SW620
(E)
Number of clones
(F)
RKO-shCtrl
RKO-sh1
RKO-sh2
SW620-shCtrl
SW620-sh1
SW620-sh2
Count
ECD-A
(H)
FITC-A
ECD-A
(G)
Percentage (%)
G0/G1
S
G2/M
(I)
Cell apoptosis (%)

FIGURE 2 Silencing of TIFA inhibits colorectal cancer (CRC) cell proliferation in vitro. (A) Quantitative PCR and western blot analysis of TIFA expression in CRC cell lines and normal colon cell lines. (B) Western blot analysis of TIFA knockdown in RKO and SW620 cell lines and the expression of Ki-67, cleaved caspase 3 (Casp3*). β-Actin was included as a loading control. (C) CCK-8 assay to detect viability in RKO and SW620 cells with TIFA silencing or control (shCtrl). (D) Cell clone formation assay to test proliferation ability in RKO and SW620 cells with TIFA silencing or shCtrl. (E) Statistical results of cell clone formation assay. (F) Propidium iodide (PI) single staining assay was used to analyze cell cycle progression in RKO and SW620 cells with TIFA silencing or shCtrl. (G) Statistical results of PI single staining assay. (H) PI–annexin V assay on shCtrl and shTIFA of RKO and SW620 cells. A representative of three experiments is shown. (I) Statistical results of PI–annexin V assay. $^{**}p < 0.01$, $^{***}p < 0.001$. n, not significant

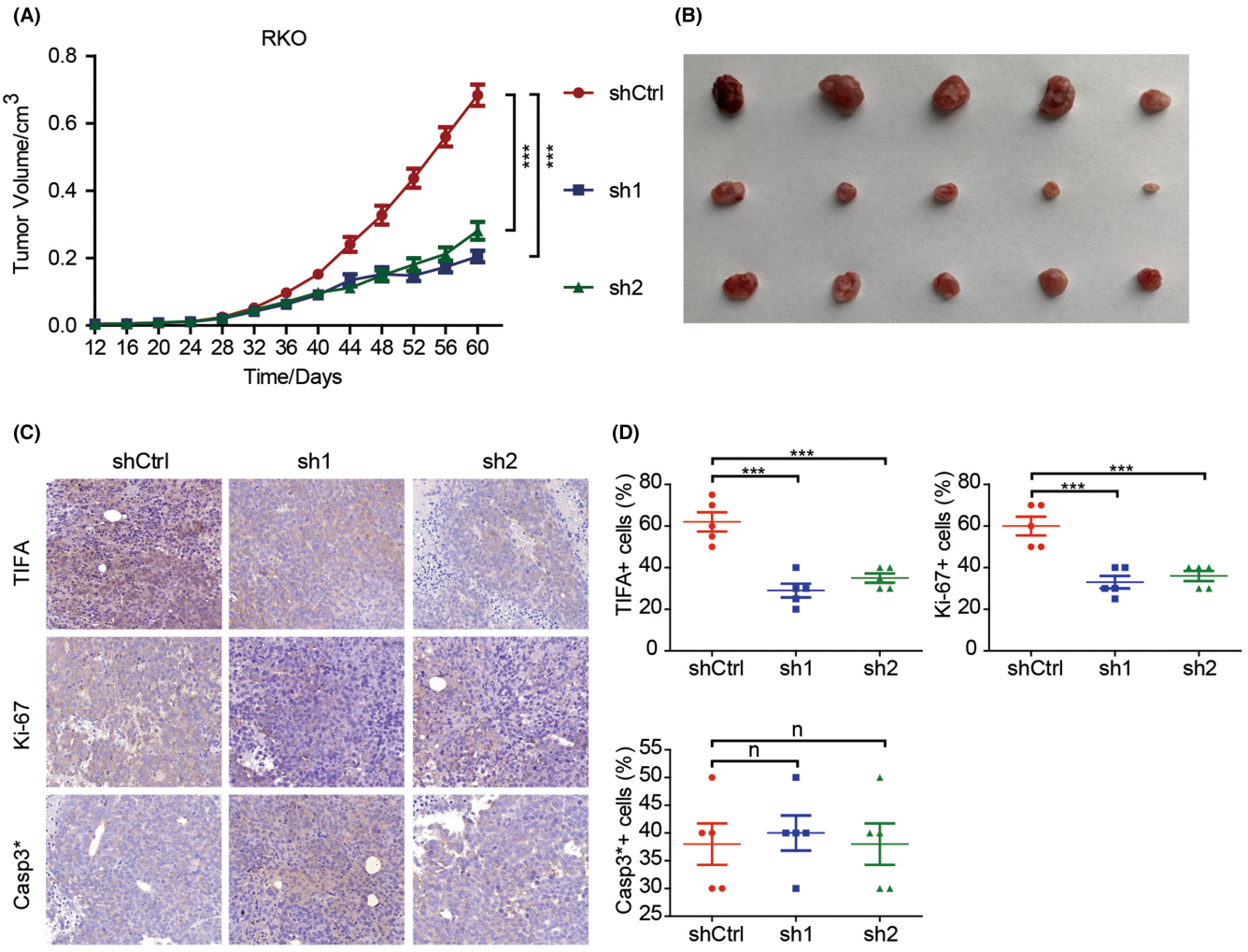
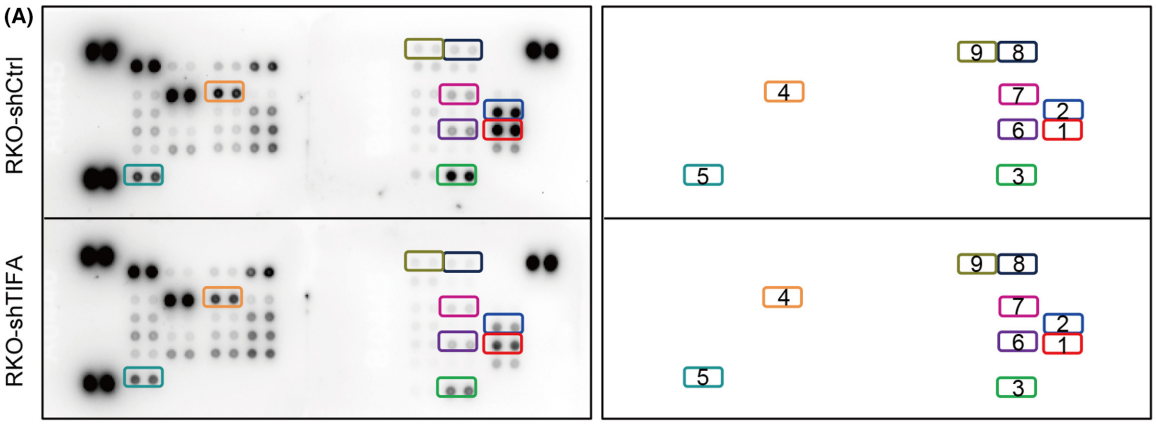


