RSK2 phosphorylates T-bet to attenuate colon cancer metastasis and growth

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Metastasis is a major cause of cancer-related deaths. Approximately 80% of patients with colorectal cancer develop liver metastasis and 20% develop lung metastasis. We found that at different stages of colon cancer, IFNγ secretion from peripheral blood mononuclear cells was decreased compared with healthy controls. The ribosomal S6 kinase 2 (RSK2) family of kinases has multiple cellular functions, and we examined their roles in this observed IFNγ decrease. Flow cytometry analysis of wild-type (WT) and RSK2 knockout (KO) mice revealed significantly lower levels of IFNγ in the RSK2 KO mice compared with the WT mice. Since IFNγ is a component of immunity, which contributes to protection against metastatic carcinomas, we conducted a colon cancer liver metastasis experiment. We found significantly greater metastases in RSK2 KO mice compared with WT mice. In vitro kinase assay results showed that RSK2 phosphorylated T-bet at serines 498 and 502. We show that phosphorylation of T-bet by RSK2 is required for IFNγ expression, because knockdown of RSK2 expression or overexpression of mutant T-bet reduces IFNγ mRNA expression. To verify the function of the phosphorylation sites, we overexpressed a constitutively active mutant T-bet (S498E/S502E) in bone marrow. Mutant T-bet restored the IFNγ mRNA levels and dramatically reduced the metastasis rate in these mice. Overall, these results indicate that phosphorylation of T-bet is required for the inhibition of colon cancer metastasis and growth through a positive regulation of RSK2/T-bet/IFNγ signaling.

Significance

Many patients with colorectal cancer die because of metastases in distant organs such as the liver and lungs, rather than from the primary tumor. A better molecular understanding of colorectal cancer has allowed for improved patient prognosis and the launching of precision medicine for treating metastatic colorectal cancer. Here we demonstrate that a deficiency of ribosomal S6 kinase 2 (RSK2) can result in dramatically decreased IFNγ secretion through an inappropriate phosphorylation status of T-bet, a modulator of IFNγ expression. Decreased IFNγ levels can lead to immune suppression, accelerating colon cancer-mediated liver and lung metastasis. We found that RSK2-mediated phosphorylation of T-bet at serines 498 and 502 is required for the inhibition of colon cancer metastasis and growth, through a positive regulation of RSK2/T-bet/IFNγ signaling.


The authors declare no conflict of interest.

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Results

IFNγ Levels Spontaneously Decreased in Peripheral Blood Mononuclear Cells from Colon Cancer Patients at Different Stages of Disease. IFNγ is a critical cytokine for immunity against viral and bacterial infections, as well as tumor surveillance (17, 18). Samples of blood from the peripheral circulation were collected from patients with colon cancer at various disease stages. Peripheral blood mononuclear cells (PBMCs) were isolated, including 70–90% lymphocytes (i.e., T cells, B cells, and NK cells) (19). The PBMCs were stimulated with phorbol-12-myristate acetate (PMA) and ionomycin (20), RSK2 mRNA levels were analyzed using human RSK2 primers, and IFNγ levels were detected in supernatant fractions using a human IFNγ ELISA kit. The results showed lower levels of RSK2 mRNA expression in the patients with colon cancer compared with normal control subjects (Fig. S1A). Furthermore, IFNγ levels decreased spontaneously, corresponding with advanced disease stage (Fig. 1A). Serum cytokine levels were also assayed in colon cancer patients and normal, healthy controls, and no significant differences were observed (Fig. S1B–G). GM09621 and GM03317 are human lymphoblast cell lines that express RSK2 WT (RSK2*) and RSK2 mutant (RSK2−) genes, respectively (7). When stimulated with PMA and ionomycin, RSK2− lymphoblasts showed decreased IFNγ mRNA levels compared with RSK2+ lymphoblasts (Fig. 1B).

RSK2 KO mice were generated for studying brain function and body size. These mice exhibit characteristics consistent with the mental retardation and reduced growth characteristics observed in persons with CLS, who lack functional RSK2 proteins (21), and thus provide a useful model for studying RSK2 function. The spleen and lymph nodes are known to be important for proper functioning of the immune system, acting as filters for foreign particles and cancer cells. Primary cells isolated from mouse spleen and lymph nodes were analyzed by flow cytometry. The results showed that CD4+, CD8+, and NK cell populations and their rates of proliferation were not significantly different between WT and RSK2 KO mice (Fig. S2); however, the IFNγ−stained cell population in these organs was dramatically decreased in the RSK2 KO mice (Fig. 1C).

