Ribosomal RACK1 promotes chemoresistance and growth in human hepatocellular carcinoma

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Coordinated translation initiation is coupled with cell cycle progression and cell growth, whereas excessive ribosome biogenesis and translation initiation often lead to tumor transformation and survival. Hepatocellular carcinoma (HCC) is among the most common and aggressive cancers worldwide and generally displays inherently high resistance to chemotherapeutic drugs. We found that RACK1, the receptor for activated C-kinase 1, was highly expressed in normal liver and frequently upregulated in HCC. Aberrant expression of RACK1 contributed to in vitro chemoresistance as well as in vivo tumor growth of HCC. These effects depended on ribosome localization of RACK1. Ribosomal RACK1 coupled with PKCβII to promote the phosphorylation of eukaryotic initiation factor 4E (eIF4E), which led to preferential translation of the potent factors involved in growth and survival. Inhibition of PKCβII or depletion of eIF4E abolished RACK1-mediated chemotherapy resistance of HCC in vitro. Our results imply that RACK1 may function as an internal factor involved in the growth and survival of HCC and suggest that targeting RACK1 may be an efficacious strategy for HCC treatment.

Introduction

Hepatocellular carcinoma (HCC) is among the most common and lethal cancers in the human population, ranked the fifth-most frequent neoplasm and the third-most common cause of cancer-related death worldwide (1). Though surveillance can lead to early diagnosis when the tumor might be resectable, most patients with HCC are diagnosed at advanced stages and can only receive palliative treatments. However, HCC generally displays inherent resistance to chemotherapeutic drugs, and systemic or selective intra-arterial administration of any chemotherapy agent, which has marginal antitumor activity and shows no benefit for survival, is not recommended in clinical practice (2–5).

Under normal circumstances, coordinated translation initiation is coupled with cell cycle progression and cell growth, whereas aberrant protein biosynthesis has been associated with tumor transformation and survival (6–8). In general, translation initiation, which is the rate-limiting step in protein synthesis, is tightly regulated by eukaryotic initiation factors (eIFs), including eIF2, which controls loading of the ternary complex on the 40S subunit, and eIF4E, which acts in the eIF4F complex and regulates binding of capped mRNA to 40S subunit (9–11). Increased eIF4E activity is associated with tumor formation and progression in several human malignancies, including leukemias, lymphomas, and cancers of the breast, colon, bladder, lung, prostate, and head and neck (12). While most cellular mRNAs require only minimal eIF4E to be efficiently translated, elevated eIF4E activity preferentially enhances translation of select mRNAs, many of which encode potent growth and survival factors, such as cyclin D1, MYC, ODC, VEGF, Survivin (also known as BIRC5), and BCL-2 (12–14). Therapeutic repression of eIF4E expression induces apoptosis in tumor cell lines (14). Several drugs that suppress translation initiation by preventing eIF4F assembly (such as rapamycin) or blocking eIF4F activity (such as silvestrol), enhance the chemosensitivity in human and experimental cancers (15–19).

RACK1 was originally identified based on its ability to bind to the activated C kinase (PKC) isoform βII and is highly conserved among all eukaryotic species (20, 21). As a member of the Trp-Asp (WD) repeat protein family, it serves as a scaffold protein for many kinases and receptors and plays a pivotal role in a wide range of biological responses, including signal transduction and immune response as well as cell growth, migration, and differentiation (21). Recent studies have shown that RACK1 is a component of the 40S subunit of ribosome and present in both ribosome- and nonribosome-bound form (22–24). RACK1 is found to be upregulated in several kinds of tumors and considered as an excellent marker of oral squamous carcinoma, breast cancer, and pulmonary adenocarcinomas (25–29). Herein, we demonstrate that RACK1 is highly expressed in normal liver and frequently upregulated in HCC and that the ribosome localization is essential for RACK1-mediated in vitro chemoresistance and in vivo growth of HCC. Our results suggest that ribosomal RACK1 might function as an internal factor contributing to the growth and survival of HCC and that targeting RACK1 may be an efficacious strategy for HCC treatment.

Results

RACK1 is highly expressed in normal liver and frequently upregulated in HCC. RACK1 is a classic scaffold protein ubiquitously expressed

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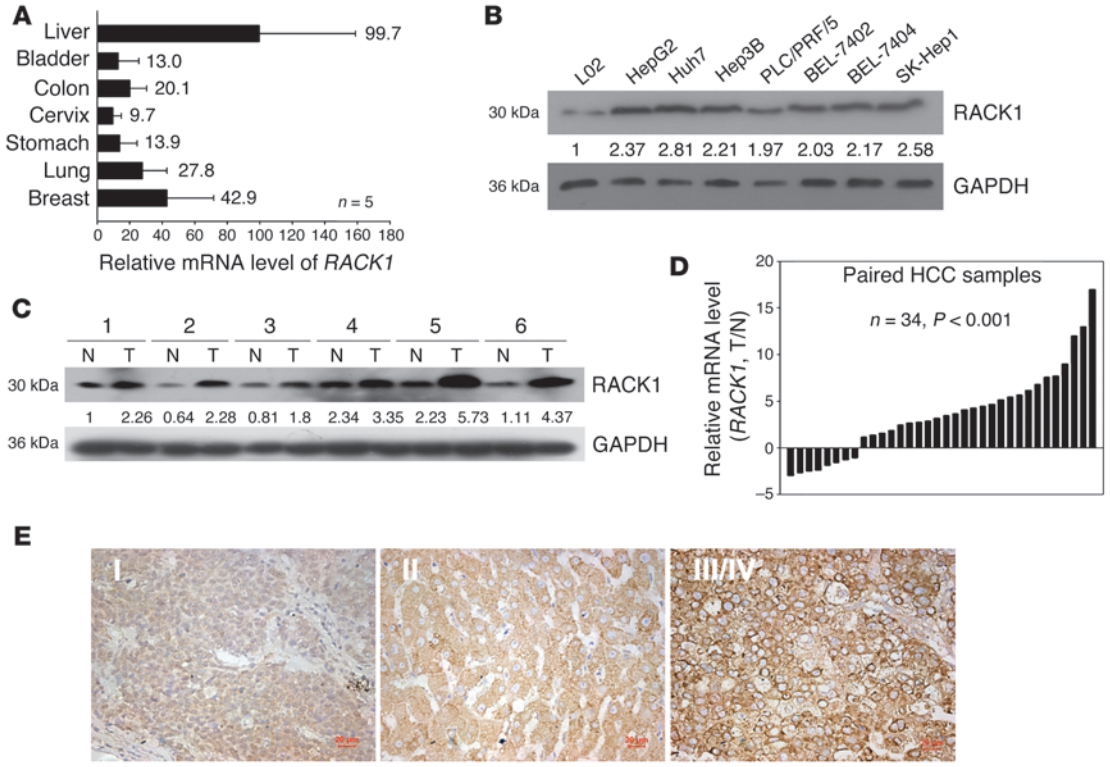

Figure 1

RACK1 is preferentially expressed in normal liver and frequently upregulated in HCC. (**A**) Assessment of RACK1 transcripts in a variety of normal human tissues. Total RNA was extracted from frozen fresh normal tissues, and quantitative real-time PCR was performed to assess RACK1 transcripts, with β-actin mRNA as the internal control. Numbers listed are mean of each group. (**B**) Expression of RACK1 in an immortalized liver cell line and HCC cell lines. (**C**) Expression of RACK1 in paired liver tissue samples. N, adjacent nontumor sections; T, tumor sections. (**D**) MRNA levels of *RACK1* in paired liver tissue samples. Total RNA was extracted from paired liver tissue samples, and *RACK1* mRNA levels were detected by quantitative real-time PCR, with β-actin as internal control. (**E**) Expression of RACK1 in HCC samples at different stages. RACK1 expression was detected by immunohistochemistry in 162 HCC samples. In **B** and **C**, numbers represent relative expression of RACK1, which was quantified by comparing it with GAPDH. Original magnification, ×400; scale bars: 20 μm.

in the tissues of higher mammals and humans (21). We first examined the expression pattern of *RACK1* mRNA in various normal human tissues. As shown in Figure 1A, the highest mRNA level of *RACK1* was detected in normal liver. Western blot analysis of the tissues of adult mice also confirmed the highest protein level of RACK1 in liver (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI58488DS1). These results suggest that, while ubiquitously distributed in the tissues of mammals, RACK1 is highly expressed in the liver.

To understand whether RACK1 was involved in the hepatocarcinogenesis, we first examined the expression of RACK1 in HCC cell lines (HepG2, Huh7, Hep3B, PLC/PRF/5, BEL-7402, BEL-7404, and SK-Hep1) and the immortalized liver cell line (L02). The endogenous expression of RACK1 in HCC cell lines was much higher than that in the L02 cell line (Figure 1B). Moreover, the expression of RACK1 was higher in HCC samples compared with that in their adjacent nontumor tissues (Figure 1C). Quantitative real-time PCR also confirmed the increased mRNA levels of *RACK1* in most HCC cases (Figure 1D). These results suggest that RACK1 is involved in the tumorigenesis of HCC.