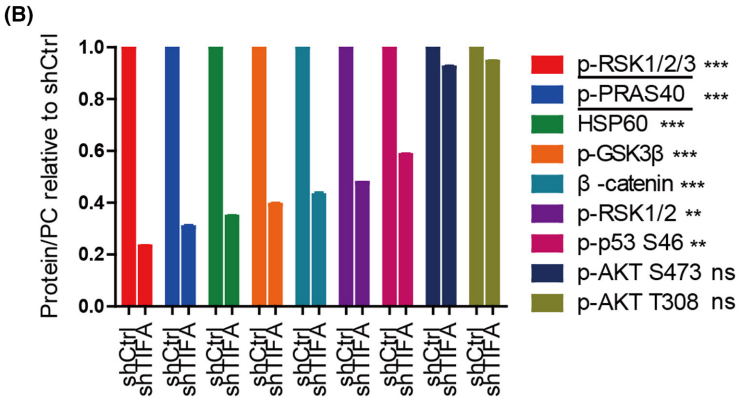
FIGURE 3 TIFA deficiency suppresses colorectal cancer cell proliferation in vivo. (A) stable RKO-shCtrl, RKO-shTIFA-1 (sh1), and RKO-shTIFA-2 (sh2) cells were injected into the fourth fat pad of nude mice, and tumor growth curves were measured. (B) Tumors from mice from different treatment groups. (C) Immunohistochemistry staining assay to detect the expression of TIFA, Ki-67, and cleaved caspase 3 (Casp3*) in various groups of mouse tumors. (D) Statistical results of the TIFA-, Ki-67-, and Casp3*-positive cells in various tumors. $^{***}p < 0.001$. n, not significant

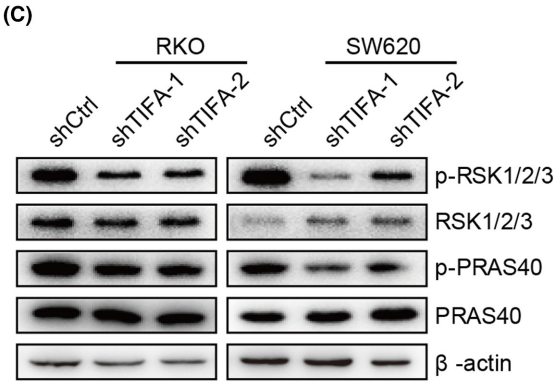
and TIFA-D6 abolished this effect (Figure 5F,G). In agreement, the western blot analysis results showed that the expression of proliferation-specific protein Ki-67, p-RSK, and p-PRAS40 were increased in the TIFA ectopic expression group but did not change in the TIFA-T9A and TIFA-D6 groups (Figure 5B). In summary, these results indicated that ectopic expression of TIFA fosters CRC cell proliferation and downstream signaling activation depends on its oligomerization site and TRAF6 binding site in vitro.