The Rate of Colon Cancer Liver Metastasis Is Significantly Increased in RSK2 KO Mice. IFNγ is a critical component of immunity to metastatic carcinomas (22), and our results indicate that RSK2 deficiency is associated with decreased IFNγ secretion (Fig. 1B and C). This observation led us to investigate whether RSK2 depletion could promote cancer metastatic growth. To do so, we implanted CT26 colon carcinoma cells, a mouse cancer cell line widely used in metastasis studies, into the spleens of WT and RSK2 KO mice (Fig. S2). On necropsy, we found that RSK2 KO mouse livers were fully occupied with cancer cells compared with WT mice (Fig. 2A). Histological examination of H&E-stained liver tissues revealed that very large areas of the liver contained metastases (Fig. 2B, Left). Tumor cell proliferation was examined by immunostaining tissue sections using an antibody against proliferating cell nuclear antigen
(PCNA), a proliferation marker. Staining results showed that the average level of PCNA in the liver metastases of RSK2 KO mice was more than five times higher than that of WT mice (Fig. 2B, Right). In addition, the average tumor weight in the liver was more than twofold greater in the RSK2 KO mice compared with the WT mice at 11 d after implantation (Fig. 2C, Left), indicating that RSK2 deficiency promotes liver metastatic growth in mice. Moreover, the average weight of the spleen was more than threefold greater in the RSK2 KO mice (Fig. 2C, Center), but average body weight did not differ between the RSK2 KO and WT mice (Fig. 2C, Right). Consistent with our ex vivo data, the liver metastasis results demonstrate that the absence of RSK2 promotes both tumor growth and metastasis in mice.

**IFNγ Levels Are Down-Regulated in RSK2 KO Mice.** Previous studies have identified CD4\(^+\), CD8\(^+\), and NK cells as critical components of immunity against metastatic tumor growth (24, 25). IFNγ is produced by CD4\(^+\) and CD8\(^+\) T cells and plays important roles in inhibiting and killing tumor cells, thereby impeding tumor growth (17). Liver cells were collected from RSK2 KO and WT mice and analyzed by flow cytometry. CD4\(^+\) and CD8\(^+\) cell populations were significantly higher in the RSK2 KO mice compared with WT controls (Fig. S3A). In addition, because antigen-specific cells are developed within the draining lymph nodes (26), we collected lymph nodes and spleens from mice with liver metastases. Primary cells were isolated and stained with IFNγ/CD4, IFNγ/CD49b (i.e., NK cell marker), or IFNγ/CD8. The data show significantly lower IFNγ expression in CD4\(^+\), NK, and CD8\(^+\) cells in RSK2 KO mice compared with WT mice even under CT26 colon tumor cell-challenging conditions (Fig. 3 and Fig. S3B).

As reported previously (27), IL-2 is one of the major cytokines regulated by RSK2. Analysis of IL-2 mRNA expression in primary cells from the spleen and lymph nodes showed less IL-2 expression in the RSK2 KO mice compared with WT mice (Fig. S3C).

Taken together, the foregoing results suggest that RSK2 KO mice may have an immune-suppressed environment in vivo that accelerates colon cancer metastasis and growth (28) (Fig. 2). We explored this idea in additional experiments.

**RSK2 Bounds and Phosphorylates T-Bet.** RSK2 is a serine/threonine kinase that phosphorylates numerous substrates involved in mediating multiple cellular functions. The role of RSK2 in the immune system has not been well studied, however. IFNγ cannot be directly regulated by kinases; however, T-bet, a member of the T-box family of transcription factors, can directly activate gene transcription (11). In addition, as reported previously, T-bet protein posttranslational modification may play an important role in its function (12).

