Cellular Signalling 53 (2019) 102–110

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Ribosomal RACK1 promotes proliferation of neuroblastoma cells independently of global translation upregulation

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ARTICLE INFO

Keywords:
Neuroblastoma
RACK1
Proliferation
Translation
Ribosomes
Cell cycle

ABSTRACT

Neuroblastoma is the most frequent solid tumor among those diagnosed during infancy and like most tumors, it is characterized by elevated rates of cell proliferation, migration and invasion. RACK1 is among the top 10 genes identified for unfavorable prognosis at 5 years in neuroblastoma cases and its depletion negatively affects proliferation, invasion and migration. Here, we show that the ribosomal localization of RACK1 modulates the proliferation of SH-SY5Y neuroblastoma cells by regulating the expression of cell cycle genes, such as Cyclin D1, D3 and B1 independently of global translation increase. Ribosomal RACK1 is not involved in general protein synthesis, which is instead dependent on total RACK1 and PKC but independent from mTOR. Thus, ribosomal RACK1 may represent a new target to develop more efficient therapies for neuroblastoma treatment.

1. Introduction

Neuroblastoma is among solid tumors with the highest incidence during infancy [1]. Despite remarkable advances in therapeutic protocols and basic research investigations, the cellular and molecular mechanisms triggering the onset and progression of neuroblastoma still remain elusive. As for other tumors, the major hallmarks of neuroblastoma are the extraordinary high rates of cell proliferation, invasion and migration [2].

Receptor for Activated C-Kinase 1 (RACK1) is expressed in all tissues [3]. RACK1 has been initially isolated as a protein scaffold for activated PKCβII [4]. Subsequently, many other proteins and kinases have been found to interact with RACK1 through its seven WD domains [5–9]. With such a broad interactome, RACK1 influences numerous cellular processes, such as proliferation, adhesion and migration [10]. Moreover, the interaction with elements regulating apoptosis [11] and autophagy [12] allows RACK1 also to participate in the modulation of these cellular processes. In addition, RACK1 has also been isolated from 40S ribosomal subunits [13,14]. On ribosomes, RACK1 acts by recruiting several mRNA-binding proteins (RBPs), such as ZBP1 [15] and TDP-43 [16], components of RISC, such as AGO-1 [17] and kinases, such as PKC [18], Src [15] and JNK [19]. In so doing RACK1 contributes both to the translation of specific RBP-bound mRNAs [20] and to the control of de-novo polypeptide synthesis [19]. Moreover, emerging evidence reports that RACK1 participates in global translation [21]. In RACK1 heterozygous mice it was shown that RACK1 modulates global translation through PKCβII interaction, independently of mTOR [3] and in hepatocellular carcinoma, this modulation was shown to require the phosphorylation of eIF4E by PKCβII [22]. RACK1 seems also to collaborate in the translation of both viral IRES-mRNAs [23], and CAP-dependent mRNAs.

Studies on RACK1 expression revealed high RACK1 protein levels in various tumor tissues and a strong correlation between RACK1 expression and pathology progression [10,24]. These findings led many scientists to investigate on the role of RACK1 in tumors. As expected, the main cellular processes affected by modulation of RACK1 expression in oral, breast and lung cancers are proliferation, adhesion, migration and invasion [25–27]. Not surprisingly, migration and proliferation are influenced by differential expression of RACK1 also in neuroblastoma, in which RACK1 is among to the top 10 genes identified for unfavorable 5-year prognosis [28,29]. However, all the above-listed effects on cellular processes are related to down or up-regulation of total RACK1 abundance and do not account for any differential contribution of ribosomal and non-ribosomal RACK1. So far, in hepatocellular carcinoma cells it was found that the ribosomal localization of RACK1 promotes chemoresistence and cellular growth [22]. In other

Abbreviations: RACK1, Receptor for Activated C Kinase 1; PKC, Protein Kinase C; RBPs, RNA-binding proteins; ZBP1, *Z*-binding protein; TDP-43, TAR DNA binding protein; eIF, eukaryotic initiation factor; SUnSET, surface sensing of translation

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https://doi.org/10.1016/j.cellsig.2018.09.020
Received 17 June 2018; Received in revised form 26 September 2018; Accepted 26 September 2018
Available online 01 October 2018
0898-6568/

cell lines, depletion of RACK1 from ribosomes was shown to induce non-canonical autophagy [12]. Although these results clearly demonstrate the role of RACK1 in regulating major tumor hallmarks, the function attributed to ribosomal RACK1 in cancer has not been fully defined yet.

In this study, we investigated the effects of the ribosomal localization of RACK1 on cell proliferation in neuroblastoma cells. We found that the expression of a RACK1 mutant which is unable to bind to ribosomes leads to a decreased proliferation rate. Such effect is obtained by altering the progression through the G0/G1 phase and by specifically affecting the expression of cyclin D1, D2 and B1. Furthermore, we determined that ribosomal RACK1 is not involved in general protein synthesis regulation, which is instead dependent on overall levels of total RACK1 and on PKC, but independent of mTOR and eIF4E phosphorylation. Establishing the contribution of ribosomal-localized RACK1 to cell proliferation in neuroblastoma cells may permit the discovery of new and specific targets in order to design more efficient therapeutic strategies.

2. Materials and methods

2.1. Cell culture, transfection and stable clones

Human neuroblastoma SH-SY5Y and MCF-7 cells, obtained from the American Type Culture Collection (ATCC, Rockville, MD), were cultured in DMEM/F12 medium containing 10% FBS and antibiotics (50 U/ml penicillin and 50 mg/ml streptomycin) at 37 °C in 5% CO2. To produce stable clones overexpressing wild type RACK1 and RACK1 mutants, cells were transfected with Lipofectamine 2000 (Thermofisher) following manufacturer's instructions, with plasmids carrying cDNA encoding for wild type or mutant forms of RACK1. Wt RACK1 and the R36D/K38E mutant (termed $RACK1_{WT}$ or $RACK1_{DE}$, MRDE or MRDE) were tagged with histidine-myc at the C-terminus [22], the Y246F mutant was fused to the GFP tag (termed $RACK1_{Y246F}$) [30] while the Y302F mutant was fused to the HA tag (termed $RACK1_{Y302F}$) [31]. Transfected cells were selected using G418 500 μg/ml (Gentamycin, Sigma). SH-SY5Y and MCF-7 cells transfected with the empty vector were used as control cells (called CTR1).

To produce the RACK1 −/+ cell line (called C4 cells), SH-SY5Y cells were transfected with the CRISPR/Cas9 double-nickase plasmid (sc-400800 nic, Santa Cruz) and then selected with 1 μg/ml puromycin (Thermofisher) for 1 week. SH-SY5Y cells transfected with the empty vector were used as control cells (called CTR2).