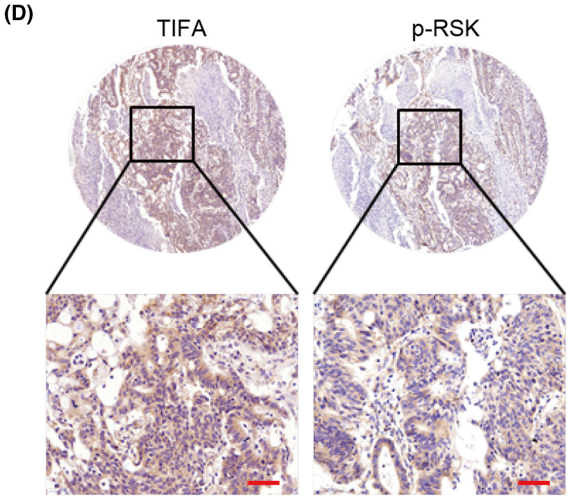
3.6 | TIFA mediates p-RSK/p-PRAS40 activation to foster CRC tumor progression rely on its oligomerization site and TRAF6 binding site in vivo

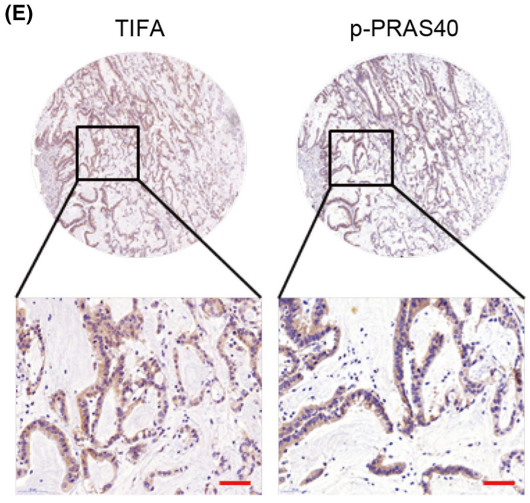
As TIFA mediates p-RSK/p-PRAS40 activation to contribute to CRC cell proliferation in vitro, we next tested the results in a xenograft animal model. To this end, stable SW480-OECtrl, SW480-TIFA,

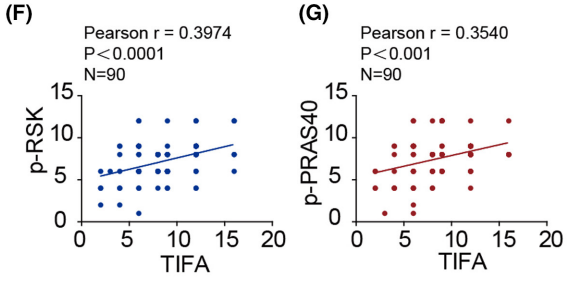

(A)
RKO-shCtrl
RKO-shTIFA
9
8
7
2
6
1
4
5
3


(B)
Protein/PC relative to shCtrl
1.0
0.8
0.6
0.4
0.2
0.0
shCtrl
shTIFA
p-RSK1/2/3 ***
p-PRAS40 ***
HSP60 ***
p-GSK3β ***
β -catenin ***
p-RSK1/2 **
p-p53 S46 **
p-AKT S473 ns
p-AKT T308 ns


(C)
RKO
SW620
shCtrl
shTIFA-1
shTIFA-2
p-RSK1/2/3
RSK1/2/3
p-PRAS40
PRAS40
β -actin


(D)
TIFA
p-RSK


(E)
TIFA
p-PRAS40


(F)
Pearson r = 0.3974
P<0.0001
N=90
p-RSK
TIFA
0
5
10
15
20

(G)
Pearson r = 0.3540
P<0.001
N=90
p-PRAS40
TIFA
0
5
10
15
20

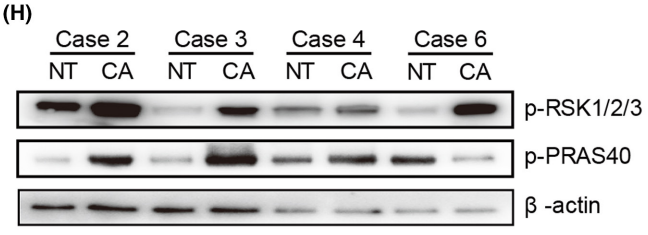

(H)
Case 2
Case 3
Case 4
Case 6
NT
CA
p-RSK1/2/3
p-PRAS40
β -actin

FIGURE 4 TIFA mediates RSK and PRAS40 signaling activation in vitro. (A) Human phosphokinase array was used to detect activation of 43 kinases in RKO cells with TIFA silencing or control (shCtrl). (B) Seven kinases or proteins were selected and statistical results are shown. (C) Western blot analysis of RSK, p-RSK, PRAS40, and p-PRAS40 expression; β-actin was included as a loading control. (D, F) Representative immunohistochemistry of TIFA and p-RSK and correlation graphs in a human colorectal cancer (CRC) tissue array. Scale bar, 50 μm (E, G) Representative immunohistochemistry of TIFA and p-PRAS40 and correlation graphs in a human CRC tissue array. Scale bar, 50 μm. (H) Western blot analysis of p-RSK and p-PRAS40 expression in fresh colon cancer tissue (CA) with high expression of TIFA; β-actin was included as a loading control. $^{**}p < 0.01$, $^{***}p < 0.001$. n, not significant; NT, normal tissue

SW480-TIFAT9A, or SW480-TIFAD6 cells were injected into the fourth fat pad of nude mice. As shown in Figure 6A,B, the tumor growth and tumor volume in the SW480-TIFA group showed a marked increase versus the SW480-OECtrl control; however, a significant reduction of the tumor growth and tumor volume was observed in SW480-TIFAT9A and SW480-TIFAD6 groups versus the SW480-TIFA group. Moreover, the IHC staining results also revealed that TIFA reconstitution increased the expression of Ki-67, p-RSK, and p-PRAS40, whereas these effects were abolished in SW480-TIFAT9A and SW480-TIFAD6 groups (Figure 6C,D). These findings collectively suggested that TIFA mediates p-RSK/p-PRAS40 activation to foster CRC tumor progression relying on its oligomerization site and TRAF6 binding site in vivo.