To examine whether RSK2 works as an upstream kinase for T-bet, we carried out LTO Orbitrap hybrid MS analysis to identify T-bet sites potentially phosphorylated by RSK2. The results indicate that RSK2 phosphorylated serines 498 and 502 on T-bet (Table S1). Furthermore, an in vitro kinase assay proved that full-length T-bet was strongly phosphorylated by RSK2 (Fig. 4A, Upper, lane 3). However, mutation of T-bet serines 498 and 502 to alanine (T-bet S498A/S502A) almost completely abolished the phosphorylation by RSK2 (Fig. 4A, Upper, lane 4). To examine the phosphorylation levels ex vivo, we stimulated GM03317 (RSK2\(^+\)) and GM03317 (RSK2\(^−\)) cells with PMA and ionomycin. We found a significantly higher T-bet phosphorylation level in GM03317 (RSK2\(^+\)) cells compared with GM03317 (RSK2\(^−\)) cells (Fig. 4B). To study the interaction of T-bet and RSK2, we pulled down the endogenous RSK2 with an RSK2 antibody and also detected endogenous T-bet in a communoprecipitation assay (Fig. 4C).

These results provide further confirmation that RSK2 binds T-bet.

Our laboratory previously solved the crystal structure of the N-terminal domain of RSK2 (29), which is critical for downstream activation (6). Using a computational homology method, we constructed a T-bet fragment (residues 490–510) that includes serines 498 and 502. After docking with the RSK2 N terminal and conducting molecular dynamics, the binding model showed that the RSK2 ATP-biding cavity specifically recognizes the phosphorylation sites, serines 498 and 502 (Fig. 4D). Based on these overall results, we conclude that T-bet is a phosphorylation target of RSK2.

**RSK2 Promotes T-Bet-Mediated IFNγ mRNA Expression.** T-bet binds the *Ifny* promoter region and regulates *Ifny* expression (13, 30). Using Jurkat cells, a human T lymphocyte cell line, we found that ectopic overexpression of T-bet could significantly promote *Ifny* mRNA expression (Fig. 5A). We also found that lentivirus expression of sh-RSK2 in these cells blocked RSK2 protein expression (Fig. 5B, Left), and *Ifny* mRNA expression was suppressed compared with sh-mock-transfected cells (Fig. 5B, Right). Finally, to elucidate the function of T-bet S498/S502 phosphorylation, we overexpressed T-bet\(^{S498A/S502A}\) or T-bet\(^{S498A/S502A,\text{wild-type}}\) (Fig. 5C, Left). The results indicated that T-bet\(^{\text{wild-type}}\) could be phosphorylated by RSK2, whereas the mutant form of T-bet could not be phosphorylated (Fig. 4A, lanes 3 and 4). As expected, the mutant T-bet\(^{S498A/S502A}\)–promotion of *Ifny* expression was dramatically decreased compared with that of WT T-bet (Fig. 5C, Right). These data further confirm that RSK2 phosphorylation of T-bet is required for IFNγ expression ex vivo.

**RSK2 Promotes Ifny Transcription Activity.** T-bet is a transcription factor regulating *Ifny* gene transcription (13, 30). A reporter gene assay was conducted using the *Ifny-luc* reporter plasmid...
Peripheral blood was withdrawn from mice for identification of successful reconstitution of recipient mice. The data show that the sry (male-specific) gene (32) was detected in all female recipient mouse blood samples (Fig. S4A). We used these RSK2 KO mice with bone marrow cells overexpressing mock, T-bet<sup>wt</sup>, or T-bet<sup>S498A/S502A</sup> as our study model and implanted CT26-luciferase mouse colorectal cancer cells into the spleen of each mouse. As in a previous study of liver metastasis (33), the implanted CT26-luciferase cells quickly multiplied and occupied the mouse liver (by spleen injection) or lung (by tail vein injection) (34) in vivo. In vivo Xenogen imaging (35), which measures the bioluminescence of CT26 cells, revealed that starting on day 7 after tumor implantation, significantly more CT26 cells were detected in RSK2 KO mock-transfected mice than in RSK2 KO T-bet<sup>wt</sup> or T-bet<sup>S498E/S502E</sup>-transfected mice (Fig. 7A and B and Fig. S4 B and C). In addition, RSK2 KO T-bet<sup>S498E/S502E</sup>-transfected mice had significantly less CT26 liver and lung metastasis compared with RSK2 KO T-bet<sup>wt</sup>-transfected mice (Fig. 7A–C and Fig. S4 B–E).