For pharmacological treatments, serum-starved cell lines were treated with 500 nM Rapamycin for 1 h, 10 μM Enzastaurin for 48 h, PMA 80 nM or 10 μM Mnk1/2 inhibitor II ETP45835 for 1 h.

2.2. Polysomal profiling

Polysomal profiles were performed as previously described [32]. Briefly, wild type or transfected SH-SY5Y cells were lysed in polysomal buffer (10 mM Tris-HCl, 50 mM KCl, 10 mM MgCl2 and 0,5% NP-40). Total lysates were clarified by centrifugation at 14,000 r.p.m. for 5 min at 4 °C and the supernatants were loaded on continuous 15–50% sucrose gradients in 10 mM Tris-HCl, 50 mM KCl, 10 mM MgCl2 or 10 mM EDTA. After ultracentrifugation (Beckman) at 37,000 rpm for 2 h at 4uC, sucrose gradients were collected in fractions and the profiles were obtained by total RNA analysis at 254 nm in a Bio-rad Biologic LP. Half the amounts of the collected fractions were used to precipitate proteins with 10% trichloroacetic acid (TCA) and were then used for immunoblotting as reported below.

2.3. Histidine pull-down

The pull down was performed as previously published [16]. Briefly, cells were lysed and protein extracts were clarified as described above. Extracts were then incubated 2 h with a nickel affinity resin (BioRad), pre-equilibrated with polysomal buffer. The resin was then extensively washed with 20 mM Imidazole dissolved in polysomal buffer and finally eluted with 500 mM Imidazole in polysomal buffer. The eluted proteins were then precipitated with 10% TCA, as described for for polysomal profiles and used in immunoblotting as reported below.

2.4. Immunofluorescence and antibodies

Immunofluorescence staining was performed as previously described [16]. Briefly, after two rinses with PBS 1×, SH-SY5Y and embryonic primary cells were fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature, then rinsed again with PBS and permeabilized with PBS-Triton-X 0.5% for 10 min at room temperature. After blocking in PBS-BSA 2% at 37 °C for 30 min, cells were incubated for 30 min at 37 °C with mouse monoclonal anti-RACK1primary antibodies (dissolved in blocking solution) (BD Biosciences, 1:200). After washes with PBS, goat anti-mouse (Alexa Fluor® secondary antibodies, Molecular Probes), dissolved 1:500 in PBS-DAPI were incubated for 30 min at 37 °C in the dark. After mounting with 80% glycerol, cells were visualized by confocal microscopy on a Zeiss LSM-710 microscope; images were captured by a Zeiss EC Plan-Neofluar 40×/1.30 Oil DIC M27 objective, using the "ZEN" software. Images were processed using ImageJ and Adobe Photoshop.

2.5. SUnSET

The rate of protein synthesis was measured using the SUnSET assay [16]. Briefly, cells were treated for 10 min with 5 μg/ml of puromycin (Thermofisher), then lysed and subjected to immunoblotting analysis as described below.

2.6. Immunoblotting and antibodies

SDS-PAGE was performed on protein extracts obtained with lysis buffer (Tris-HCl 0,01 M pH 7.6, NaCl 0,1 M, MgCl 0,01 M, Triton 0.1%). Cells were washed two times with PBS 1×, the lysis buffer was then added and cells were lysed with a scraper. The lysate was clarified by centrifugation at 14.000 r.p.m. for 5 min at 4 °C. Protein concentration was determined with BCA analysis (Thermo Fisher Scientific). Equal amounts of proteins were loaded on each lane and separated on a 12% polyacrylamide gel, then transferred on a PVDF membrane. Membranes were blocked in 5% Bovine Serum Albumin in PBS1X with Tween (0.01%) for 30 min at 37 °C. The following primary antibodies were used: mouse anti-RACK1 (BD Biosciences, 610178, 1:2000), mouse anti-β-actin (Sigma, A2228, 1:1000), rabbit polyclonal anti-rpS6 (Cell Signaling, 5610, 1:1000), mouse anti-puromycin (Millipore, MABE343, 1:10000), rabbit polyclonal anti-4E-BP1 (Cell Signaling, 9452, 1:1000), rabbit anti-eIF4E (Cell Signaling, 9742, 1:1000), rabbit polyclonal anti-phospho-eIF4E (Cell Signaling, 9741, 1:1000), rabbit anti-phospho-ERKs (Cell Signaling, T202/Y204, 1:2000), rabbit polyclonal anti-phospho-Mnk1 (Cell Signaling 1:1000), rabbit polyclonal anti-cyclin B1 (Abcam, 1:500), rabbit polyclonal anti-cyclin D1 (Abcam, 1:1000), rabbit anti-phospho-AKT (Cell Signaling, 1:1000), rabbit anti-cyclin D3 (Millipore 1:1000), monoclonal anti-myc 9B11 (Cell Signaling, 1:5000), monoclonal anti-HA (SIGMA, 1:1000), polyclonal anti-GFP (Thermofisher, 1:500) and monoclonal anti-PKCβII (Santa Cruz, 1:1000). Secondary HRP-conjugated anti-mouse or anti-rabbit antibodies and ECL reagent (1:5000, GE Healthcare) were used. For RACK1 detection, secondary mouse HRP-conjugated anti-IgM (1:5000, Sigma) was used.

2.7. MTT assay

The MTT assay was performed according to the protocols previously described [16]. A total of 15,000 cells for each genotype were plated in