To further determine the role of RACK1 in the development of HCC, RACK1 expression was detected in HCC cases at different TNM stages (Supplemental Table 1). As shown in Figure 1E and Supplemental Table 2, RACK1 expression was well correlated with the clinical progression of HCC ($P < 0.001$). Moreover, the protein level of RACK1 was strongly related to the expression of Ki67 and the serum level of AFP ($P < 0.001$ and $P = 0.002$, respectively) as well as the poor prognosis of patients with HCC ($P < 0.001$) (Supplemental Tables 3 and 4 and Supplemental Figure 2). These results indicate that RACK1 is frequently upregulated in HCC and suggest that RACK1 may contribute to the tumorigenesis and progression of HCC.

Ribosomal RACK1 modulates the chemosensitivity of HCC in vitro. Since the antiapoptotic effect of RACK1 has been characterized in several kinds of tumors, including breast cancer (30) and melanoma (31), we next examined the role of RACK1 in the chemosensitivity of HCC in vitro. Overexpression of wild-type RACK1 significantly inhibited doxorubicin-induced apoptosis in HepG2 (p53 wild-type), Huh7 (p53-mutated), and Hep3B (p53-null) cells, while depletion of RACK1 enhanced the sensitivity to doxorubicin-induced apoptosis (Figure 2, A and B). Similar results were also observed in CDDP- and 5-FU–treated cells (data not shown). Moreover, overexpression of wild-type RACK1 showed little effect on doxorubicin-induced transcriptional activity of p53 in HepG2 cells (Supplemental Figure 3). These results imply that RACK1

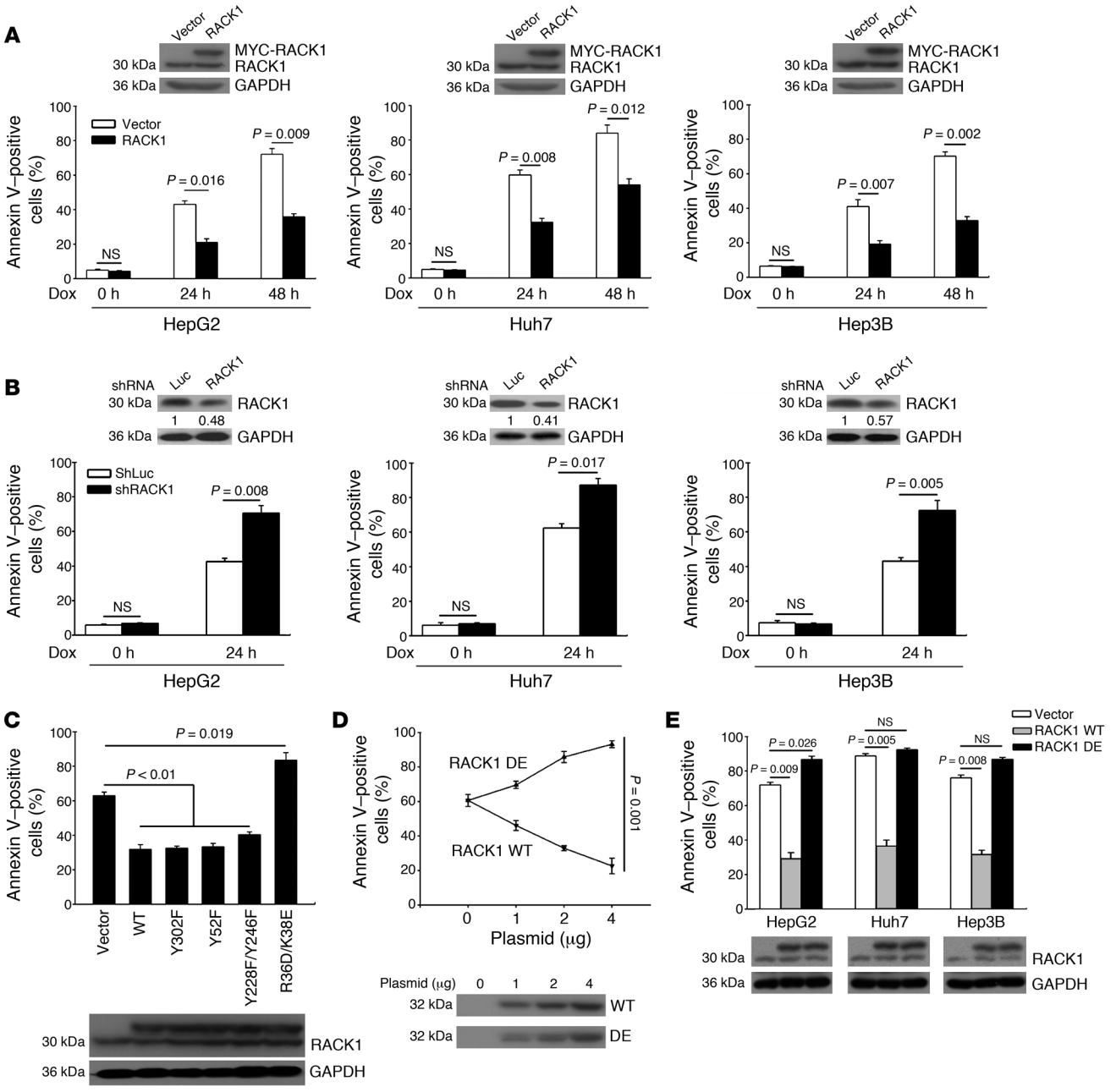

Figure 2

Ribosomal RACK1 contributes to the chemotherapy resistance of HCC in vitro. (**A**) Overexpression of RACK1 enhances the doxorubicin (Dox) resistance of HCC cell lines. Twenty-four hours after transfection with vector or wild-type RACK1, HepG2 (100 μg/ml), Huh7 (20 μg/ml), and Hep3B (50 μg/ml) cells were treated with doxorubicin for indicated times. Cellular apoptosis was determined by annexin V staining. (**B**) Depletion of RACK1 sensitizes HCC cells to doxorubicin-induced apoptosis. Seventy-two hours after transfection with shLuc or shRACK1, cells were treated with doxorubicin for another 24 hours as in **A**. Numbers represent relative expression of RACK1, which was quantified by comparing it with GAPDH. (**C**) The ribosome localization of RACK1 is required for doxorubicin resistance of HCC. Twenty-four hours after transfection, Huh7 cells were treated with doxorubicin (20 μg/ml) for another 24 hours. (**D**) The DE mutant sensitizes Huh7 cells to doxorubicin-induced apoptosis. Huh7 cells were transiently transfected with increasing doses of wild-type RACK1 or the DE mutant and treated as in **C**. (**E**) Wild-type RACK1, but not the DE mutant, restores the chemoresistance of RACK1-depleted HCC cells. HCC cells expressing shRACK1 were supertransfected with the constructs as indicated and treated with doxorubicin as in **A** for 24 hours. All assays were performed in triplicate.