Finally, we isolated immune cells from the thymus of mice from each group and found that the mRNA level of Ifnγ was significantly up-regulated in both T-bet<sup>wt</sup>- and T-bet<sup>S498E/S502E</sup>-transfected groups. Interestingly, Ifnγ levels were higher in the T-bet<sup>S498E/S502E</sup>-transfected mice than in the T-bet<sup>wt</sup>-transfected mice (Fig. 7D). These data indicate that the inappropriate phosphorylation of T-bet accelerates colorectal tumor metastasis and growth because of an impaired immune response due to a deficiency of RSK2.

**Discussion**

Failing immunity is known to contribute to cancer development and progression (36); thus, enhancing and maintaining T-cell carrying the luciferase gene containing the T-bet promoter region. After cotransfection with a fixed amount of T-bet but with increasing amounts of RSK2, T-bet transcription activity was significantly increased dose-dependently by RSK2 in RSK2<sup>−/−</sup> murine embryonic fibroblasts (MEFs) (Fig. 6A). To examine the function of the T-bet phosphorylation sites, T-bet<sup>wt</sup>- and mutant T-bet<sup>S498A/S502A</sup> or T-bet expressing serines 498 and 502 mutated to glutamine (T-bet<sup>S498E/S502E</sup>, which mimics a constitutively phosphorylated status of T-bet), were each cotransfected with the Ifnγ-luc reporter plasmid. As expected, in RSK2<sup>+/+</sup> MEF cells, the T-bet<sup>wt</sup>-expressing T-bet showed higher transcriptional activity compared with cells expressing the T-bet<sup>S498A/S502A</sup> mutant form (Fig. 6B). Furthermore, RSK2<sup>−/−</sup> MEFs expressing the active mutant T-bet<sup>S498E/S502E</sup> showed greater IFNγ transcriptional activity compared with cells expressing the T-bet<sup>wt</sup>- Fig. 6C). These data indicate that T-bet promotion of IFNγ transcription activity relies on its phosphorylation and activation by RSK2.

**Phosphorylation of T-Bet at Serines 498 and 502 Attenuates Colon Cancer Metastasis in RSK2 KO Mice.** To further investigate the role of T-bet<sup>S498A/S502E</sup> phosphorylation by RSK2 in vivo, we conducted a bone marrow cell transplantation assay. Female RSK2 KO mice served as recipient mice, and male RSK2 KO mice were donors. After isolation of bone marrow cells, mock, T-bet<sup>wt</sup>, or T-bet<sup>S498A/S502E</sup> (i.e., mimicking phosphorylated status) was overexpressed in these bone marrow cells using an electropulse method. Simultaneously, female RSK2 KO mice were sublethally irradiated using an X-ray generator to fully abolish bone marrow function, and then received i.v. transplantation with transfected bone marrow cells within 3 h after irradiation. Peripheral lymphohematopoietic reconstitution of all cell lineages should be normal by day 21 after bone marrow transplantation (31).
activation might be an effective approach in cancer treatment (37). All immunosuppressive treatments can potentially impair the immune system defense capacity, leading to an increased incidence of cancers (38). Although RSK family proteins have been extensively studied for their involvement in multiple cellular functions, little is known of their role(s) in the immune system in vivo. As reported previously, RSK2 is catalytically activated by T-cell receptor (TCR) stimulation, and plays an essential role in T-cell activation. T, B, and NK cells from RSK2 KO mice develop normally (27), and similar observations were made in our in-house RSK2 KO mouse breeding colony. When primary cells were isolated from spleens and lymph nodes and stained to detect the CD4, CD8, or NK cell marker, no significant differences were found between WT and RSK2 KO groups (Fig. S2). Both T and B cells can recognize a diverse array of potential tumor antigens, and also can detect small antigenic differences between normal and transformed cells (36). Our RSK2 KO mice showed a significantly increased rate of liver metastasis with a colon cancer cell challenge (Fig. 2A).

All of the foregoing effects seem to be due to the inappropriate phosphorylation of T-bet, which impairs the immune response (Fig. 7 and Fig. S4); however, how RSK2 enhances antitumor CD8+ T-cell responses directly or indirectly through CD4+ T cells or another mechanism requires further study. Clinically, heterogeneous loss-of-function mutations in the hRSK2 gene (RPS6KA3) cause CLS, and ~70-80% of diagnosed patients have no family history (9). An RSK2-null mouse has been created as a model for CLS. Current clinical studies of CLS are focused mainly on patients with serious developmental retardation, and the extent of immune deficiencies remains unclear.

