PRAS40 deregulates apoptosis in Ewing sarcoma family tumors by enhancing the insulin receptor/Akt and mTOR signaling pathways

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Abstract: EWS expression in Ewing sarcoma family tumors (ESFTs) is decreased due to the haploinsufficiency elicited by chromosomal translocation. The abnormal expression levels of EWS and its downstream factors contribute to the manifestation of ESFTs. Previously, we reported that increased Proline-rich Akt substrate of 40 kDa (PRAS40), which is encoded by an EWS mRNA target, promotes the development of ESFTs. However, the mechanism remains elusive. To clarify the role of PRAS40 in ESFTs, we silenced PRAS40 expression in ESFT cells using siRNAs and found increased levels of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells. Cleaved caspase 3 levels and cytochrome C release were increased simultaneously. Furthermore, with PRAS40 knockdown, the phosphorylation of Akt and mTOR downstream factors, i.e., S6K and S6, was attenuated notably. Ectopic expression of PRAS40 increased Akt and S6 phosphorylation. Activation of Akt only partially reversed the apoptosis induced by PRAS40 knockdown, and downregulation of S6 phosphorylation by PRAS40 silencing could not be sufficiently restored via Akt activation. Searching the upstream factors in this pathway, the autophosphorylation of insulin receptor (IR) was found to be inhibited significantly by PRAS40 silencing but increased by PRAS40 overexpression. Therefore, PRAS40 may enhance IR phosphorylation to facilitate Akt and mTOR signaling leading to the apoptosis deregulation in ESFTs. Moreover, in vivo results confirmed that PRAS40 deletion suppressed the growth of ESFT xenografts and downregulated IR and S6 phosphorylation. Our findings suggest a novel functioning model for PRAS40, which represents a novel therapeutic target for ESFTs.

Keywords: PRAS40, insulin receptor, Ewing sarcoma family tumors, apoptosis, signal transduction, mTOR, Akt

Introduction

Ewing sarcoma family tumors (ESFTs) are a group of aggressive and highly metastatic malignancies predominantly afflicting children and young adults. A number of studies described the (11;22)(q24;q12) chromosomal translocation that results in the EWS/FLI-1 fusion protein, which accounts for approximately 85% of ESFTs. However, the ectopic expression of EWS/FLI-1 induces growth arrest or cell death rather than cellular transformation in cells [1] and tumor formation in mice [2, 3]. Therefore, cellular context is critical to the oncogenic potential of EWS/FLI-1, and additional events are required. These additional events potentially include a deregulated p53 pathway [4] and loss of the p16 pathway [1]. Moreover, decreased EWS expression is attributed to the haploinsufficiency caused by the chromosomal translocation or the expression of splicing variants in the ESFTs [5, 6]. This abnormal expression of EWS leads to the upregulation of its downstream factors [6] and to the impairment of midzone formation, which induces aneuploidy [7], suggesting that EWS itself or its targets play important roles in the development of ESFTs.

Previously, we reported that PRAS40, which is also known as AKT1S1 and which is encoded by one of the mRNA targets of EWS, was increased in ESFTs and that cellular proliferation and metastasis were suppressed by siRNA-mediated PRAS40 knockdown [6]. PRAS40 was originally identified as a 14-3-3 binding protein in
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insulin-treated hepatoma cells [8] and is identical to the phosphorylated p39 protein in nerve growth factor (NGF)- or epidermal growth factor (EGF)-treated PC12 cells [9]. PRAS40 is characterized as a substrate for Akt [10] and functions as a regulator of mTORC1 [10-15]. PRAS40 plays an important role in cell survival in different species [6, 10, 16, 17] and is aberrantly expressed or phosphorylated in multiple tumors [6, 17-19]. PRAS40 promotes melanoma tumorigenesis by deregulating apoptosis, the details of which remain unclear [17]. PRAS40 silencing was recently reported to induce p53 upregulation in a RPL11-dependent manner [20], which could serve as a mechanism of PRAS40-mediated apoptosis deregulation. In addition, given that PRAS40 lies at the crossroads of PI3K/Akt and the mTOR pathway [15, 18], both of which are key axes controlling cellular proliferation, apoptosis and autophagy, the relationship between the PRAS40-mediated-tumorigenesis of ESFTs and PI3K/Akt and mTOR signaling pathways must be determined.