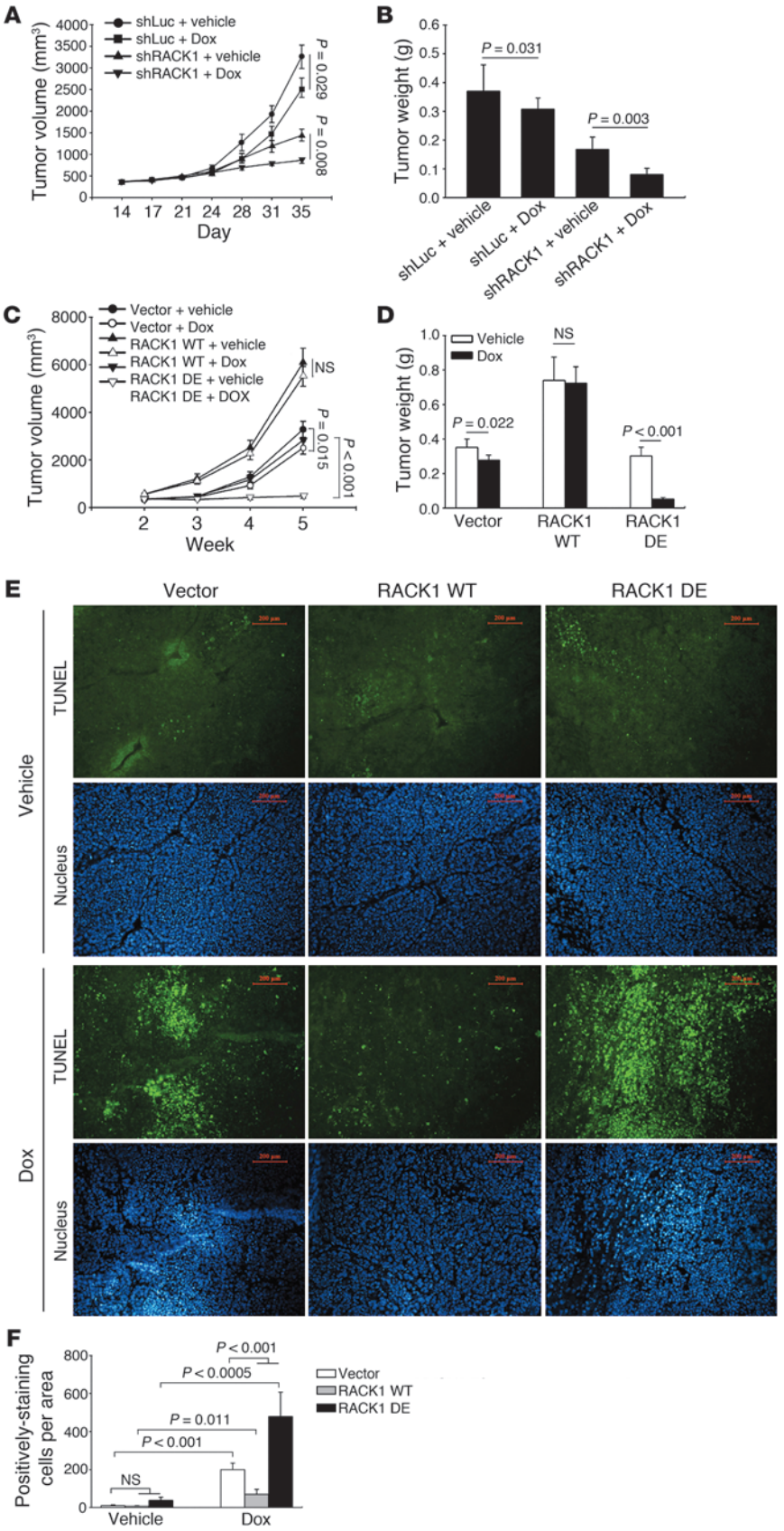

Figure 3

Ribosomal RACK1 modulates tumor growth of HCC in vivo. (**A** and **B**) RACK1 depletion inhibited tumor growth in vivo. Xenografts were generated using Huh7 cells. (**A**) Two weeks later, retroviruses carrying shRNA and doxorubicin were delivered by intratumoral injection. Tumor volume was measured at indicated time points. (**B**) Tumor weight was measured on the day of harvest, after excision of the tumor from the euthanized mouse. (**C**–**F**) Ribosomal RACK1 modulates tumor growth in vivo. Xenografts were generated using Huh7 cells stably expressing empty vector, wild-type RACK1, or the DE mutant. Two weeks later, doxorubicin was given by intratumoral injection. In **C** and **D**, tumor volume and weight were measured as in **A** and **B**. In **E** and **F**, TUNEL assay was performed to detect apoptosis. Statistical significance was determined using 1-way or 2-way repeated-measures ANOVA. Original magnification, ×200; scale bars: 200 μm.

modulates the chemosensitivity of HCC in a p53-independent manner.

Several reports have implicated RACK1 in ways that are related to apoptosis resistance, such as BimEL degradation (30) and JNK activation (31). To understand how RACK1 modulates the chemosensitivity in HCC cells, wild-type RACK1 as well as several mutants with functional defects that have been described previously (32–35), were transfected into Huh7 cells. Interestingly, the nonribosome-binding mutant of RACK1 (the R36D/K38E mutant [DE mutant]) conversely sensitized Huh7 cells to doxorubicin-induced apoptosis, while the other mutants, including Y302F (β1 integrin binding), Y52F (FAK activation), and Y228F/Y246F (Src activation), still promoted the chemotherapy resistance of HCC cells (Figure 2, C and D, and Supplemental Figure 4, A and B). Moreover, supertransfection of rescue wild-type RACK1, but not the DE mutant, restored the resistance to doxorubicin in RACK1-depleted cells (Figure 2E). These results imply that ribosomal localization is essential for RACK1-mediated chemotherapy resistance in vitro.

Ribosomal RACK1 modulates the tumor growth of HCC in vivo. We next determined the effects of RACK1 on the xenograft formation of HCC in vivo. Depletion of RACK1 alone inhibited xenograft development and induced significant suppression of tumor growth in combination with doxorubicin (Figure 3, A and B, and Sup-

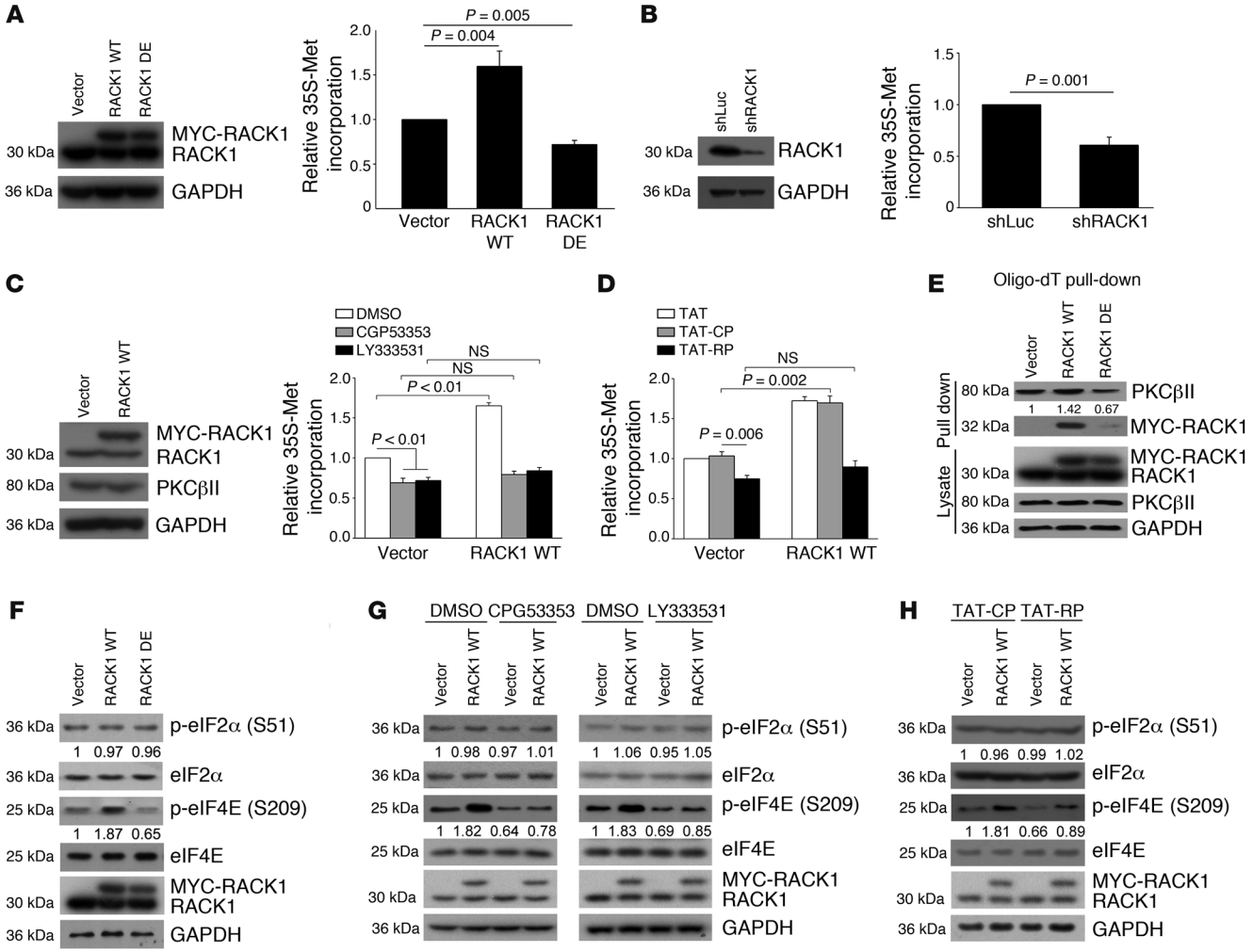

Figure 4

Ribosomal RACK1 enhances global translation and the phosphorylation of eIF4E in a PKCβII-dependent manner. (**A**) Ribosomal RACK1 promotes de novo protein synthesis. The rate of [^{35}S]-methionine (35S-Met) incorporation was assessed relative to that of cells transfected with empty vector. (**B**) RACK1 is required for efficient translation. (**C**) RACK1 stimulates translation requiring PKCβII activity. Transfected Huh7 cells were incubated with CGP53353 (5 μM) or LY333531 (50 nM) for 1 hour. (**D**) Functional association with PKCβII is required for RACK1-stimulated translation. Intracellular delivery of TAT peptides was conducted as described in the Methods. (**E**) Ribosomal RACK1 enriches PKCβII with poly(A) mRNA. Huh7 cells were transfected as indicated, followed by oligo-dT pull-down assay. (**F**) The effect of ribosomal RACK1 on the phosphorylation of eIF2α and eIF4E. (**G**) PKCβII activity is required for RACK1-mediated phosphorylation of eIF4E. Huh7 cells were transfected and treated as in **C**. (**H**) RACK1/PKCβII interaction is required for RACK1-mediated phosphorylation of eIF4E. Huh7 cells were transfected and treated as in **D**. In **A**–**D**, assays were performed in quadruplicate (n = 4). In **E**–**H**, numbers represent relative expression of PKCβII, p-eIF2α (S51), or p-eIF4E (S209), which was quantified by comparing with GAPDH.

plemental Figure 5). To examine the effect of ribosomal RACK1 on tumor growth in vivo, xenografts were also generated using HCC cells stably transfected with wild-type RACK1 or the DE mutant. Overexpression of wild-type RACK1 promoted tumor growth in nude mice; however, the DE mutant failed to promote the generation of xenografts and showed more inhibitory effect on tumor growth upon doxorubicin treatment (Figure 3, C and D, and Supplemental Figure 4C). TUNEL staining revealed that under doxorubicin challenge, xenografts overexpressing the DE mutant displayed the highest rate of cellular apoptosis (Figure 3, E and F). Taken together, these results imply that ribosomal RACK1 modulates the tumor growth of HCC in vivo.