3.7 | Ribosomal S6 protein kinase and PRAS40 activation indispensable for TIFA-mediated CRC cell proliferation in vitro

To investigate this role of RSK and PRAS40 activation in TIFA-mediated CRC cell proliferation, we used a specific inhibitor for RSK and specific shRNAs for PRAS40 to suppress their phosphorylation and activation. As shown in Figure 7A, the inhibitor BRD7389 (4 μM) effectively inhibits RSK activation in SW480-TIFA cells. As shown in Figure 7B, the PRAS40-shRNAs efficiently inhibit PRAS40 expression and activation in SW480-TIFA cells as well. Furthermore, we combined BRD7389 (4 μM) and PRAS40-shRNA to treat the SW480-TIFA cells, then checked the expression of Ki-67, p-RSK, and p-PRAS40. The western blot results showed that BRD7389 (4 μM) or PRAS40-shRNA alone or combined reduced the expression of Ki-67 (Figure 7C). Moreover, the CCK-8 assay results showed that BRD7389 and shRAS40 inhibited the growth of SW480-WT cells, whereas the proliferation ability of SW480-OETIFA cells, enhanced by TIFA expression, was abolished by BRD7389 (4 μM), PRAS40-shRNA, or combined treatment (Figure 7D). Furthermore, the cell clone formation assay results showed that the cell growth ability reinforced by TIFA expression was also reduced by the BRD7389 (4 μM), PRAS40-shRNA, or combined treatment (Figure 7E,F). The same results were proven by the PI single staining assay; the results revealed that the cell cycle acceleration mediated by TIFA was decelerated by BRD7389 (4 μM), PRAS40-shRNA, or combined treatment as well (Figure 7G,H). Together, these findings suggested that RSK and PRAS40 activation were indispensable for TIFA-mediated CRC cell proliferation. Both RSK and PRAS40 worked as the potential therapy targets for TIFA-mediated CRC progression.

3.8 | Proposed mechanism and model of TIFA promotes CRC cell proliferation

PRAS40 is a substrate of AKT and a component of mTORC1, which can activate S6K1 to regulate protein synthesis. To investigate the potential mechanism of TIFA-mediated RSK (S6K2) and PRAS40 activation, western blot analysis was undertaken to check the activation of AKT and mTOR in RKO TIFA knockdown cells and SW480 TIFA overexpression cells. The results showed that TIFA knockdown reduced p-mTOR expression and TIFA overexpression increased p-mTOR expression, while p-AKT (Ser473) expression showed no change following TIFA knockdown or overexpression (Figure S1A). The results were consistent with phosphokinase array data (Figure 4A,B, 8 and 9).

Moreover, to verify whether RSK was the down-stream target of PRAS40/mTOR signaling, SW480-OETIFA-shPRAS40 cells (Figure 7B) were used to detect the activation of RSK. The results showed that PRAS40 knockdown reduced p-mTOR expression, whereas the expression of p-RSK did not change following PRAS40 knockdown (Figure S1B).

In addition, to clarify whether the enhancement of TIFA–TRAF6 binding, which resulted in the activation of the IKK–NF-κB signaling pathway, was correlated with phosphorylation of RSK and PRAS40, we first knocked down TRAF6 in RKO-WT cells (TIFA high expression), then detected the phosphorylation of RSK and PRAS40. The results showed that TRAF6 knockdown reduced p-RSK and p-PRAS40 expression (Figure S1C). Then we used IKK-16 to suppress IKK–NF-κB signaling activation, and checked the phosphorylation of RSK and PRAS40. As shown in Figure S1D, IKK-16 (5 μM) inhibits IKK activation effectively in SW480-TIFA cells, while there was no difference in the expression of p-RSK and p-PRAS40 following IKK inhibition.

To further understand the coexpression of TIFA, TRAF6, RSK, and PRAS40, the immunoprecipitation assay was carried out. The results indicated that TIFA could bind TRAF6 and RSK to mediate RSK activation, but PRAS40 was not examined (Figure S1E). Taken together, the above results indicated that TIFA-mediated RSK signaling and PRAS40/mTOR signaling were mutually independent in an AKT/IKK-independent manner.

Based on our findings, we propose the following model (Figure 7I): TIFA acts as an adaptor protein, its oligomerization mediates the oligomerization of binding protein TRAF6; furthermore, it directly activates RSK kinase and indirectly activates PRAS40. The activated RSK and PRAS40 could facilitate protein synthesis in cell cycle, finally promoting cell proliferation and fostering CRC tumor progression.