In this report, we demonstrate that PRAS40 deregulates apoptosis in ESFTs and that the activities of the insulin receptor (IR)/Akt and mTOR pathways are potentially crucial in this process. Taken together, our findings suggest that PRAS40 promotes the development of ESFTs and may therefore represent a novel therapeutic target in these aggressive diseases.

Materials and methods

Cell lines and cell culture

The ESFT cell line A673 was purchased from American Type Culture Collection, and TC-32 was a gift from Dr. Heather Davidson, Children’s Oncology Group (COG), Cell Culture and Xenograft Repository, Texas Tech University Health Sciences Center. A673 was grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). TC-32 was grown in RPMI 1640 supplemented with 10% FBS and 2 mM glutamine. Both cell lines were incubated at 37°C with 5% CO₂.

Plasmids

The plasmids pRK5-myc-PRAS40 (Addgene plasmid #15476) and pRK5-myc-PRAS40 T246A (Addgene plasmid #15479) were gifts from Dr. Do-Hyung Kim [13]. The human wild type IR (HIR WT) was a gift from Dr. Frederick Stanley (Addgene plasmid # 24049) [21]. pcDNA3 Myr HA Akt1 was a gift from Dr. William Sellers (Addgene plasmid # 9008) [22].

Antibodies and Western blot analysis

Antibodies were purchased for the detection of β-actin (AC-15; Sigma); PRAS40 (Invitrogen); cleaved caspase 3, cytochrome C, p-PRAS40 (T246), p-PRAS40 (S183), p-Akt, Akt, p-S6, S6, p-S6K, p-IR, and IR (Cell Signaling); and p-IR (Abcam). Western blot analysis was performed as described previously [23], and the signals were detected using an ECL Plus Detection System (Thermo Fisher). Images were acquired using an Image Analyzer ChemiDoc XRS+ (Bio-Rad).

RNA interference

For the PRAS40 knockdown experiment, stealth siRNAs were synthesized by Invitrogen [6]. siRNAs (120 pmol) were introduced into A673 cells (6×10⁵) using 5 µl of Lipofectamine RNAiMAX reagent (Invitrogen).

For combined PRAS40 knockdown and IR or myr-Akt overexpression, PRAS40 siRNA (120 pmol) was introduced into A673 cells (6×10⁵) using 5 µl of Lipofectamine RNAiMAX reagent. Twenty-four hours later, 1.5 µg of the plasmid was introduced into the cells using Lipofectamine 3000 reagent.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Twenty-four hours after siRNA transfection, A673 cells were reseeded on glass slides and fixed in 4% formaldehyde. The TUNEL assay was performed using an In Situ Cell Death Detection Kit, Fluorescein (Roche) according to the manufacturer’s protocol.

In vivo tumorigenicity assay

All animals were maintained and animal experiments were conducted in the specific-pathogen-free (SPF) Laboratory Animal Center of Dalian Medical University. A673 cells (5×10⁶) were injected subcutaneously into the two posterior flanks of male BALB/c nude mice (Dalian Medical University). Mice were assessed daily. When tumors in any of the groups (mice inject-
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Figure 1. siRNA-mediated PRAS40 knockdown induces apoptosis in ESFT cell lines. A673 cells (A-C) or TC-32 cells (D-F) were transfected with or without (-) scrambled (con) or PRAS40 (#1 and #2) siRNAs. After 48 hours, the cytosol was collected, and the levels of cleaved caspase 3, cytochrome C, PRAS40, and β-actin were analyzed by Western blot (A, D). Apoptosis was analyzed by TUNEL assay (B, C, E, F). Three independent experiments were performed, and the representative images are presented. Scale bars, 100 µm. Green, TUNEL-positive cells; blue, nuclei (B, E). TUNEL-positive cells were counted from 300 total cells. The quantitative results of three independent experiments are presented. **, p<0.01; *, p<0.05. Bars, SD (C, F).
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using the formula $V = \frac{1}{2} \times \text{width}^2 \times \text{length}$. Body weights were also recorded. On day 17 after tumor cell inoculation, all mice were sacrificed, and the tumors were dissected, weighed, and measured.