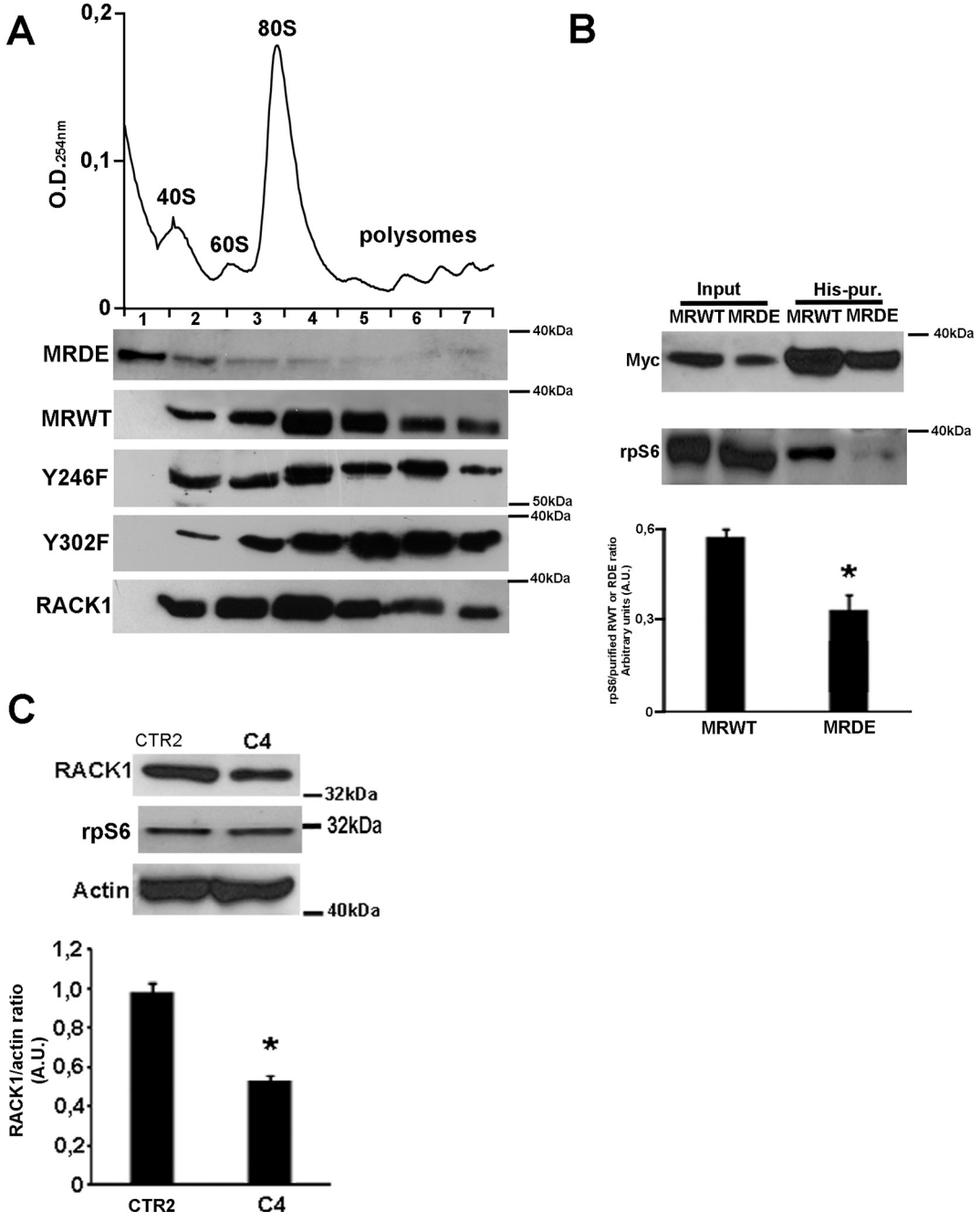

Fig. 1. The DE mutation reduces RACK1 ribosomal localization. (A) Polysomal profiles (top) are not affected by the overexpression of $RACK1_{WT,}$ $RACK1_{DE}$ $RACK1_{Y246}$ and $RACK1_{Y302F}$. Immunoblotting with anti-Myc antibodies to identify $RACK1_{WT}$ or $RACK1_{DE}$, with anti-GFP antibodies to detect $RACK1_{Y246,}$ with anti-HA antibodies to identify $RACK1_{Y246}$ and with anti- RACK1 antibodies to detect endogenous RACK1, which was also used as ribosomal marker (bottom). (B) Immunoblotting for Myc and rpS6 on imidazole eluted proteins from histidine pull-downs (C) Western blot for endogenous RACK1, rpS6 and actin on SH-SY5Y and C4 cell lines. Densitometry values for proteins are normalized on actin levels. Bar graphs represent means ± S.D. of three independent experiments; * $p \leq 0.05$ vs CTR as determined by Student's t-test.

0.2 ml in 96-well flat bottom plates. At the indicated times, 20 µl of 5 mg/ml MTT solution in PBS were added to each well for 4 h. After removal of the medium, 170 µl of DMSO were added to each well to dissolve the formazan crystals. The absorbance at 540 nm was determined using a Biokinetics plate reader (Bio-Tek Instruments Inc., Winooski, VT, USA). Triplicate wells were assayed for each condition and S.D. was determined.

2.8. Cytofluorimetric assay

Cells were seeded the day before the assay on 10 cm plates at 70% confluence. After 24 h cells were harvested with 0,05% Tripsin-EDTA solution and washed with PBS 1×. Suspended cells were fixed in 70% ethanol in PBS1× at −20 °C for 1 h. After ethanol removal, cells were then washed twice with PBS 1×. Cells were then incubated with 50 µg/ml Propidium Iodide (PI), 0,1 µg/ml RNase and 0,05% Triton for 30 min at room temperature. After PI treatment cells were washed and re-suspended in PBS 1× and then analyzed by flow cytometry.

2.9. Cell counting

20.000 cells were seeded in 24-well plates in DMEM F12 with 10%

FBS. After 7 days cells were harvested and counted using a Neubauer chamber. All counts were performed in triplicate.

2.10. Colony formation assay

300 cells were seeded in 6-well plates in DMEM F12 with 10% FBS. After 15 days cells were fixed at room temperature with PFA 4% for 10 min. After fixing cells were stained with 0,01% crystal violet in H2O for 30 min. The excess of crystal violet was washed, and colonies were then counted.

2.11. Protein turn-over

SH-SY5Y overexpressing $RACK1_{WT}$ and $RACK1_{DE}$ were treated with 10 mg/ml Puromycin for 1 and 4 h and then lysed as previously described. The immunoblotting for myc and actin were conducted as previously described.

3. Statistical analysis

All grouped data are presented as mean ± S.D. Differences between groups were analyzed using Student's t-test. P values < 0.05 were considered statistically significant. All experiments were repeated at least twice with three technical replicates.

4. Results

4.1. Ribosomal RACK1 controls the proliferation of neuroblastoma cells

To examine whether the depletion of RACK1 from ribosomes affected cell proliferation, the phenotype of SH-SY5Y human neuroblastoma cells overexpressing a RACK1 R36D/K38E mutant allele unable to associate with ribosomes (termed MRDE) [22] was compared to that of cells overexpressing either wild type RACK1 (termed MRWT) or other RACK1 mutants, including $RACK1_{Y246F}$ [30] and $RACK1_{Y302F}$ [31] (Supplemental Fig. 1A). As reported [16,22] the DE mutations affected RACK1 ribosomal localization, as shown by immunoblotting on ribosomal fractions collected through polysomal profiling (Fig. 1A). In contrast, all the other mutations did not affect RACK1 localization on the translational machinery (Fig. 1A and [32]). To further confirm that the DE mutant impaired the association of RACK1 with the translational machinery, $RACK1_{DE}$ binding with ribosomal proteins was investigated through a co-purification assay on histidine pull-downs. Immunoblotting for ribosomal protein S6, rpS6, on imidazole-eluted protein extracts of $RACK1_{WT}$ or $RACK1_{DE}$ pull-downs revealed that the amount of rpS6 co-purifying with $RACK1_{DE}$ was reduced when compared to that co-purifying with $RACK1_{WT}$ (Fig. 1B). In addition, the immunoblot in Fig. 1B shows a reduced expression of $RACK1_{DE}$ when compared to $RACK1_{WT}$. Experiments on protein stability using 10 mg/ml puromycin revealed that, in the short term,$RACK1_{DE}$ was less abundant than $RACK1_{WT}$. This suggests that the $RACK1_{DE}$ protein is less stable than $RACK1_{WT}$ (Supplemental Fig. 1B). Despite $RACK1_{DE}$protein instability, this might only partially affect RACK1 ribosomal localization.