Ribosomal RACK1 modulates the rate of translation through PKCβII. Ribosomal RACK1 is a component of the 40S small subunit involved in the regulation of translation initiation (36–38). To explore the potency of ribosome-associated RACK1 on translation regulation in HCC, metabolic labeling studies were carried out. Overexpression of wild-type RACK1 promoted [^{35}S]-methionine incorporation, whereas DE mutant overexpression or RACK1 depletion partly inhibited de novo protein synthesis (Figure 4, A and B). The effect of ribosomal RACK1 on cap- and IRES-mediated translation was further assessed by using the bicistronic reporter pcDNA/Fluc/IRES/Rluc construct that we previously described (Supplemental Figure 6A and ref. 39). Introduction of wild-type

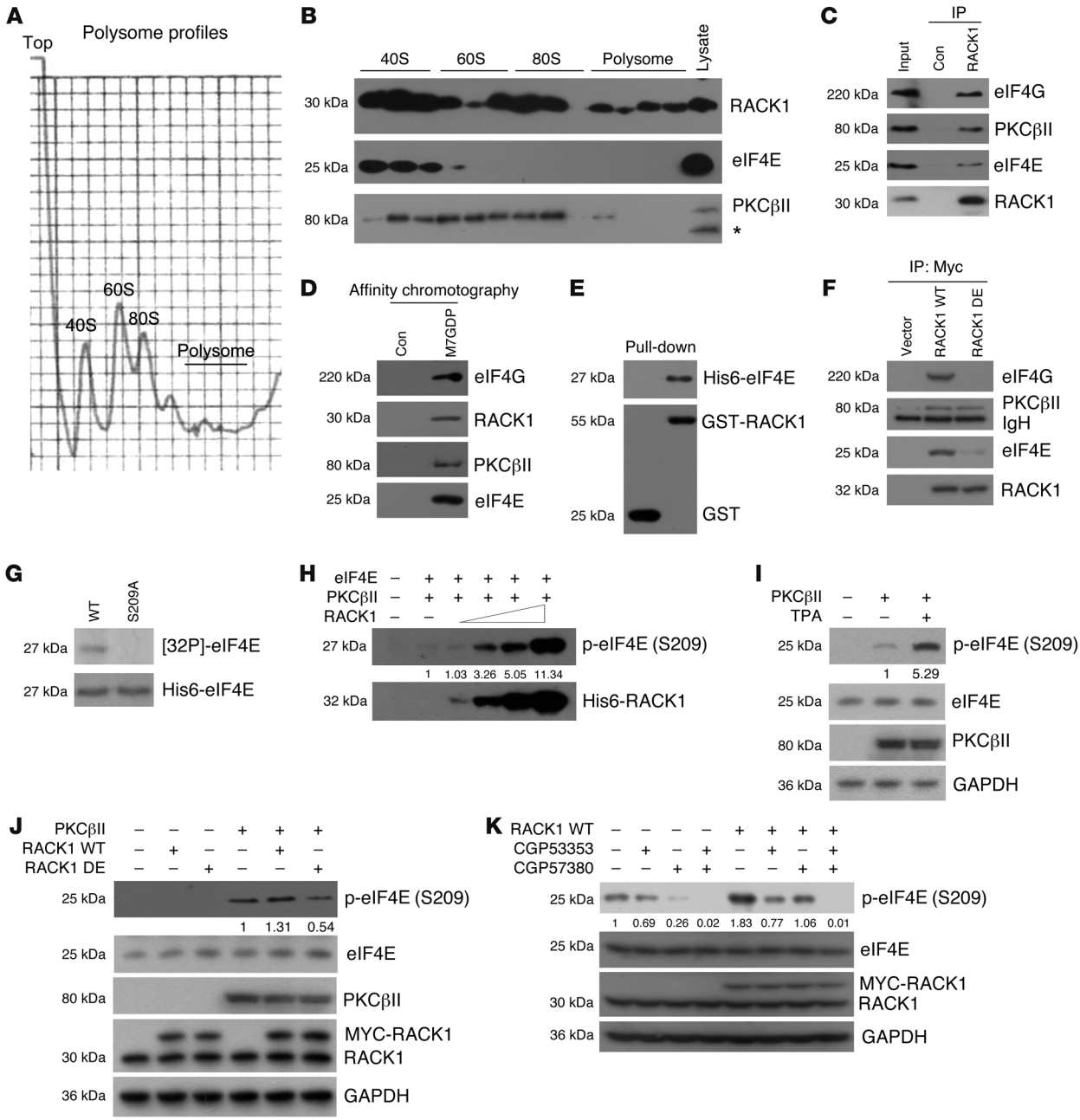

Figure 5

Ribosomal RACK1/PKCβII complex acts on eIF4E. (**A** and **B**) Polysome profiling and association of RACK1 and PKCβII with ribosomes in Huh7 cells. The asterisk indicates a nonspecific band. (**C**) Ribosomal association between eIF4E and RACK1. Purified ribosomes were digested with RNase and subjected to immunoprecipitation. Con, control. (**D**) RACK1 associates with eIF4F complex. (**E**) In vitro association between eIF4E and RACK1. (**F**) Ribosome localization is required for eIF4E and RACK1 association. Cell lysates of transfected Huh7 cells were applied to immunoprecipitation assay. (**G**) eIF4E is phosphorylated in vitro by PKCβII. (**H**) RACK1/PKCβII complex acts on eIF4E. In vitro phosphorylation assay was performed as described in the Supplemental Methods. (**I**) PKCβII restores eIF4E phosphorylation in *Mnk1*$^{-/-}$ *Mnk2*$^{-/-}$ MEFs. *Mnk1*$^{-/-}$*Mnk2*$^{-/-}$ MEFs were transfected with PKCβII and treated with or without TPA (100 nM) for 1 hour. (**J**) Effect of ribosomal RACK1 on PKCβII-mediated eIF4E phosphorylation. *Mnk1*$^{-/-}$*Mnk2*$^{-/-}$ MEFs were transfected with PKCβII, along with or without wild-type RACK1 or the DE mutant. (**K**) Effect of MNK inhibition on RACK1-mediated eIF4E phosphorylation. Transfected Huh7 cells were treated with CGP53353 (5 μM) and/or CGP57380 (10 μM). In **H**–**K**, numbers represent relative expression of p-eIF4E (S209), which was quantified by comparing with GAPDH (**I**–**K**).