All animal maintenance and procedures were carried out in strict accordance with the recommendations established by the Animal Care and Ethics Committee of Dalian Medical University as well as the guidelines by the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Animal Care and Ethics Committee of Dalian Medical University. In animal study, all efforts were made to minimize suffering of mice.

**Immunohistochemistry**

Formalin-fixed, paraffin-embedded xenograft tumor sections were deparaffinized, dehydrated, and treated with 0.3% hydrogen peroxide. Slides were incubated with anti-p-IR (Abcam) or anti-p-S6 (Cell Signaling) antibodies overnight at 4°C followed by incubation with biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA, USA) for 1 hour at room temperature. Signals were detected using a dianinobenzidine substrate kit (Vector Laboratories). Slides were counterstained with hematoxylin.

**Statistical analyses**

All experiments were repeated thrice. The data are represented as the mean ± standard deviation (SD). Analysis of variance and Student’s t-test were used to compare the values of the test and control samples in vitro and in vivo. $p<0.05$ was considered statistically significant. SPSS 17.0 software was used for all statistical analyses.

**Results**

**PRAS40 deletion induces ESFT cell apoptosis**

We previously reported that siRNA-mediated PRAS40 knockdown suppressed the cellular proliferation of ESFT cells. To further investigate the role of PRAS40 in the development of ESFTs and to determine whether the cell growth inhibition caused by PRAS40 knockdown in ESFTs is due to apoptosis, we performed a TUNEL assay. PRAS40 expression was silenced by siRNAs in A673 cells. PRAS40 protein levels were significantly reduced in the cells transfected with PRAS40 siRNAs (#1 or #2) but not in those transfected with scrambled siRNA (con; Figure 1A). Simultaneously, we found the activated form of caspase 3, i.e., cleaved caspase 3, was markedly increased in the PRAS40-
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knockdown cells. The release of cytochrome C into the cytosol was also notably increased in the PRAS40-knockdown cells. A673 cells transfected with PRAS40 siRNA exhibited remarkable increases in TUNEL-positive cells of 7.4% and 14.3% (#1 and #2, respectively), whereas cells with no siRNA transfection (-) exhibited only 1% TUNEL-positive cells. The cells transfected with scrambled siRNA (con) did not show a significant change (2.8%; Figure 1B, 1C).

Similar results were obtained in another ESFT cell line; i.e., TC-32 (Figure 1D-F). The percentages of TUNEL-positive cells increased remarkably from 2.1% (-) to 8.7% and 22.6% (#1 and #2, respectively) upon PRAS40 siRNA transfection but not scrambled siRNA transfection (con; 4.4%). These results demonstrate that PRAS40 silencing induces apoptosis, thus suggesting a mechanism by which PI3K inhibitors augment apoptosis in ESFT cells induced by actinomycin D [24] and doxorubicin [25].

**PRAS40 facilitates Akt phosphorylation in response to insulin stimulation in ESFTs**

PRAS40 is the substrate of Akt, which controls the mitochondrial pathway of apoptosis as an anti-apoptotic factor [26]. To investigate the mechanism of apoptosis induced by PRAS40 silencing, we first examined the effects of PRAS40 on Akt activation. Akt was phosphorylated in both A673 and TC-32 cells in response to insulin treatment, and this phosphorylation was greatly decreased in PRAS40-knockdown cells (#1 and #2). However, extensive downregulation was not observed in cells transfected with scrambled siRNA (con; Figure 2).