Therefore, taken together these results indicate that DE mutations specifically inhibit the ribosomal localization of RACK1.

To further examine the role of ribosomal RACK1 in cellular processes, the phenotype of $RACK1_{DE}$ cells was also compared to that of cells downregulating RACK1. To delete RACK1, the CRISPR-CAS9 Nickase method was used. Unfortunately, after puromycin selection, no RACK1 knockout cells were obtained, although, prior to selection, a small number of RACK1-depleted cells was present, as revealed by immunofluorescence analysis (Supplemental Fig. 1C). Thus, for this study, we decided to use a cell line with downmodulation of about half the amounts of endogenous RACK1 (called C4 in Fig. 1C). Since RACK1 is a component of the 40S ribosomal subunit, we checked whether RACK1 downregulation affected the levels of 40S ribosomal proteins. Immunoblotting on C4 cells showed that the amount of rpS6 was unaffected by RACK1 downregulation (Fig. 1C), suggesting that 40S ribosomal protein composition was unchanged.

Since cell proliferation was shown to be affected by RACK1 protein levels, we initially examined the increase in cell number after 5 days of culture. Overexpression and down-regulation of RACK1 affected positively or negatively, respectively, the overall number of cells compared to the control (Fig. 2A). Intriguingly overexpression of $RACK1_{DE}$ determined a reduction of cell proliferation comparable to that caused by downregulation of RACK1 (Fig. 2A), while RACK1 Y246F and Y302F mutations increased the proliferation rate when compared to controls (Supplemental Fig. 2A). In particular, $RACK1_{Y302F}$ cells proliferated more than those carrying the Y246F substitution. To exclude that the effects induced by the DE mutant might be cell specific, we tested the proliferation rate in MCF-7 cells overexpressing $RACK1_{WT}$ or $RACK1_{DE}$. As expected, MCF-7 cells overexpressing $RACK1_{WT}$ proliferated more than control cells. (Supplemental Fig. 2B). In line with the results obtained in SH-SY5Y cells, overexpression of $RACK1_{DE}$ in MCF-7 cells caused a reduction in cell number compared to $RACK1_{WT}$ and control cells (Supplemental Fig. 2B).

Since cell proliferation was affected by the DE mutations, we further explored this effect performing a colony formation assay in $RACK1_{DE}$ overexpressing cells. In accordance with the results on cell proliferation, overexpression of $RACK1_{WT}$ promoted the formation of colonies (Fig. 2B), while, on the contrary, both the downregulation of RACK1 and the overexpression of the DE mutant inhibited the colony-forming capacity of cells. Next, we determined how ribosomal RACK1 depletion inhibited cell proliferation analyzing the cell cycle by flow cytometry. 28,4% of control cells were detected in the G0-G1 phase, while 6,9% and 4,5% were found in the S- and G2-M phases, respectively (Fig. 2C). Up-regulation of $RACK1_{WT}$ caused a reduction in the number of cells in the G0-G1 and G2-M phases but an increase in the percentage of cells in S-phase. Overexpression of the RACK1 MRDE mutant reduced cell proliferation just like suppression of RACK1 (Fig. 2C) and was accompanied by impaired progression through the S and G2/M phases of the cell cycle. To exclude that the decrease in proliferation could be ascribed to a toxic effect of RACK1 delocalization from the ribosome, an MTT assay was performed after 48 h of cell culturing. As shown in Fig. 1D, $RACK1_{WT}$ overexpression slightly improved cell viability, while both downmodulation of RACK1 and its depletion from the ribosome exhibited only a minimal effect, indicating that no toxic effect occurred in these cells. Therefore, these results suggest that RACK1 may control cell proliferation through its ribosomal localization.

4.2. A specific PKCβII/RACK1 axis stimulates global translation independently of RACK1 association with the ribosome

Since ribosomal RACK1 is involved in protein synthesis, we examined whether the proliferation defect seen in $RACK1_{DE}$ overexpressing cells correlated with alterations of global translation. To examine this possibility, the rate of protein synthesis was measured using the SUnSET assay. Immunoblotting for puromycilated proteins showed that cells overexpressing $RACK1_{WT}$ incorporated more puromycin than control cells (Fig. 3A), while cells downmodulating RACK1 incorporated less puromycin compared to the control (Fig. 3B). Interestingly, cells overexpressing $RACK1_{DE}$ revealed a high level of puromycilated proteins which was comparable to that detected in cells overexpressing $RACK1_{WT}$ (Fig. 3A). The finding that $RACK1_{DE}$ overexpression increases general protein synthesis, induced us to postulate that this event may be a prerogative of total RACK1 up-regulation. To investigate this hypothesis, we performed the SUnSET assay also in cells overexpressing the Y246F and Y302F RACK1 mutants. The SUnSET experiment revealed that both mutants incorporated more puromycin than control cells (Supplemental Fig. 3A) suggesting that the overexpression of these mutant proteins stimulated global translation just like the overexpression of $RACK1_{WT}$ and $RACK1_{DE}$. In addition, we