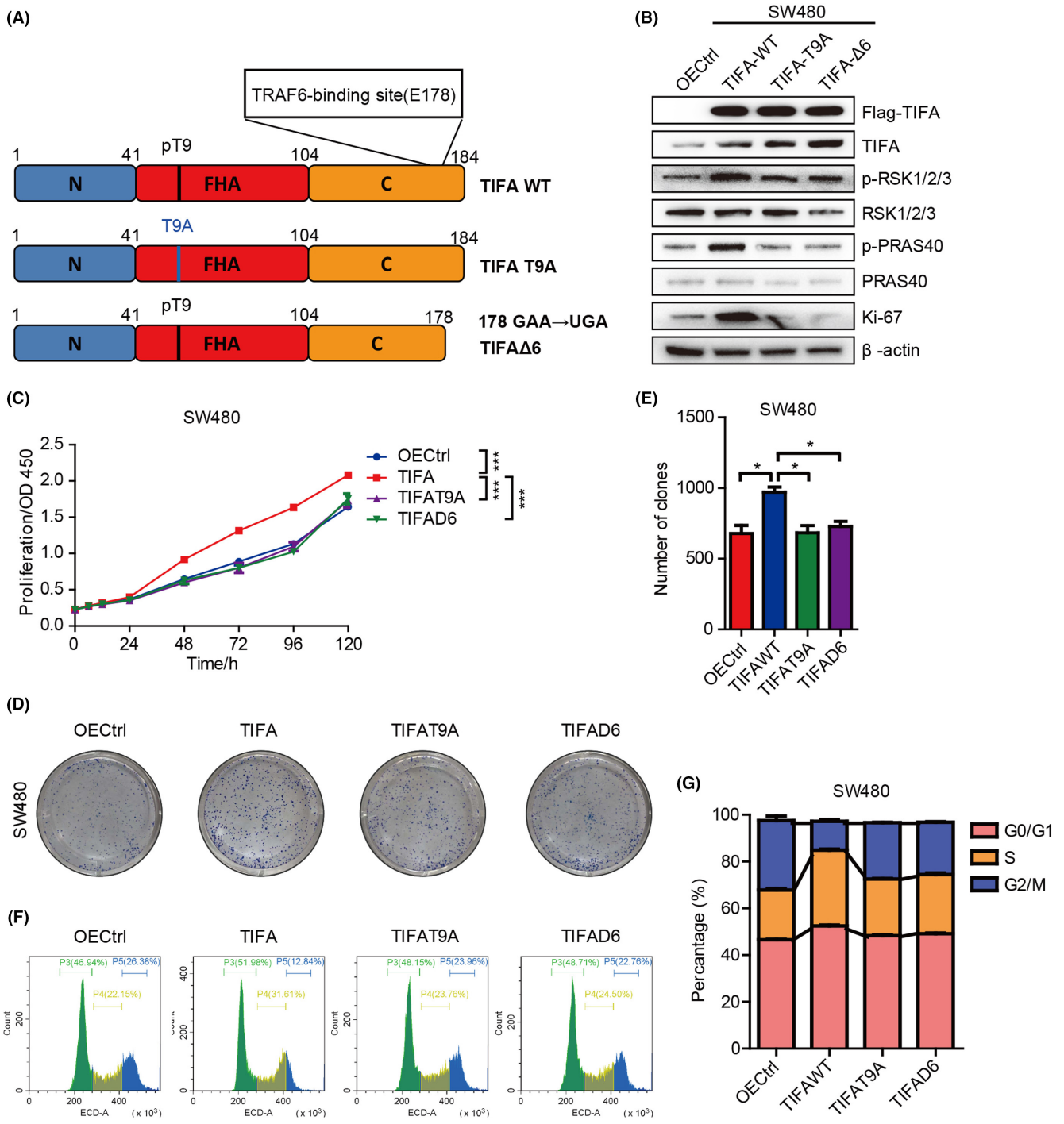


FIGURE 5 TIFA reconstitution, which facilitates colorectal cancer cell proliferation, relies on its oligomerization site (T9) and TRAF6 binding site (E178) in vitro. (A) Schematic structure of oligomerization site mutant TIFA (T9A) and TRAF6 binding site mutant TIFA (D6). FHA, Forkhead-associated domain. (B) Western blot analysis of TIFA overexpression in SW480 cell line and the expression of Ki-67, RSK, p-RSK, PRAS40, and p-PRAS40; β-actin was included as a loading control. (C) CCK-8 assay to detect viability in SW480 cells with reconstituted TIFA or OECtrl. (D) Cell clone formation assay to test the proliferation ability of SW480 cells with reconstituted TIFA or OECtrl. (E) Statistical results of cell clone formation assay. (F) Propidium iodide (PI) single staining assay analyze the cell cycle progression in SW480 cells with reconstituted TIFA or OECtrl. (G) Statistical results of PI single staining assay. *$p<0.05$, ***$p<0.001$

4 | DISCUSSION

As one of the major common human malignancies, CRC has been ranked as the third most commonly diagnosed cancer in men and the second most common in women according to morbidity and mortality.[1] Moreover, clinical success in the pharmacological treatment of CRC patients has been limited. With the development of precision medicine, there is an urgent need to explore