To confirm the influence of PRAS40 on Akt activation, we introduced wild type PRAS40 or PRAS40246A plasmid into A673 cells and assessed Akt phosphorylation. In response to insulin stimulation, wild type PRAS40 transfection resulted in an increased level of Akt phosphorylation compared with empty vector transfection, while cells ectopically expressing PRAS40246A, wherein threonine 246 was mutated to alanine and could not be phosphorylated by Akt, also exhibited a significant increase in Akt phosphorylation (Figure 3). In addition, both wild type PRAS40 and PRAS40246A were phosphorylated similarly at serine 183. These results suggest that PRAS40 facilitates Akt phosphorylation in response to insulin treatment in ESFTs regardless of phosphorylation by Akt.

**PRAS40 induces mTOR signaling in response to insulin stimulation in ESFTs**

PRAS40 is a modulator of mTOR, which is important for apoptosis [27, 28]. To investigate the mechanism of apoptosis caused by PRAS40 knockdown, we next examined mTOR activation. When PRAS40 expression was reduced by siRNAs (#1 and #2) in the A673 cells treated with insulin, the phosphorylation of mTOR downstream factors, i.e., S6K and S6, was significantly decreased. In contrast, the levels of phosphorylation were not altered in cells transfected with scrambled siRNA (con; Figure 2A). Similar results were obtained in TC-32 cells (Figure 2B). These data suggest that the down-regulation of mTOR signaling is involved in the
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Apoptosis induced by PRAS40 silencing in ESFTs.

To confirm the influence of PRAS40 on mTOR activation, we introduced wild type PRAS40 or PRAS40<sup>246A</sup> plasmid into A673 cells and assessed S6 phosphorylation. In response to insulin stimulation, both wild type PRAS40 and PRAS40<sup>246A</sup> transfection resulted in significantly increased S6 phosphorylation compared with empty vector transfection (Figure 3). Therefore, PRAS40 is necessary for mTOR activation in response to insulin stimulation in ESFTs.

Akt activation cannot sufficiently reverse the PRAS40 silencing-induced apoptosis and S6 phosphorylation suppression in ESFTs.

Given that Akt is the important factor in both PI3K/Akt and mTOR pathways, we introduced an activated Akt (myr-Akt) to investigate the mechanism of the apoptosis caused by PRAS40 knockdown in detail. We transfected an empty vector or myr-Akt together with (#1, #2) or without (-) PRAS40 siRNA or with scrambled siRNA (con) into A673 cells and analyzed apoptosis via TUNEL assay (Figure 4A, 4B). In the cells transfected with the empty vector, PRAS40...
knockdown increased the percentages of TUNEL-positive cells from 1.8% (-) to 7.4% and 16.5% (#1 and #2, respectively). In the cells transfected with myr-Akt, the percentages of TUNEL-positive cells increased from 0.7% (-) to approximately 4.9% (#1 and #2) upon PRAS40 knockdown. These data demonstrate that myr-Akt can only partially rescue the apoptosis caused by PRAS40 knockdown.

Furthermore, S6 phosphorylation was downregulated by PRAS40 deletion likewise in myr-Akt-transfected cells. These phosphorylation levels were slightly higher than those observed in empty vector-transfected cells, indicating that downregulation of S6 phosphorylation induced by PRAS40 knockdown could be only partially restored by myr-Akt transfection (Figure 4C). These data suggest that Akt cannot fully activate mTOR signaling in A673 cells with PRAS40 knockdown and that mTOR and Akt signaling is essential for apoptosis caused by PRAS40 knockdown.