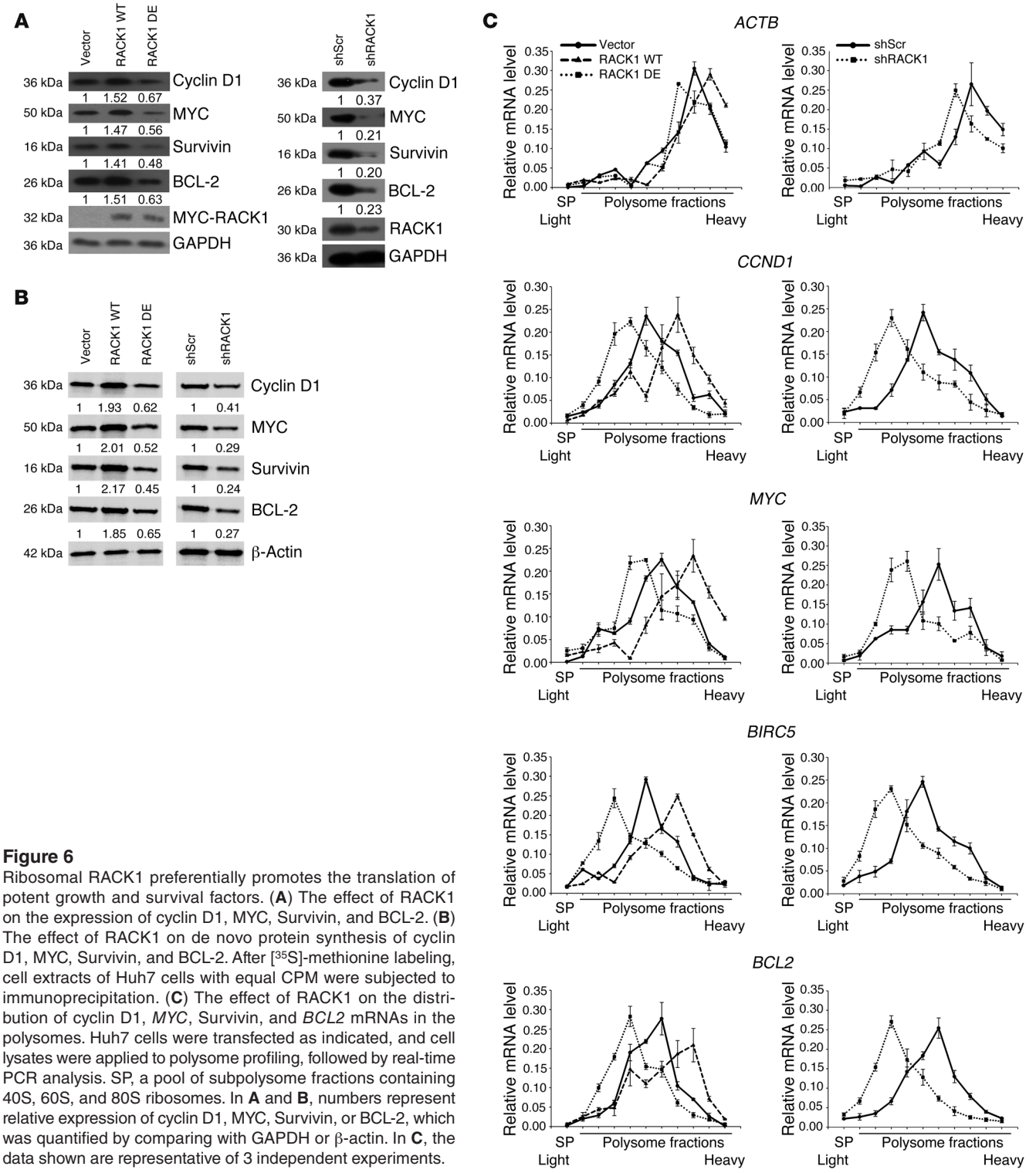

Figure 6

Ribosomal RACK1 preferentially promotes the translation of potent growth and survival factors. (**A**) The effect of RACK1 on the expression of cyclin D1, MYC, Survivin, and BCL-2. (**B**) The effect of RACK1 on de novo protein synthesis of cyclin D1, MYC, Survivin, and BCL-2. After [^{35}S]-methionine labeling, cell extracts of Huh7 cells with equal CPM were subjected to immunoprecipitation. (**C**) The effect of RACK1 on the distribution of cyclin D1, *MYC*, Survivin, and *BCL2* mRNAs in the polysomes. Huh7 cells were transfected as indicated, and cell lysates were applied to polysome profiling, followed by real-time PCR analysis. SP, a pool of subpolysome fractions containing 40S, 60S, and 80S ribosomes. In **A** and **B**, numbers represent relative expression of cyclin D1, MYC, Survivin, or BCL-2, which was quantified by comparing with GAPDH or β-actin. In **C**, the data shown are representative of 3 independent experiments.

RACK1 increased activities of both Firefly (cap dependent) and Renilla luciferase (ECMV IRES mediated), while DE mutant transfection or RACK1 depletion showed the opposite effect (Supplemental Figure 6, B–E). These results suggest that ribosomal RACK1, which possibly targets both cap- and IRES-mediated translation, contributes to translational regulation in HCC cells.

We next determined whether RACK1 stimulated translation through PKCβII, the known signaling molecule with which RACK1 interacts on ribosome (40). Administration of selective PKCβII inhibitor CGP53353 or PKCβ inhibitor LY333531 attenuated RACK1-mediated translation enhancement (Figure 4C). Moreover, delivery of recombinant peptide, which mimics

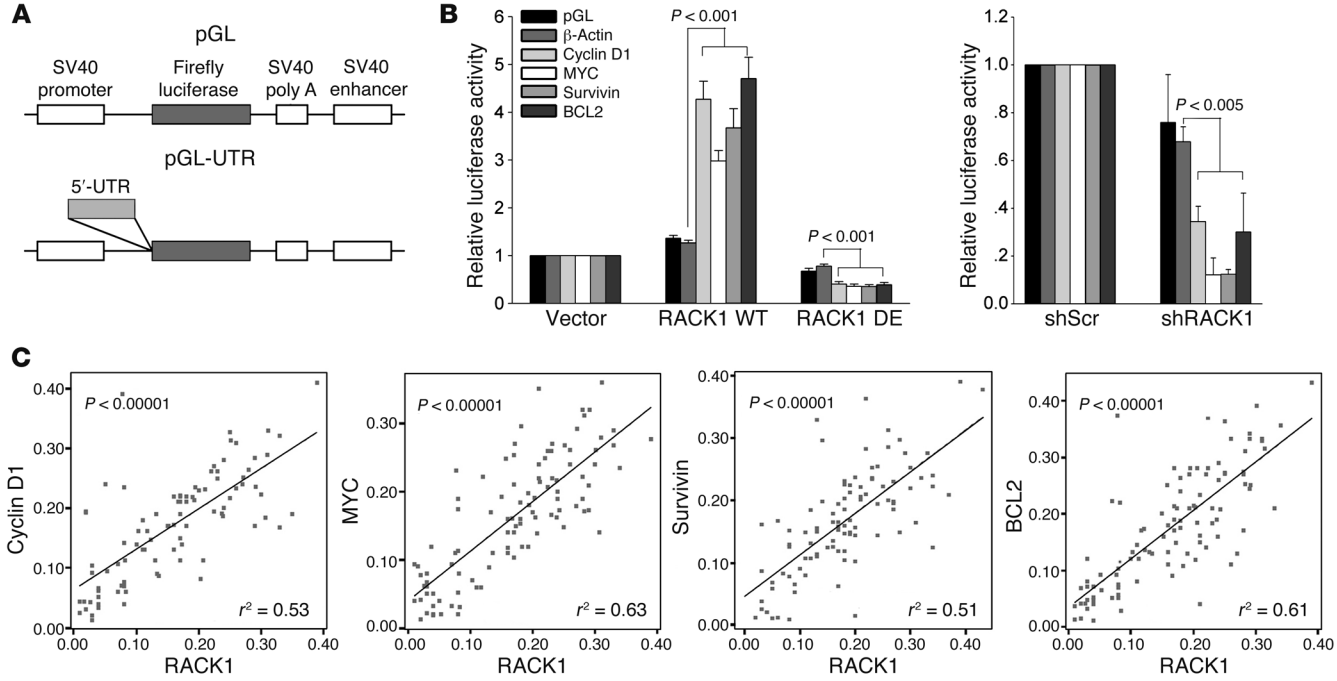

Figure 7
Ribosomal RACK1 preferentially promotes the translation of potent growth and survival factors. (**A**) Schematic diagram of SV40-based monocistronic constructs. (**B**) The effect of RACK1 on the translational activity of the 5′-UTR of cyclin D1, MYC, Survivin, and BCL-2. The relative luciferase activities of empty vector were normalized to 1. (**C**) Correlation of RACK1 with the expression of cyclin D1, MYC, Survivin, and BCL-2 in HCC cases. The expression profiles of cyclin D1, MYC, Survivin, and BCL-2 were examined by immunohistochemistry, quantified, and applied to Pearson's correlation analysis. In **B**, assays were performed in triplicate.

the RACK1 binding site and inhibits the interaction of RACK1 to PKCβII, also suppressed RACK1-stimulated translation (Figure 4D). These results suggest that RACK1 modulates translation in a PKCβII-dependent manner.

RACK1 binds to PKCβII with high affinity and is recognized as a shuttling protein that moves PKCβII from one intracellular site to another (41). To explore whether RACK1 stimulated translation by recruiting PKCβII to ribosome, oligo-dT pull-down assay was performed. Overexpression of wild-type RACK1 induced more enrichment of PKCβII with poly(A) mRNA, whereas overexpression of the DE mutant suppressed PKCβII enrichment, probably by competing with endogenous RACK1 in binding with PKCβII (Figure 4E and Supplemental Figure 7A). These results suggest that ribosomal RACK1 contributes to the recruitment of PKCβII to ribosome in HCC.

Ribosomal RACK1 modulates the phosphorylation of eIF4E through PKCβII. We next examined the effect of RACK1 on the rate-limiting factors of translation, whose activities play a central role in controlling translation initiation. Wild-type RACK1 promoted the phosphorylation of eIF4E on serine 209, whereas the DE mutant suppressed eIF4E phosphorylation (Figure 4F and Supplemental Figure 7B). Both RACK1 constructs showed little effect on the phosphorylation of eIF2α (Figure 4F and Supplemental Figure 7B). Overexpression of RACK1 mutants (Y302F, Y52F, and Y228F/Y246F) also stimulated the PKCβII recruitment and eIF4E phosphorylation, further demonstrating the critical role of ribosomal RACK1 on eIF4E activation (Supplemental Figure 7C). Inhibiting PKCβII or abolishing the RACK1/PKCβII interaction suppressed the regulatory effect of wild-type RACK1 on eIF4E phosphorylation, suggesting that PKCβII is required for RACK1-stimulated activity of eIF4E (Figure 4, G and H, and Supplemental Figure 7D). Moreover, overexpression of wild-type RACK1 stimulated the phosphorylation of eIF6 (36), a target of RACK1/PKCβII complex on ribosome, whereas the DE mutant suppressed eIF6 phosphorylation (Supplemental Figure 8).