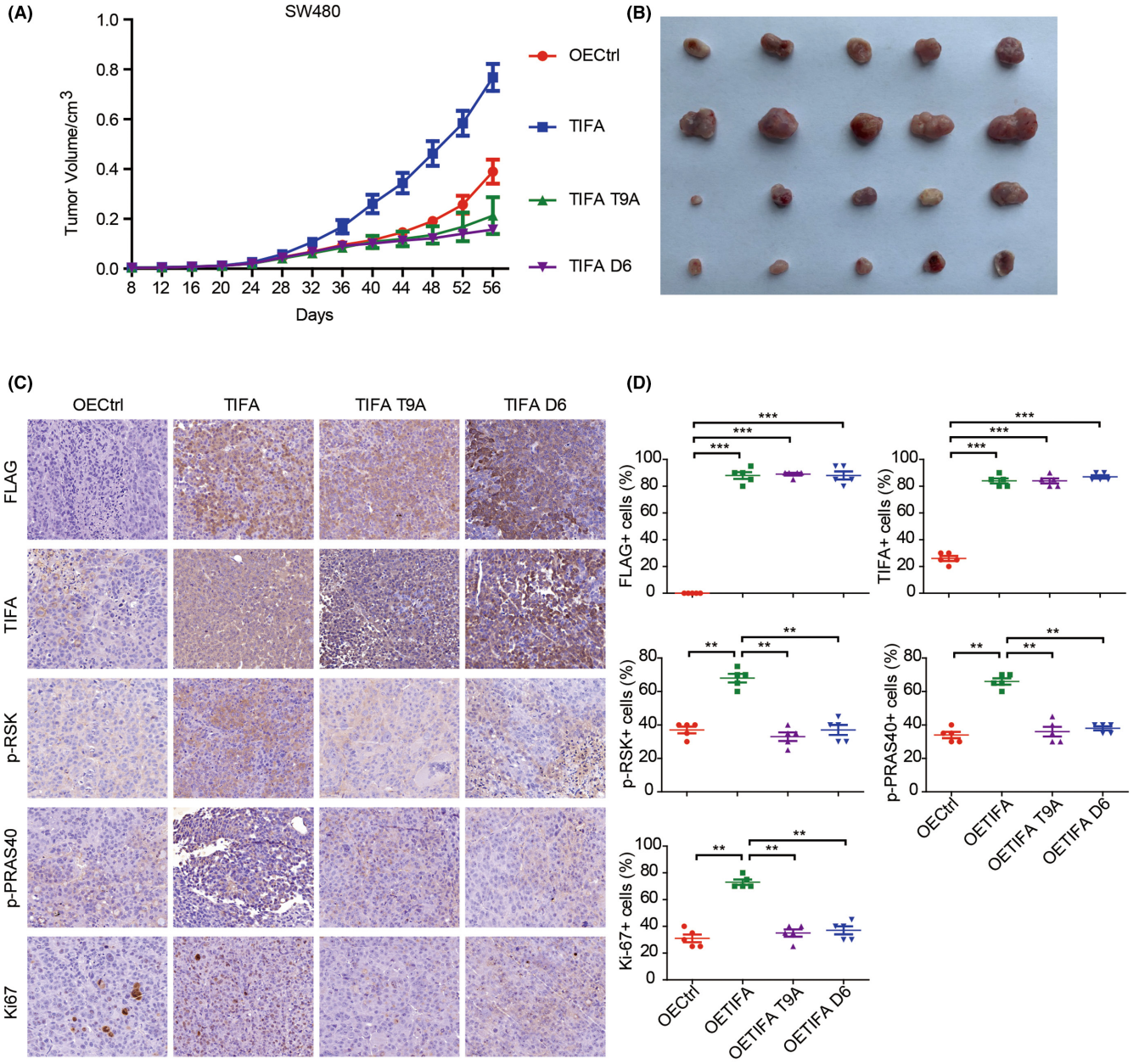


FIGURE 6 TIFA mediates p-RSK/p-PRAS40 activation to foster colorectal cancer tumor progression and relies on its oligomerization site and TRAF6 binding site in vivo. (A) Stable SW480-OECtrl, SW480-OETIFA, SW480-OETIFA T9A, and SW480-OETIFA D6 cells were injected into the fourth fat pad of nude mice. Tumor growth curves were measured. (B) Tumors from different treatment groups. (C) Immunohistochemistry staining assay to detect the expression of FLAG, TIFA, Ki-67, p-RSK, and p-PRAS40 in tumors from various treatment groups. (D) Statistical results of the FLAG-, TIFA-, Ki-67-, p-RSK-, and p-PRAS40-positive cells in tumors from various treatment groups. $^{**}p<0.01$, $^{***}p<0.001$

new prognostic biomarkers or therapeutic targets for CRC clinical treatment.