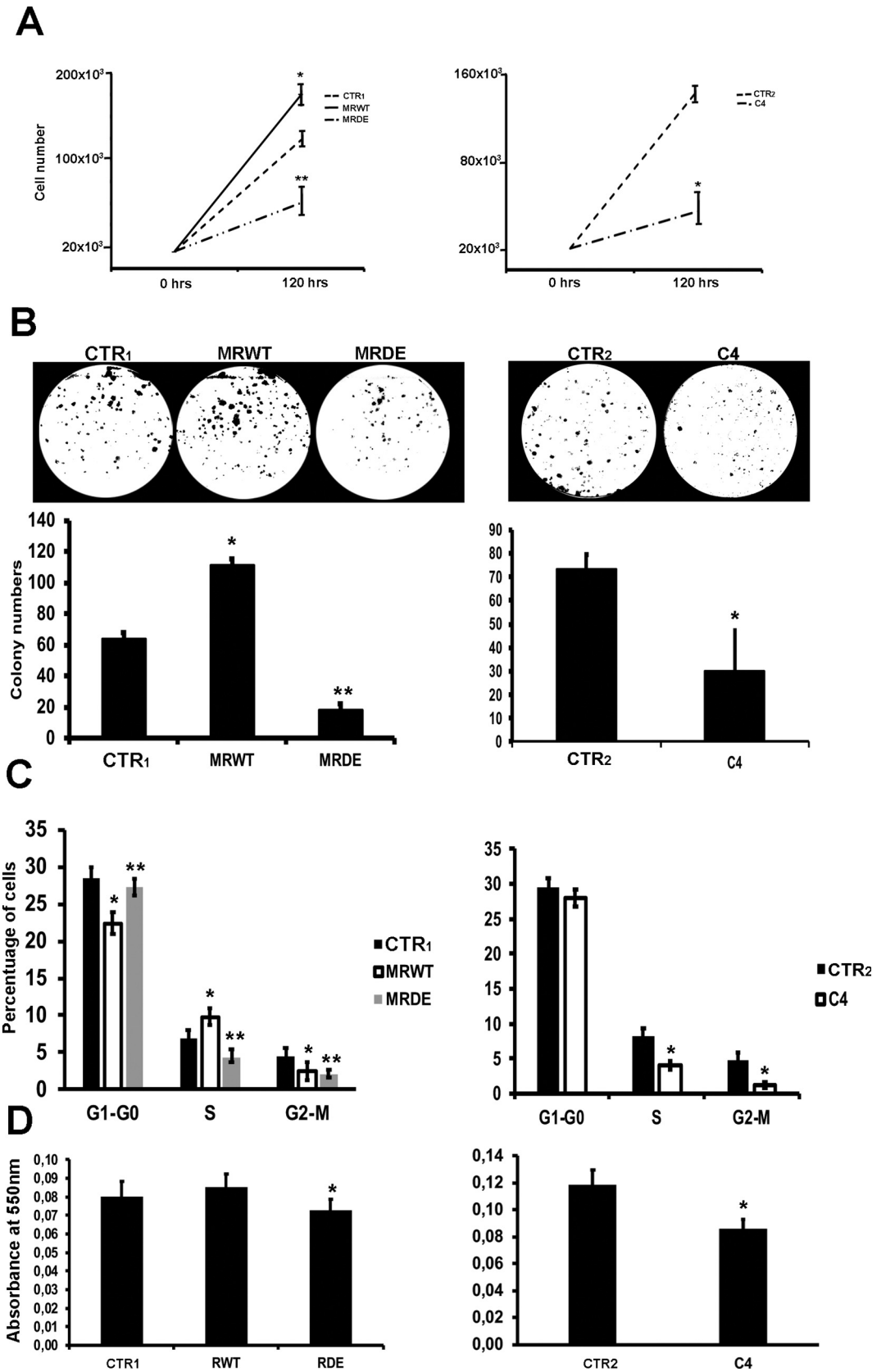

Fig. 2. Ribosomal RACK1 controls cell proliferation. (A), Overexpression of the $RACK1_{WT}$ (MRWT) protein enhances cell proliferation (left) while RACK1 down-regulation (C4 clone, right) causes a decrease in cell proliferation. The $RACK1_{WT}$ (MRDE) cell line (left) behaves like the C4 cell line and shows a decrease in cell proliferation. (B), In the colony formation assay, overexpression of RACK1 (left) increases the capacity of cells to form colonies. Expression of the DE mutant protein (left) causes a reduction in the capacity of cells to form colonies and the same effect is seen in the C4 cell line (right). (C), flow cytometry reveals that the overexpression of $RACK1_{WT}$ causes an increase in the percentage of cells in S phase (left), whereas MRDE (left) and C4 (right) cells show an arrest in the G0/G1 phase. (D), the MTT assay reveals that both overexpression of the DE mutant (left) and depletion of total RACK1 (right) affect cell viability. In all experiments, bar graphs represent means ± S.D. of three independent experiments; * $p \leq 0.05$ or ** $p \leq 0.01$ vs CTR_1 or CTR_2 as determined by Student's t-test.

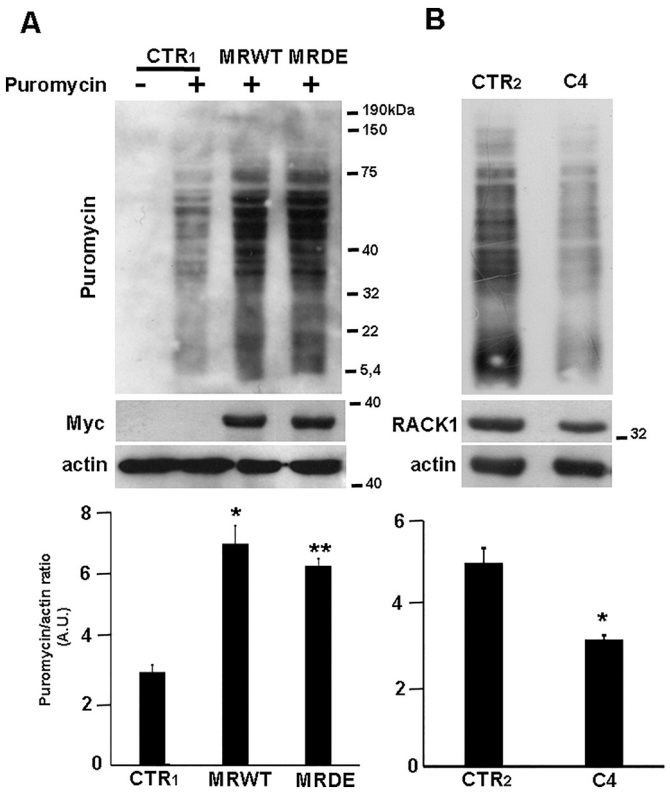

Fig. 3. Up-regulation of RACK1 induces global protein synthesis. (A), immunoblotting using anti-puromycin antibodies. Translation levels were measured as the ratio, expressed in Arbitrary Units (A.U.), between the densitometry of bands of puromycilated proteins and that of total actin. The MRWT and MRDE lysates show more puromycin incorporation than myc cells, as also shown in the bar graphs. (B), while the C4 cell line shows less puromycin incorporation compared to the control. Cells not treated with puromycin are shown (first line, −). Bar graphs represent means ± S.D. of three independent experiments; * $p \leq 0.05$ or **$p \leq 0.01$vs CTR_1 or CTR_2 as determined by Student's t-test.

measured global translation also in MCF-7 cells overexpressing $RACK1_{WT}$ or the $RACK1_{DE}$mutant, since they showed a proliferation rate similar to that of SH-SY5Y cells. The SUnSET assay revealed that overexpression of either $RACK1_{WT}$ or $RACK1_{DE}$ caused an increase in the translation rate also in MCF-7 cells (Supplemental Fig. 3A). Thus, all together, these results indicate that ribosomal RACK1 is not essential for the proceeding of global translation, an event which is surprisingly enhanced by up-regulation of total RACK1.