Ribosomal RACK1/PKCβII complex associates with eIF4E. To understand how ribosomal RACK1/PKCβII complex modulated the activity of eIF4E, we first examined its effect on phosphorylation of MAP kinase-interacting serine/threonine kinase 1 (MNK1) and 4E-BP1, which are involved in the regulation of eIF4E phosphorylation and eIF4F formation, respectively (42, 43). However, inhibition of PKCβII or overexpression of RACK1 showed little effect on MNK1 or 4E-BP1 phosphorylation (Supplemental Figure 9).

We next examined whether ribosomal RACK1/PKCβII complex acted on eIF4E. Ribosome profiling confirmed the association of RACK1 with ribosomes and polysomes, while PKCβII was detected in 40S, 60S, and 80S ribosomes but not in polysomes, suggesting a role of ribosomal RACK1/PKCβII complex in the regulation of translation initiation (Figure 5, A and B). To explore whether ribosomal RACK1 coupled with eIF4E to allow the regulation of its phosphorylation, ribosomes were purified to perform coimmunoprecipitation. Results demonstrated that eIF4E, as well as eIF4G and PKCβII, was detected in the immunoprecipitates of RACK1 (Figure 5C). Affinity chromatography performed using the m^7GDP Sepharose also confirmed that RACK1 associated with eIF4F complex (Figure 5D). GST pull-down assay further revealed

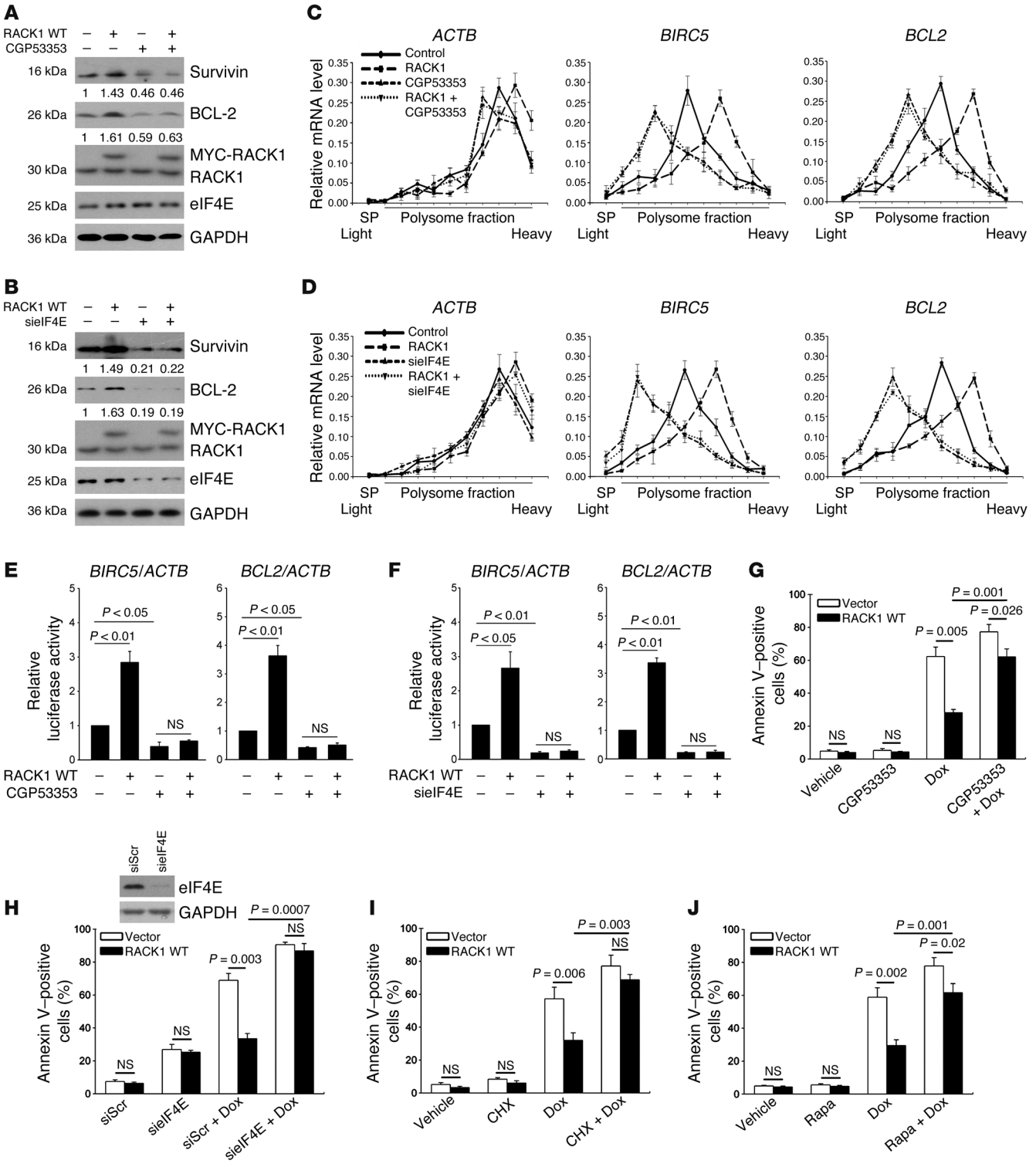
A
RACK1 WT – + – +
CGP53353 – – + +
16 kDa
Survivin
1 1.43 0.46 0.46
26 kDa
BCL-2
1 1.61 0.59 0.63
30 kDa
MYC-RACK1
RACK1
25 kDa
eIF4E
36 kDa
GAPDH
B
RACK1 WT – + – +
sieIF4E – – + +
16 kDa
Survivin
1 1.49 0.21 0.22
26 kDa
BCL-2
1 1.63 0.19 0.19
30 kDa
MYC-RACK1
RACK1
25 kDa
eIF4E
36 kDa
GAPDH
C
ACTB
BIRC5
BCL2
Control
RACK1
CGP53353
RACK1 + CGP53353
Relative mRNA level
SP
Polysome fraction
Light
Heavy
D
ACTB
BIRC5
BCL2
Control
RACK1
sieIF4E
RACK1 + sieIF4E
Relative mRNA level
SP
Polysome fraction
Light
Heavy
E
BIRC5/ACTB
BCL2/ACTB
Relative luciferase activity
P < 0.05
P < 0.01
NS
RACK1 WT – + – +
CGP53353 – – + +
F
BIRC5/ACTB
BCL2/ACTB
Relative luciferase activity
P < 0.01
P < 0.05
NS
RACK1 WT – + – +
sieIF4E – – + +
G
Vector
RACK1 WT
Annexin V–positive cells (%)
P = 0.001
P = 0.026
P = 0.005
NS
Vehicle
CGP53353
Dox
CGP53353 + Dox
H
siScr
sieIF4E
eIF4E
GAPDH
P = 0.0007
Vector
RACK1 WT
Annexin V–positive cells (%)
P = 0.003
NS
siScr
sieIF4E
siScr + Dox
sieIF4E + Dox
I
Vector
RACK1 WT
Annexin V–positive cells (%)
P = 0.003
P = 0.006
NS
Vehicle
CHX
Dox
CHX + Dox
J
Vector
RACK1 WT
Annexin V–positive cells (%)
P = 0.001
P = 0.02
P = 0.002
NS
Vehicle
Rapa
Dox
Rapa + Dox

Figure 8
RACK1 promotes the in vitro chemotherapy resistance of HCC in a PKCβII- and eIF4E-dependent manner. (**A** and **B**) RACK1 promotes the expression of Survivin and BCL-2 in a PKCβII- and eIF4E-dependent manner. Huh7 cells were treated with (**A**) 5 μM CGP53353 for 24 hours or (**B**) sieIF4E for 72 hours and then cell lysates were applied to Western blot analysis. In **A** and **B**, numbers represent relative expression of Survivin or BCL-2, which was quantified by comparing with GAPDH. (**C** and **D**) RACK1 promotes the right shift of Survivin and *BCL2* mRNA in a PKCβII- and eIF4E-dependent manner. Huh7 cells were treated as in **A** or **B** and applied to polysome profiling, followed by real-time PCR analysis. (**E** and **F**) RACK1 preferentially upregulates the 5′-UTR activities of Survivin and *BCL2* mRNAs in a PKCβII- and eIF4E-dependent manner. Huh7 cells were transfected and treated as in **A** or **B**, followed by luciferase assay. Relative luciferase activities were calculated by Survivin or BCL-2 compared with β-actin, and the ratio of empty vector was normalized to 1. (**G**–**J**) Huh7 cells were pretreated with or without (**G**) CGP53353 (1 μM), (**I**) cycloheximide (CHX) (50 nM), or (**J**) rapamycin (rapa) (10 nM) for 24 hours or (**H**) sieIF4E for 48 hours. Then cells were treated with doxorubicin (20 μg/ml) for another 24 hours, collected, and applied to annexin V staining. In **C** and **D**, the data shown are representative of 3 independent experiments. In **E**–**J**, assays were performed in triplicate.

the direct interaction between RACK1 and eIF4E in vitro (Figure 5E). However, the DE mutant of RACK1 failed to bind to eIF4E in vivo, but not in vitro, suggesting that ribosomal localization is required for RACK1 to associate with eIF4E (Figure 5F and Supplemental Figure 10A).