T-bet directly activates the *Ifnγ* gene by binding to the IFNγ promoter and to multiple distal regulatory elements located upstream and downstream of the *Ifnγ* gene (39). T-bet expression has been correlated with increasing IFNγ expression (40). Similar results were also obtained in our study (Figs. S4 and S4A). A high percentage of T cells produce IFNγ after TCR stimulation, and T-bet deficiency results in reduced IFNγ production (41). Selective expression of T-bet accounts for TH1 cell development and for the TH1 cell-specific expression of IFNγ (11). T-bet expression is rapidly induced by TCR and IL-12R signaling, and is required for the early production of IFNγ by antigen-specific CD8+ T cells (42). T-cell kinase-mediated phosphorylation of T-bet at Tyr525 promotes the interaction of T-bet with GATA3, which interferes with the binding of GATA3-3 to its target DNA (43). T-bet phosphorylation at Thr302 is crucial for the interaction of T-bet with NFAT1, and loss of this interaction abrogates the ability of

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**Fig. 6.** RSK2 promotes IFNγ transcription activity. (A) The *Ifnγ-luc* reporter plasmid and pCMV-HA-T-bet plasmid were cotransfected with pcDNA4-mock (a control vector) or different doses of pcDNA-Xpress-RSK2 into RSK2−/− MEF cells. The luciferase activity was measured and normalized against Renilla luciferase activity (Left), and T-bet and RSK2 expression levels were detected by Western blot analysis (Right). The *Ifnγ-luc* reporter plasmid and pCMV-HA-T-betS498A/S502E, pCMV-HA-T-betS498E/S502E, or pCMV-HA-T-betS498E/S502E plasmid were cotransfected with pCMV-HA-mock (a control vector) into RSK2−/− cells (B) or RSK2+/− cells (C). The luciferase activity was measured and normalized against Renilla luciferase activity (Left), and T-bet expression level was detected by Western blot analysis (Right). *P < 0.05. Panels represent individual blots.

**Fig. 7.** Phosphorylation of T-betS498E/S502E attenuates colon cancer liver metastasis in RSK2 KO mice. RSK2 KO mice overexpressing mock, T-betS498A/S502E, or T-betS498E/S502E were established by bone marrow transplant assay. CT26 cells (1 x 10^6) tagged with firefly luciferase were injected into the spleens of these mice, and bioluminescence of CT26 cells was visualized using in vivo Xenogen imaging at different days after tumor implantation. (A) Representative images from each group (*n = 5*) are shown. (B) Data were analyzed using Bruker Mi SE software. *P < 0.05, ANOVA. (C) Representative photographs of spleen and liver metastases (Mets) of mice. (D) Gene expression of *Ifnγ* and T-bet was analyzed by PCR with specific primers, and gapdh was used as an internal control to verify equal amounts of cDNA. *P < 0.05.
T-bet to suppress NFAT1-dependent cytokine expression (44). T-bet phosphorylation at Ser508 by casein kinase I and glycogen synthase kinase 3 (GSK3) promotes the interaction of T-bet with RelA, which impairs RelA binding to the IL2 promoter and the subsequent transcriptional activation of the IL2 gene (45). Mutation of lysine 313 (K313) decreases ubiquitination-mediated T-bet degradation and completely abrogates T-bet functions involving DNA binding and transcriptional activation of IFNγ (44). Based on recent studies, we hypothesized that T-bet activity might be regulated by posttranslational modification, specifically phosphorylation. We found that T-bet is phosphorylated by RSK2 at serines 498 and 502, and plays an important role in regulating IFNγ mRNA (Figs. 5C and 7D) and transcription level (Fig. 6 B and C).

Overall, our results show that RSK2 deficiency can result in dramatically decreased IFNγ secretion through inappropriate phosphorylation of T-bet. This can lead to immune suppression, which accelerates colon cancer metastasis and growth. The clinical relevance of these findings requires additional study. In addition, analysis of the cancer incidence and immune function in CLS patients could provide valuable information.

Materials and Methods

The in vivo Xenogen imaging of mice was performed using the Xtreme Imaging system (Carestream Health), and bioluminescence was quantified using Bruker MI software. The materials and methods used in this study are described in detail in SI Materials and Methods.

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