Discovering that increased levels of total RACK1 caused an upregulation of overall translation prompted us to investigate on how this phenomenon was occurring. Recently, different authors found that RACK1 stimulates translation through the AKT/mTOR pathway [27,33], while others suggested that RACK1 promotes protein synthesis through PKC [21]. To establish which pathways were modulated by RACK1 in neuroblastoma cells, we initially investigated the activation of AKT and mTOR kinases. By immunoblotting for phosphorylated AKT we showed that the up or downregulation of RACK1 caused no alteration to the phosphorylation status of AKT (Supplemental Fig. 4A and B). Similarly, up or downmodulation of RACK1 did not affect the phosphorylation of two mTOR targets, rpS6 and eIF4E-Binding protein 1, 4E-BP1, (Supplemental Fig. 4A and B). To further exclude that the up-regulation of RACK1 triggers global translation by affecting mTOR activity, we administered rapamycin, a specific mTOR inhibitor, to cells overexpressing RACK1 and we monitored the translation rate using the SUnSET assay. Rapamycin caused a dramatic decrease of protein synthesis in control cells (Supplemental Fig. 5A), but only a slight reduction in $RACK1_{WT}$ or $RACK1_{DE}$ overexpressing cells, although mTOR activity, was equally reduced in all cases, as determined by a comparable reduction of rpS6 phosphorylation (Supplemental Fig. 5A). Therefore, these results rule out the possibility that up-regulation of RACK1 can promote overall translation through the AKT/mTOR axis.

Next, we tested whether RACK1 overexpression up-regulated overall protein synthesis by controlling PKC activity. Administration of the PKC inhibitor Enzastaurin [34,35] dramatically attenuated the increase in global translation induced by $RACK1_{WT}$ and $RACK1_{DE}$ overexpression (Fig. 4). To further confirm the role of PKC in RACK1-mediated translation, we attempted to administrate PMA, a PKC stimulator, or to overexpress wild type PKCβII in C4 cells. Unfortunately, the SUnSET assay on SH-SY5Y cells revealed that both PMA administration and PKCβII overexpression failed to induce translation (Supplemental Fig. 6A and B), suggesting to exclude this type of treatment in C4 cells due to its inefficiency. Therefore, the results from the experiments with the PKC inhibitor confirmed that the PKC/RACK1 axis might control global translation.

In hepatocarcinoma cells, the PKC/RACK1 axis stimulates eIF4E phosphorylation [22]. Thus we explored whether up-regulation of RACK1 could modulate PKCβII/eIF4E phosphorylation. We initially examined the phosphorylation of eIF4E on serine 209 in cells overexpressing or downmodulating RACK1. Both the overexpression of $RACK1_{WT}$ and of $RACK1_{DE}$ induced the phosphorylation of eIF4E, while downregulation of RACK1 suppressed it (Fig. 5A and B). Enzastaurin administration failed to suppress phosphorylation of eIF4E in RACK1 overexpressing and control cells. In addition, PKC inhibition also failed to reduce the phosphorylation of ERK kinases, up-stream regulators of eIF4E phosphorylation, which were up-regulated in $RACK1_{WT}$ and $RACK1_{DE}$ overexpressing cells (Fig. 5C). Moreover, as ERK activity induces the phosphorylation of eIF4E stimulating Mnk1/2 activity [36], we investigated whether the overexpression of $RACK1_{WT}$ and $RACK1_{DE}$ promoted Mnk1/2 phosphorylation. Both $RACK1_{WT}$ and $RACK1_{DE}$ overexpressing cells showed increased Mnk1 phosphorylation (Supplemental Fig. 7). In addition, inhibiting Mnk1/2 activity by administration of the Mnk1/2 inhibitor II ETP45835 [37], caused a decrease of Mnk1 and eIF4E phosphorylation in both $RACK1_{WT}$ and $RACK1_{DE}$ overexpressing cells without affecting global translation, as previously reported in [36]. Thus, taken together, these results indicate that the up-regulation of RACK1 promotes protein synthesis through PKCβII, independently of mTOR and that eIF4E phosphorylation depends on Mnk1/2 activation.

4.3. Ribosomal RACK1 promotes the expression of cyclins

Having excluded that ribosomal RACK1 modulates global translation and phosphorylation of eIF4E, we next examined whether overexpression of the $RACK1_{DE}$ mutant caused a cell proliferation defect through specifically influencing the expression of genes involved in cell cycle regulation, such as cyclin B1, D1 and D3. Immunoblotting revealed that the levels of cyclin B1, D1 and D3 increased in $RACK1_{WT}$ overexpressing cells when compared to control cells and decreased in cells downmodulating RACK1, exception made for cyclin B1 whose levels were not altered (Fig. 6). Consistent with the above-mentioned cell proliferation defects, the overexpression of $RACK1_{DE}$ caused a reduction in the protein levels of cyclins B1, D1 and D3. All together these results demonstrate that the ribosomal localization of RACK1 controls the expression of cell cycle genes.

5. Discussion

Receptor for activated C kinase 1 (RACK1) is highly expressed in most tumors, exception made for gastric and intestinal malignancies [38]. In neuroblastoma, RACK1 has been identified among the top 10 genes selected for unfavorable prognosis at 5 years [28]. Most studies regarding the role of RACK1 in tumors are related to the expression of