We also examined the interface for RACK1 and eIF4E association. A competitive binding assay performed using the eIF4G peptides demonstrated that RACK1 and eIF4G do not compete for binding to eIF4E (Supplemental Figure 10B). Mutation analysis of the conserved residues localized within the dorsal surface of eIF4E revealed that the binding interface of eIF4E for RACK1 was possibly localized at the end of helix 2 (G139, E140, and D143) (Supplemental Figure 10C and ref. 44). GST pull-down assay performed using the truncated form of RACK1 also demonstrated that the WD repeats 5–7 were involved in the interaction with eIF4E (Supplemental Figure 10D). Since the WD repeat 5 of ribosomal RACK1 is largely obscured by ribosome proteins (45), the binding surface of RACK1 for eIF4E was possibly localized within WD repeats 6 and 7.

RACK1/PKCβII complex acts on eIF4E. We next detected whether RACK1/PKCβII complex acted on eIF4E. In vitro phosphorylation assay suggested that PKCβII directly phosphorylated eIF4E on serine 209 and RACK1 promoted PKCβII-mediated eIF4E phosphorylation in dose-dependent manner (Figure 5, G and H).

To understand whether eIF4E was a physiological substrate of PKCβII in vivo, we examined the effect of PKCβII on the phosphorylation of eIF4E in MNK1/2 double-knockout mouse embryo fibroblasts (MEFs), which was reported to show no phosphorylation of eIF4E (46). Transfection of wild-type PKCβII restored eIF4E phosphorylation, and this effect was enhanced upon tissue-type plasminogen activator (TPA) treatment (Figure 5I). Moreover, introduction of wild-type RACK1 moderately enhanced PKCβII-mediated eIF4E phosphorylation, while a contrary effect was observed in the DE mutant–transfected cells (Figure 5J).

We also examined the effect of MNK inhibition on RACK1-induced eIF4E phosphorylation in HCC. Treatment with the MNK inhibitor CGP57380, though it substantially suppressed the basal level of eIF4E phosphorylation in control cells, did not abolish RACK1-induced eIF4E phosphorylation (Figure 5K). Moreover, inhibition of MNK and PKCβII synergistically abrogated the eIF4E phosphorylation, both in normal and RACK1-transfected Huh7 cells (Figure 5K). These results suggest that RACK1 stimulates eIF4E phosphorylation in MNK-independent manner and that MNK and PKCβII are both involved in the regulation of eIF4E phosphorylation in vivo.

Ribosomal RACK1 preferentially promotes the translation of potent growth and survival factors. While most cellular mRNAs require only minimal eIF4E to be efficiently translated, elevated eIF4E activity preferentially enhances translation of select mRNAs with lengthy G + C-rich 5′-UTRs, many of which encode potent growth and survival factors, such as cyclin D1, MYC, Survivin, and BCL-2 (12–14). Western blot analysis also revealed the upregulation of cyclin D1, MYC, Survivin, and BCL-2 induced by wild-type RACK1, whereas contrary results were observed in the DE mutant–transfected or RACK1-depleted cells (Figure 6A). Real-time PCR showed no statistically significant changes in cyclin D1, *MYC*, Survivin, and *BCL2* mRNA levels in cells transfected with either construct (Supplemental Figure 11). To understand whether RACK1 modulated their protein expression at translational level, [^{35}S]-methionine incorporation assay was performed. Consistent with the alteration of protein expression, transfection of wild-type RACK1 upregulated the de novo protein level of cyclin D1, MYC, Survivin, and BCL-2, while transfection of the DE mutant or depletion of RACK1 attenuated their protein synthesis, as compared with that of β-actin (Figure 6B). Polysome profiling also showed that transfection of wild-type RACK1 induced more right shift of cyclin D1, *MYC*, Survivin, and *BCL2* mRNAs from light polysomes to heavier polysomes, in comparison with that of β-actin mRNA (Figure 6C). In contrast, the distribution of cyclin D1, *MYC*, Survivin, and *BCL2* mRNAs in the DE mutant–transfected or RACK1-depleted cells remarkably changed toward the gradient's fractions containing lighter polysomes, as compared with that of β-actin mRNA (Figure 6C).

To further evaluate the effect of ribosomal RACK1 on the 5′-UTR activity of select mRNAs, SV40-based monocistronic constructs were generated with the mRNAs' 5′-UTR to drive the translation of luciferase (Figure 7A). Overexpression of wild-type RACK1 induced more increases in the 5′-UTR activities of cyclin D1, MYC, Survivin, and BCL-2 compared with those of β-actin (Figure 7B), while, in contrast, a more inhibitory effect was observed upon transfection of RACK1 DE or shRACK1. Similar results were also observed in 7402 and PLC/PRF/5 HCC cell lines (Supplemental Figure 12). Together, these results suggest that, though ribosomal RACK1 is involved in regulation of global protein synthesis, it preferentially promotes the translation of potent growth and survival factors.

We also examined the correlation of RACK1 protein level with the expression of cyclin D1, MYC, Survivin, and BCL-2 in clinical HCC samples. As shown in Figure 7C, the protein level of RACK1 was statistically significantly correlated with the expression of cyclin D1, MYC, Survivin, and BCL-2. Taken together, these results suggest that ribosomal RACK1 may contribute to the growth and survival of tumor cells in HCC cases.

RACK1 promotes the chemoresistance of HCC in vitro in a PKCβII- and eIF4E-dependent manner. Since RACK1 synergized with PKCβII to modulate the phosphorylation of eIF4E, which is the key regulator of the translation of several potent survival factors, we next examined whether PKCβII and eIF4E were involved in RACK1-induced expression of survival factors in HCC cells. As shown in Figure 8, A and B, inhibition of PKCβII by CGP53353, or deple-

tion of eIF4E by siRNA specifically targeting eIF4E (sieIF4E), substantially blocked RACK1-induced upregulation of Survivin and BCL-2. Real-time PCR analysis showed no statistically significant changes in Survivin and *BCL2* mRNA levels in CGP53353- or sieIF4E-treated cells (data not shown). Polysome profiling further confirmed that CGP53353 or sieIF4E treatment substantially suppressed the right shift of Survivin and *BCL2* mRNAs from light polysomes to heavier polysomes induced by wild-type RACK1, while the distribution pattern of β-actin mRNA in polysomes was little or moderately affected (Figure 8, C and D). Moreover, luciferase assay also revealed that the preferential regulatory effect of wild-type RACK1 on the 5′-UTR activities of Survivin and *BCL2* mRNA was remarkably inhibited under CGP53353 or sieIF4E treatment, as compared with that of β-actin mRNA (Figure 8, E and F). These results suggest that RACK1 modulates the expression and translation of survival factors Survivin and BCL-2 in a PKCβII- and eIF4E-dependent manner.

We also examined whether PKCβII and eIF4E were involved in RACK1-mediated chemotherapy resistance in HCC in vitro. As shown in Figure 8, G and H, inhibition of PKCβII, or depletion of eIF4E, attenuated RACK1-mediated doxorubicin resistance. In addition, administration of cycloheximide, an inhibitor of translation elongation, or rapamycin, an inhibitor of cap-dependent translation, also abolished RACK1-mediated chemoresistance in vitro (Figure 8, I and J). It is consistent with previous reports that inhibiting translation can modulate the response of transformed cells to chemotherapeutic drugs (16–19, 47, 48). Taken together, these results imply that translational stimulation is involved in the drug resistance of HCC mediated by RACK1 in vitro and that RACK1 promotes the survival of HCC in a PKCβII- and eIF4E-dependent manner.

Discussion

HCC is a major health problem worldwide, with more than 500,000 new cases currently diagnosed yearly, and our previous investigations have elucidated possible mechanisms for HBV-associated hepatocarcinogenesis (49–51). We herein demonstrate that RACK1, a scaffold protein highly expressed in normal liver and frequently upregulated in HCC, contributes to the in vitro chemoresistance and in vivo growth of HCC and preferential translation of potent growth and survival factors. Our data support the fact that normal liver cells display a high rate of protein synthesis and mitosis and imply that the robust machinery of translation in normal liver may potentiate the growth and survival of HCC.

Translational control plays a crucial role in cancer development and progression, directing both global protein synthesis and selective translation of specific mRNAs that promote tumor survival and growth (6, 7). Altered levels and activities of translation initiation factors, which contribute to aberrant translational control, have been reported in a wide range of cancers (7). In this study, we observed that RACK1, which was involved in the regulation of eIF4E phosphorylation through PKCβII, was frequently upregulated in HCC. However, we did not detect remarkable expression alteration of the translation initiation factors (eIF2α, eIF3, eIF4A, eIF4E, and eIF6) in our clinical samples, suggesting that translational control of cancer is multifaceted and may be unique to different types of cancers (our unpublished observations). Previous results as well as our results suggest eIF6 and eIF4E as the substrates of PKCβII (36); however, since the ribosomal position of RACK1 shows that the WD repeats responsible for receptor/kinase binding are exposed to the solvent (40), other ribosomal substrates of RACK1/PKCβII complex may exist.