Acting as an inflammation-related adaptor protein, TIFA plays a vital role in various biological processes that include signal transduction in innate immunity,[9,38–41] inflammation,[42,43] hypoxia reoxygenation,[44] and cancer. Previous studies proved that TIFA plays different roles in various cancer types. As our previous work described, TIFA expression is downregulated in HCC and TIFA reconstitution promotes HCC cell apoptosis and suppresses cell proliferation among surviving cells.[12,13] However, other studies revealed that TIFA is highly expressed in lung adenocarcinoma, and could promote lung adenocarcinoma cell survival and migration.[11] Moreover, TIFA is reported to be overexpressed in acute myeloid leukemia, correlated with poor prognosis, and also contributed to chemoresisitance.[45] However, the function of TIFA in CRC was not dissected. In this study, we showed that the expression of TIFA was markedly upregulated in CRC tissues and cell lines versus normal tissue or normal cell lines. TIFA expression was also positively correlated with CRC TNM stages. This evidence suggests that TIFA acts as a promising biomarker for CRC prognosis.

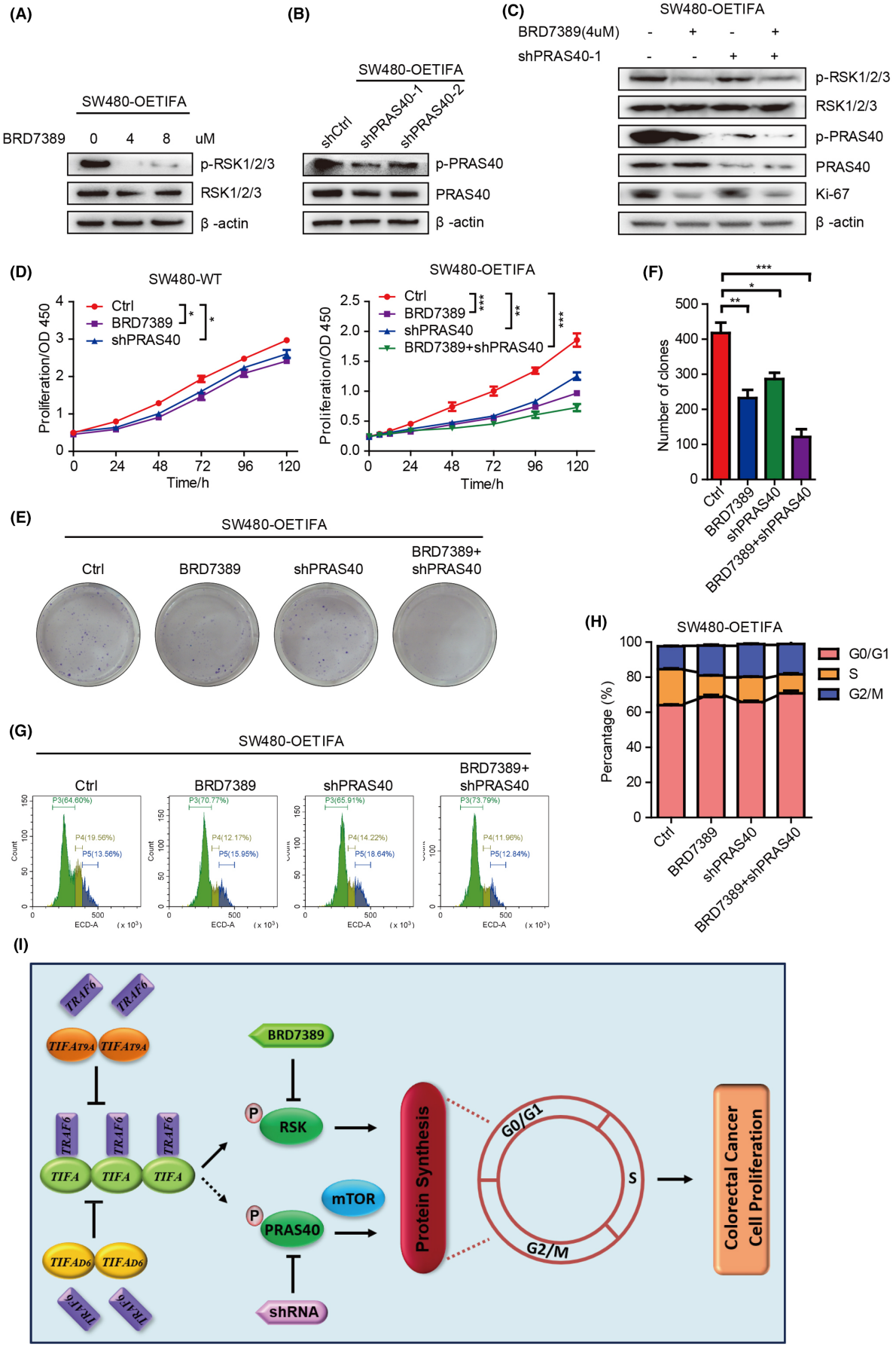

(A)
SW480-OETIFA
BRD7389 0 4 8 uM
p-RSK1/2/3
RSK1/2/3
β -actin
(B)
SW480-OETIFA
shCtrl
shPRAS40-1
shPRAS40-2
p-PRAS40
PRAS40
β -actin
(C)
SW480-OETIFA
BRD7389(4uM) - + - +
shPRAS40-1 - - + +
p-RSK1/2/3
RSK1/2/3
p-PRAS40
PRAS40
Ki-67
β -actin
(D)
SW480-WT
Ctrl
BRD7389
shPRAS40
Proliferation/OD 450
Time/h
SW480-OETIFA
Ctrl
BRD7389
shPRAS40
BRD7389+shPRAS40
Proliferation/OD 450
Time/h
(F)
Number of clones
Ctrl
BRD7389
shPRAS40
BRD7389+shPRAS40
(E)
SW480-OETIFA
Ctrl
BRD7389
shPRAS40
BRD7389+ shPRAS40
(H)
SW480-OETIFA
Percantage (%)
G0/G1
S
G2/M
Ctrl
BRD7389
shPRAS40
BRD7389+shPRAS40
(G)
SW480-OETIFA
Ctrl
BRD7389
shPRAS40
BRD7389+ shPRAS40
P3(64.60%)
P4(19.56%)
P5(13.56%)
P3(70.77%)
P4(12.17%)
P5(15.95%)
P3(65.91%)
P4(14.22%)
P5(18.64%)
P3(73.79%)
P4(11.96%)
P5(12.84%)
Count
ECD-A
(I)
TRAF6
TIFAT9A
TIFA
TIFAD6
BRD7389
RSK
mTOR
PRAS40
shRNA
Protein Synthesis
G0/G1
S
G2/M
Colorectal Cancer Cell Proliferation

FIGURE 7 Activation of RSK and PRAS40 was indispensable for TIFA-mediated colorectal cancer (CRC) cell proliferation in vitro. (A) Western blot analysis to check the inhibition of BRD7389 to RSK activation in SW480-OETIFA cells (0, 4, and 8 μM concentrations). (B) Western blot analysis of the inhibition of PRAS40-shRNAs on PRAS40 activation in SW480-OETIFA cells. (C) Western blot analysis of the expression of RSK, p-RSK, PRAS40, p-PRAS40, and Ki-67 in SW480-OETIFA cells treated with BRD7389 or PRAS40-shRNA or BRD7389and PRAS40-shRNA combined. (D) Clone formation assay to test the cell proliferation ability in SW480-WT or SW480-OETIFA cells treated with BRD7389, PRAS40-shRNA, or BRD7389 and PRAS40-shRNA combined. (E) Clone formation assay to test the cell proliferation ability in SW480-OETIFA cells treated with BRD7389, PRAS40-shRNA, or BRD7389 and PRAS40-shRNA combined. (F) Statistical results of cell clone formation assay. (G) Propidium iodide (PI) single staining assay to analyze the cell cycle progression in SW480-OETIFA cells treated with BRD7389, PRAS40-shRNA, or BRD7389 and PRAS40-shRNA combined. (H) Statistical results of PI single staining assay. (I) Proposed model of CRC cell proliferation promoted by TIFA. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