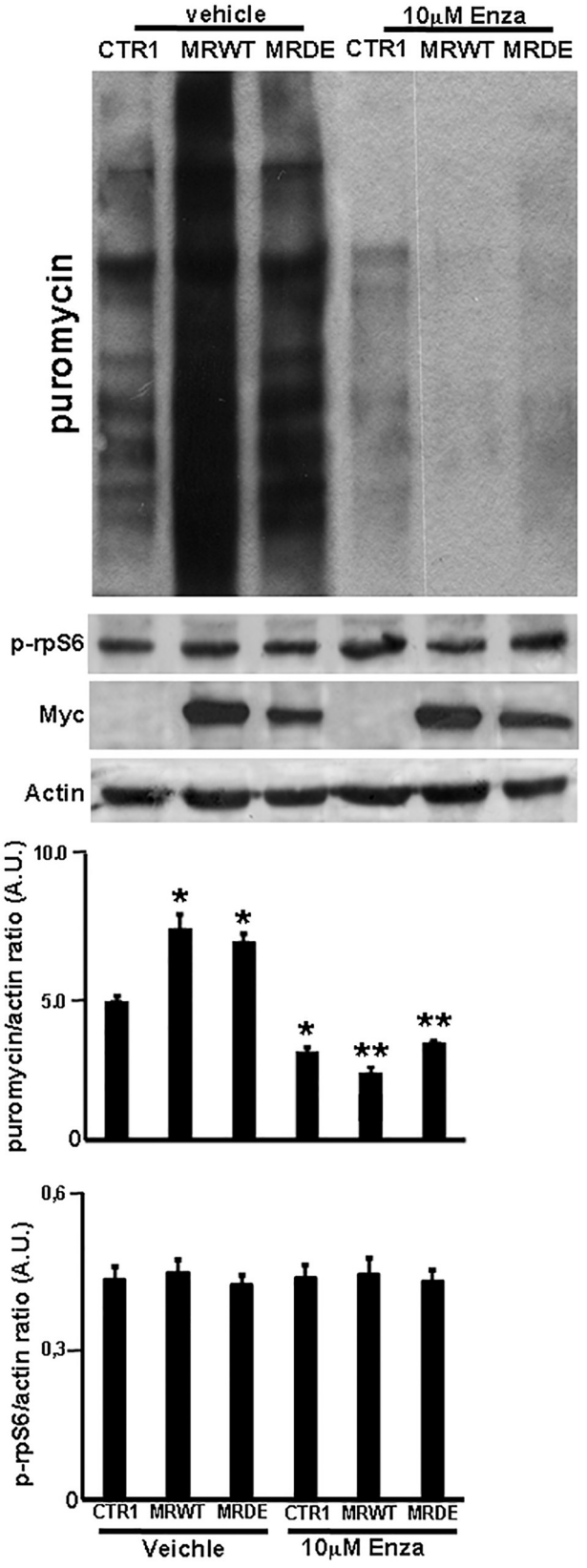

Fig. 4. RACK1 enhances the overall protein synthesis rate through PKCβII. Administration of Enzastaurin, a specific inhibitor of the PKC βII isoform, causes a decrease in protein synthesis, as measured by the SUnSET assay, in both MRWT and MRDE cell lines. Enzastaurin administration does not affect the phosphorylation of rpS6 in cells overexpressing either MRWT or MRDE. Densitometry values for phosphorylated proteins are normalized on actin levels. Bar graphs represent means ± S.D. of three independent experiments; * $p \leq 0.05$ or ** $p \leq 0.01$vs CTR_1 or CTR_2 as determined by Student's t-test.

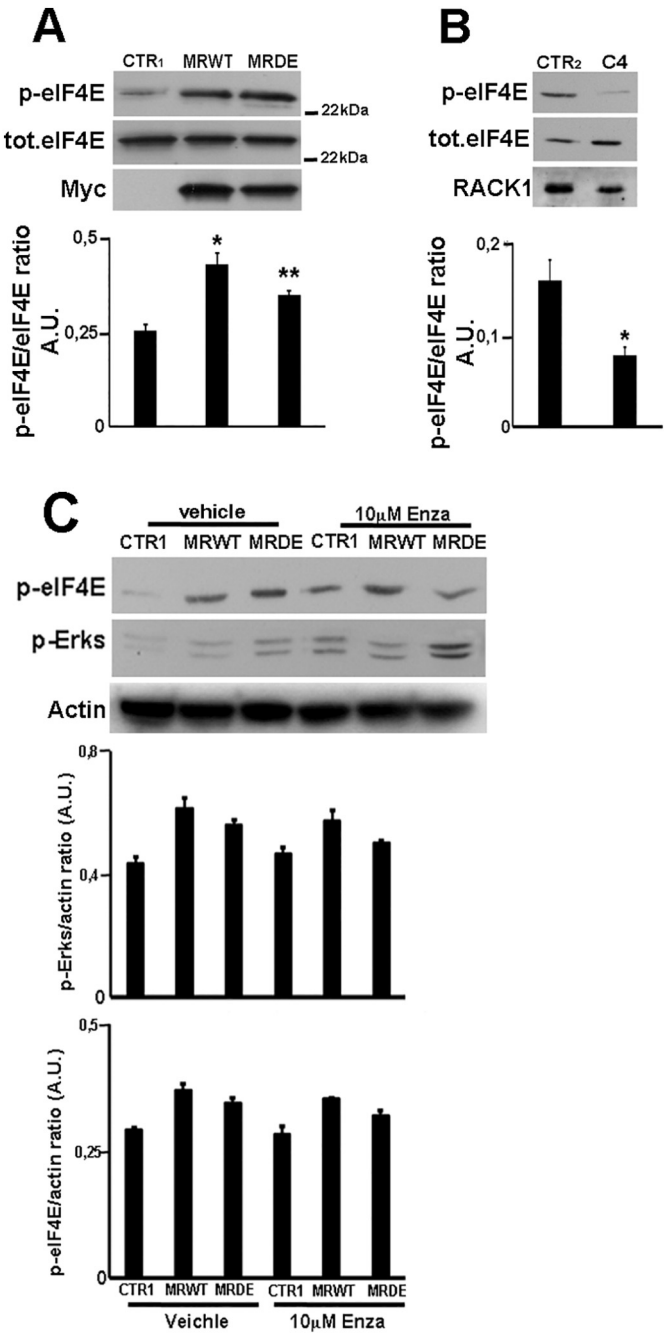

Fig. 5. eIF4E phosphorylation is stimulated by the up-regulation of RACK1. (A), overexpression of MRWT and MRDE induces the phosphorylation of eIF4E. (B), depletion of RACK1 reduces the phosphorylation of eIF4E. The values of densitometry for phosphorylated proteins are normalized on total eIF4E levels (C) Enzastaurin administration does not affect the phosphorylation of neither ERKs nor eIF4E. The values of densitometry for phosphorylated proteins are normalized on actin levels. Bar graphs represent means ± S.D. of three independent experiments; * $p \leq 0.05$ or ** $p \leq 0.01$vs CTR_1 or CTR_2 as determined by Student's t-test.

total RACK1, while the impact of the pool of RACK1 associated to ribosomes has not been fully understood. In this study, our results reveal that the ribosomal localization of RACK1 is required to control the cell proliferation of the neuroblastoma SH-SY5Y cell line. Here, we found that ribosomal RACK1 regulates the expression of cell cycle genes without affecting overall protein synthesis. This study displays that up-regulation of RACK1, independently of its ribosomal localization, stimulates global translation, which is per se irrelevant to promote