Though RACK1 participates in multiple biological processes, the role of ribosome-associated RACK1 has not been extensively defined. A previous report suggested that ribosomal RACK1/PKCβII phosphorylated eIF6 to induce its release from 60S subunit and allow 80S ribosome assembly (36). Recent studies demonstrate that ribosomal RACK1 is a component of stress granules and responsible for stress-mediated chemotherapy resistance (35, 52). Interestingly, many mRNAs that contain IRESs are sensitive to stress and encode proteins critical to cell growth, differentiation, and survival, suggesting that IRES-mediated translation provides means for selective translation of specific mRNAs and allows the regulation of cell death and survival upon stress (11, 53, 54). Our results demonstrate that ribosomal RACK1 promoted the activity of ECMV IRES (Supplemental Figure 6, C and E), and the possibility that IRES-mediated translation is involved in the progrowth and prosurvival effect of ribosomal RACK1 cannot be excluded. Our data demonstrated that ribosomal RACK1 modulated tumor growth in vivo (Figure 3, A–D), which could possibly in part be explained by the regulatory effect of ribosomal RACK1 on the translation and expression of the potent growth factors cyclin D1 and MYC (Figure 6, A–C). Moreover, since TUNEL staining indicated that the DE mutant xenografts displayed a higher basal rate of apoptosis than other 2 groups (though not statistically significant), it is likely that enhanced tumor cell apoptosis in vivo was also involved in the growth defect of the DE mutant xenografts (Figure 3, C–F). Whether ribosomal RACK1 modulates cellular survival of HCC in vivo needs further investigation.

eIF4E is a potent oncogene whose activity correlates with the phosphorylation on serine 209; phosphorylated eIF4E promotes tumorigenesis primarily by suppressing apoptosis (55). Previous research has demonstrated that the phosphorylation of eIF4E on serine 209 is tightly regulated by MAPK/ERK/MNK signaling (42) and that MNK1 and MNK2 are essential for constitutive and inducible phosphorylation of eIF4E in mice (46). This is also observed in our results: *Mnk1*$^{-/-}$*Mnk2*$^{-/-}$ MEFs displayed undetectable levels of phosphorylation of eIF4E (Figure 5, I and J). However, transfection of PKCβII restored the phosphorylation of eIF4E in *Mnk1*$^{-/-}$*Mnk2*$^{-/-}$ MEFs, suggesting PKCβII also as a physiological kinase for eIF4E in vivo (Figure 5I). We assumed that MNKs are predominant kinases for eIF4E under normal conditions, and, with the elevation of RACK1 expression and/or PKCβII activity, eIF4E might become more accessible to RACK1/PKCβII complex as a substrate. It should also be noted that the undetectable expression of PKCβII is observed in several kinds of cell lines (56) as well as in the *Mnk1*$^{-/-}$*Mnk2*$^{-/-}$ MEFs (Figure 5, I and J), and how RACK1 acts on the translational machinery other than through PKCβII is unknown and needs further investigation. In addition to phosphorylation, eIF4E is also regulated at the level of availability controlled by AKT/mTOR/4E-BP pathway (43), which plays a pivotal role in pathogenesis of HCC (57). Though RACK1 has been reported to be involved in the regulation of ERK and AKT activation (58–60), introduction of RACK1 in HCC exhibited little effect on ERK/MNK and AKT/4E-BP phosphorylation (Supplemental Figure 9 and our unpublished observations). Our results suggest that aberrant expression of RACK1 in HCC contributes to vigorous protein synthesis through functioning on ribosome, and the underlying mechanism that regulates the binding of RACK1 to ribosome needs further investigation.

Currently, there is no standard treatment for unresectable HCC. Transarterial chemoembolization, which is widely used in nonsurgical cases, shows benefits in survival for patients with preserved liver function and absence of extrahepatic spread (61, 62). In recent years, sorafenib, a multikinase inhibitor that blocks Raf signaling as well as that of VEGF, PDGF, and c-kit, has shown antiproliferative and antiangiogenic activity and increased the median overall survival from 7.9 to 10.7 months (63, 64). Moreover, among patients with advanced HCC, treatment with sorafenib plus doxorubicin, compared with doxorubicin monotherapy, resulted in greater median time to progression, overall survival, and progression-free survival (65). Therefore, it has great potential in developing new agents and strategies for this group of patients. Our data suggest RACK1 as a new biomarker to establish the risk and prognosis of HCC and to help in the selection of therapeutic modalities in clinical practice and propose a strategy to target RACK1 as a potential adjuvant therapy in combination with other methods for HCC treatment.

Methods

Patients and tissue samples. Tumor tissues from 162 primary HCC cases, surgically resected at Huashan Hospital of Fudan University (Shanghai, China), were fixed in 10% formalin and embedded in paraffin. Fresh HCC tissues and their peripheral nontumor tissues after surgical resection were collected from patients with primary HCC at Nantong Tumor Hospital (Nantong University, Jiangsu, China). None of the patients received clinical treatment before surgery. The frozen normal tissues were also collected in the Nantong Tumor Hospital (Nantong University, Jiangsu, China).

Cell lines and reagents. See the Supplemental Methods for details.

Real-time PCR analysis. See the Supplemental Methods for details.

Plasmid construction, RNA interference, and virus packaging. The human RACK1 cDNA was a gift from Jean-Luc Parent (Université de Sherbrooke, Sherbrooke, Québec, Canada). See Supplemental Methods for more details.

Evaluation of cell apoptosis. See the Supplemental Methods for details.

Oligo-dT pull-down. Oligo-dT pull-down assay was performed as described previously (36). Briefly, equal amounts of transfected cells were collected and lysed in hypotonic buffer, and lysates were incubated with oligo-dT tablets (Invitrogen) for 1 hour at room temperature. After incubation, mixtures were pelleted, washed, resuspended in Laemmli buffer, and analyzed.

Coimmunoprecipitation and GST pull-down assay. See the Supplemental Methods for details.

M^{7}GDP affinity chromatography. See the Supplemental Methods for details.

TAT-peptide delivery. The plasmid expressing TAT-RACK1 peptide (TAT-RP) was constructed by inserting the coding sequence of RACK1 (aa 94–136), which mimics the binding site of RACK1 for PKCβII, into pET22b-PTD vector, as described previously (21). The plasmid expressing TAT–control peptide (TAT-CP) was constructed by inserting the coding sequence of RACK1 (aa 270–317), which is not required for RACK1 binding to PKCβII, into pET22b-PTD. Recombinant proteins were expressed in *Escherichia coli* BL21 cells and purified using an Ni-NTA agarose column (Qiagen). For suppressing RACK1/PKCβII interaction, Huh7 cells were treated with the TAT peptides at the concentration of 10 μg/ml for 4 hours before further analysis.

[^{35}S]-methionine incorporation assay. See the Supplemental Methods for details.

Polysome profiles. Polysome profiling was carried out as described previously (36). Briefly, Huh7 cells were harvested and lysed in lysis buffer (5 mM Tris-HCl [pH 7.4], 4 mM $MgCl_2$, 1.5 mM KCl, 1 mM DTT, 1% Triton X-100, 0.5% sodium deoxycholate) in the presence of 100 μg/ml cycloheximide. The lysates were clarified, loaded onto 10%–50% sucrose gradients, and ultracentrifuged. For Western blot analysis, equal volumes of fractions were precipitated with trichloroacetic acid. For real-time PCR analysis, RNA was isolated as described previously (66). Briefly, individual fractions were digested with proteinase K, followed by phenol/chloroform extraction, and total RNA was recovered by ethanol precipitation. Equal volumes of RNA from each fraction were used to generate cDNA, followed by real-time PCR analysis.

In vitro phosphorylation assay. See the Supplemental Methods for details.

Luciferase activity assay. See the Supplemental Methods for details.

The MNK1/2 double-knockout MEFs. The MNK1/2 double-knockout mice were gifts from Rikiro Fukunaga (Osaka University, Osaka, Japan) and have been described previously (46). MEFs were prepared from 13.5-days postcoitus embryos as described previously (67).

Tumor xenograft experiments. See the Supplemental Methods for details.

Immunohistochemical staining and scoring. See the Supplemental Methods for details.

TUNEL assay. See the Supplemental Methods for details.

Statistics. Results are presented as mean ± SD. Differences between 2 groups were tested using Student's 2-tailed *t* test. One-way ANOVA was used for comparison of multiple groups. Two-way repeated-measures ANOVA was used to assess the statistical significance of 2-factor interactions. Pearson's correlation analysis was used to determine the correlation between RACK1 and cyclin D1, MYC, Survivin, or BCL-2. Survival data were analyzed with Kaplan-Meier test. Statistical significance was determined at the level of $P < 0.05$.

Study approval. The use of human tissue samples and clinical data was approved by the ethics committee of Fudan University. All donors were informed of the aim of the study and gave consent to donate their samples. Animal experiments were performed according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals, prepared by the National Academy of Sciences and published by the National Institutes of Health, and also approved by the ethics committee of Fudan University.

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