Cell cycling and protein synthesis are both key physiological processes for tumor cell proliferation.[14] Protein synthesis occurs in G_0/G_1 (enzymes like CDKs) and G_2/M phases (cyclins and others) of the cell cycle. The RSK family comprises serine/threonine protein kinases proven to regulate the stability of eIF to mediate protein synthesis.[20] PRAS40, identified as a component and substrate of mTORC1, was also shown to regulate the protein synthesis process.[24] Although some previous studies have reported that RSK and PRAS40 activation was involved in colon inflammation and CRC progression, in this study, it should be highlighted that TIFA mediates RSK and PRAS40 activation, which not only reveals a novel mechanism for TIFA function but also provides a possible therapeutic strategy for CRC. In the exploration of the underlying mechanism, we found that the oligomerization site (T9) and TRAF6 binding site (E178) were indispensable for RSK and PRAS40 activation. Moreover, we showed that the TIFA–TRAF6 complex binds and activates RSK directly. However, PRAS40 activation was indirectly mediated by the TIFA–TRAF6 complex, which needs to be investigated in the future study. In addition, we used RSK inhibitor and PRAS40 shRNAs to prevent RSK and PRAS40 activation and found that they significantly suppressed TIFA-mediated CRC cell proliferation. These results could assist in identifying potential therapeutic targets and strategies for CRC progression.

In conclusion, we discovered that TIFA promotes cell proliferation in CRC cell lines in vitro and in a xenograft nude mouse model in vivo. We revealed that TIFA mediates RSK and PRAS40 activation to facilitate protein synthesis, which relied on TIFA oligomerization (T9) and TRAF6 binding (E178) in vitro and in vivo. We also showed that RSK inhibitor and PRAS40 shRNAs, which prevent RSK and PRAS40 activation, could significantly suppress TIFA-mediated CRC cell proliferation. Finally, we take the position that TIFA could serve as a novel therapeutic target in CRC treatment.

AUTHOR CONTRIBUTIONS

S.W.Z. and L.Y.P. designed the experiments, H.Y.M., D.W.F., J.X.Y., and Y.C.L. prepared the materials and performed the experiments, T.J.P. and L.H. analyzed the data, Z.X.Y. and Z.Z.X. helped to collect the CRC tumor tissue samples, S.W.Z. wrote the manuscript, and L.N., Z.X.Y. and Z.Z.X. prepared the manuscripts.

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DISCLOSURE

The authors declare no potential conflicts of interest.

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SUPPORTING INFORMATION

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