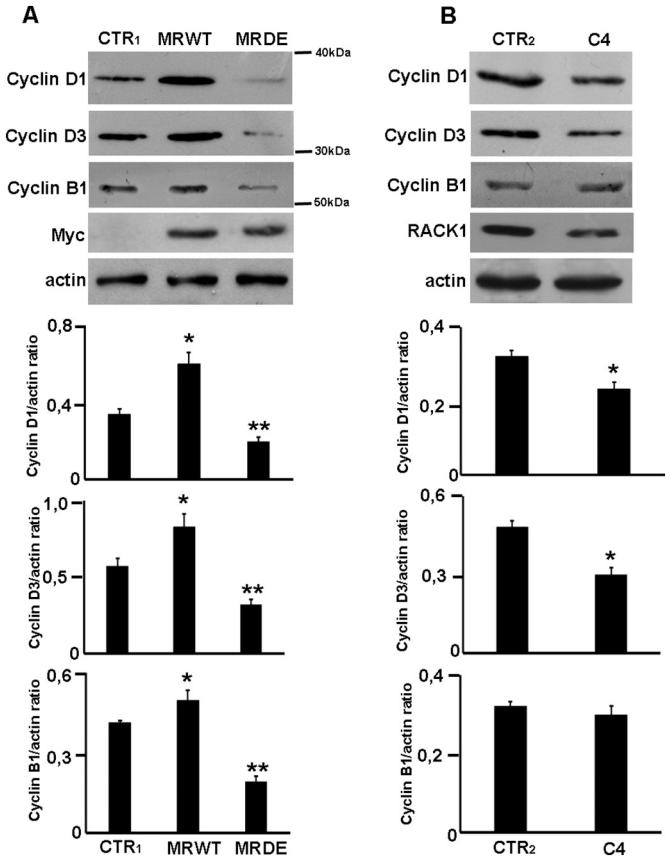

Fig. 6. Ribosomal RACK1 regulates the expression of cyclin genes. (A) cyclins D1, D3 and B1 are upregulated in MRWT cells and downregulated MRDE mutant cells (B), only cyclin D1 and D3 are downregulated in the C4 cell line. Densitometry values for proteins are normalized on actin levels. Bar graphs represent means ± S.D. of three independent experiments; * $p \leq 0.05$ or **$p \leq 0.01$vs CTR_1 or CTR_2 as determined by Student's t-test.

neuroblastoma cell proliferation.

To understand what cellular processes are modulated by ribosomal RACK1, we compared the effects caused by overexpressing a RACK1 mutant (RACK1 R36D/K38E), defective in ribosome binding, to those due to either up- or down-regulation of wild type RACK1 or overexpression of other mutants of RACK1, such as the Y246F and Y302F mutants. The effects of the DE mutations on RACK1 binding to the ribosomes are controversial. In *Saccharomyces cerevisiae*, Coyle et al. [39] showed that the RACK1DE mutant is defective in binding to the translational machinery. In contrast, the DE mutations do not cause delocalization of the yeast orthologue of RACK1, Asc1, from ribosomes [40,41]. As in *Saccharomyces cerevisiae*, the DE mutations affect ribosome localization of RACK1 also in mammalian cells [16,22]. From these findings, it appears that DE mutations cause a slight binding defect which renders RACK1 more prone to dissociate from the translational apparatus.

To delete RACK1, we attempted to repress its expression by using the CRISPR-Cas9 Nickase system. However, we were capable to select only cells expressing half the levels of endogenous RACK1, although, prior to selection, we actually found knocked-out RACK1 cells, demonstrating that our CRISPR system worked. Our hypothesis is that the deletion of RACK1 is lethal and therefore reconstitution of RACK1 KO clones is not achievable. This idea is supported by the effects of RACK1 deletion in mice. $RACK1^{-/-}$ homozygous mice die at gastrulation, while RACK1 heterozygotes are viable and pigmented [3]. Therefore, our results confirm that RACK1 is an essential gene.

Emerging evidence indicates that expression of RACK1 positively correlates with progression of many different tumors. Experiments in vitro on cancer cell lines demonstrated that RACK1 downregulation reduces adhesion, invasion, migration and proliferation rates [26]. In some cases, RACK1 modulates the cell cycle by affecting the duration of the G1/S- and G2/M phases [42]. In other cases, such as in neuroblastoma cells, RACK1 regulates proliferation and migration via its Src-binding site at tyrosine 416 [29]. However, so far, the function of RACK1 in cancers concerned only its non-ribosome-bound form, while the activity of ribosome-bound RACK1 was scantily investigated. Only Ruan et al. [22] demonstrated that the ribosomal localization of RACK1 confers chemoresistance and cell growth in human hepatocarcinoma cells. In this context, ribosomal-bound RACK1 recruits PKCβII on ribosomes in order to promote the phosphorylation of eIF4E which leads to translation of specific mRNAs encoding for growth factors and pro-survival proteins. In accordance with previous findings in hepatocarcinoma cells, in our study ribosome-associated RACK1 regulates the proliferation of neuroblastoma cells through the expression of cyclins D1, D3 and B1. However, the levels of cyclins are modulated by ribosomal RACK1 independently of PKCβII-dependent eIF4E phosphorylation. Indeed, the overexpression of the DE mutant in neuroblastoma diminished the amounts of cyclin D1, D2 and B1, while the general translation rate and phosphorylation of eIF4E increased at values similar to those induced by the overexpression of $RACK1_{WT}$ or other RACK1 mutant proteins. Moreover, administration of Enzastaurin, a specific inhibitor of PKCβII, reduced only the translational rate but did not affect ERK and eIF4E phosphorylation in neither $RACK1_{WT}$ nor $RACK1_{DE}$ overexpressing cells. In this context, our results on mTOR activation indicate that the up-regulation of total RACK1 did not stimulate the phosphorylation of its downstream targets, such as rpS6 and 4E-BP1, supporting previous findings which indicated that the protein synthesis promoted by RACK1 is mTOR-independent.

As mentioned before, the overexpression of $RACK1_{DE}$ caused a reduction in the expression of cyclin D1, D2 and B1. However, whether this occurs at the transcriptional or translational level has not been investigated. Recently, in neuroblastoma, ribosomal-bound RACK1 has been proposed to contribute to the translation of specific mRNAs through differential interaction with RBPs [15]. In hepatocarcinoma cells, it was reported that RACK1-induced expression of cell cycle regulator genes takes place at the translational level [22]. On these bases, we believe that, also in our conditions, ribosome-bound RACK1 modulates the expression of cyclin D1, D2 and B1 more likely at the translational level. Although here we have described the role of ribosomal RACK1 only in cell proliferation, we cannot exclude that the DE mutant may be also involved in other cellular processes, such as adhesion.

6. Conclusion

In conclusion, this study demonstrates that the main role of ribosomal RACK1 is to control the proliferation of neuroblastoma cells, possibly by regulating the translation of specific mRNAs encoding for proteins involved in cell cycle regulation. This evidence may provide a novel tool to discover new therapeutic targets in neuroblastoma.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2018.09.020.

Fundings

This work is supported by University of Tuscia funds.

Acknowledgements

We thank prof. Scapigliati Giuseppe for excellence support with flow cytometry.

Conflicts of interest

The authors declare no conflicts of interests.

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