**PRAS40 enhances the autophosphorylation of IR in response to insulin stimulation in ESFTs**

To determine the step at which PRAS40 facilitates Akt and mTOR signaling, we introduced the IR plasmid into A673 cells and analyzed IR autophosphorylation. In response to insulin stimulation, IR phosphorylation in PRAS40 siRNA-transfected cells was reduced significantly (#1 and #2, Figure 5A) compared with that in non-transfected cells (-), whereas IR phosphorylation was not altered markedly in scrambled siRNA-transfected cells (con; Figure 5A). However, no significant change was observed regarding IR expression in PRAS40 knockdown cells compared with control cells. Akt and S6 phosphorylation accordingly decreased in PRAS40 siRNA-transfected cells.

To confirm the influence of PRAS40 on IR phosphorylation, we co-transfected IR plasmid together with wild type PRAS40 or PRAS40<sup>246A</sup> plasmid into A673 cells. IR phosphorylation was remarkably increased by both PRAS40 and PRAS40<sup>246A</sup> overexpression even when PRAS40 threonine 246 phosphorylation was inhibited by API-2 (an Akt inhibitor) and LY294002 (a...
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PI3K inhibitor treatments (Figure 5B). Interestingly, the serine 183 phosphorylation of PRAS40 was consistent with IR phosphorylation, indicating that the serine 183 phosphorylation of PRAS40 could contribute to promoting IR phosphorylation. Taken together, these results indicate that PRAS40 enhances IR autophosphorylation in ESFTs, leading to the facilitation of Akt and mTOR signaling.

In vivo tumorigenesis is inhibited by PRAS40 deletion

Based on the in vitro results, we further explored the possibility that PRAS40 knockdown inhibits the growth of ESFT xenografts in mice. At 17 days after tumor cell injection, growth was notably inhibited in the tumor xenografts formed by the cells transfected with PRAS40 siRNAs (#1 and #2). However, growth inhibition was not observed in cells transfected with scrambled siRNA (con; Figure 6A, 6B). Reduced tumor volume (Figure 6B) and weight (Figure 6C, 6D) in the PRAS40-knockdown groups were noted compared with the control groups. Moreover, immunohistological analyses indicated that anti-p-IR or anti-p-S6 antibody staining of tumor tissues from mice of the PRAS40 knockdown (#1, #2) groups was considerably weaker compared with the control groups (-, con; Figure 6E). These results indicate that the phosphorylation of S6 and IR was suppressed by PRAS40 knockdown in vivo. These data suggest that PRAS40 knockdown significantly suppressed the tumor growth of ESFT xenografts in mice by inhibiting IR and mTOR activation.

Discussion

ESFTs are characterized as a group of extremely malignant tumors. Systemic chemotherapy in combination with surgery and/or radiotherapy has increased the 5-year disease-free survival rate for patients with localized ESFTs to approximately 70%; however, the rate for individuals with metastases is less than 20% [29]. Therefore, novel treatments are urgently needed. Numerous targeted therapies demonstrated modest efficacy, and pronounced responses have been characterized in a small subset of patients, including those who were treated with insulin-like growth factor I receptor inhibitors [30, 31]. However, numerous patients developed resistance to therapy and exhibited disease recurrence within several months [32]. To identify a novel approach for the treatment of ESFTs, understanding tumorigenesis is important.

As a downstream factor of EWS, PRAS40 expression is increased in ESFTs, and deletion of PRAS40 results in decreased cell growth and motility [6]. Here, we report that PRAS40 plays a major role in the development of ESFTs by regulating apoptosis (Figure 1), which is consistent with results in melanoma [17]. We demonstrated that Akt phosphorylation is downregulated when PRAS40 is deleted (Figures 2 and 5A) and is upregulated when PRAS40 is overexpressed (Figures 3 and 5B). Moreover, activated Akt partially reversed the apoptosis induced by PRAS40 deletion (Figure 4). Akt negatively regulates the release of cytochrome C from the mitochondria and the activation of caspase cascades. Akt has also been implicated as an anti-apoptotic factor in many different cell death paradigms [26], and Akt phosphorylation is frequently observed in ESFT samples [33]. Therefore, Akt could play an important role in apoptosis deregulation by PRAS40.

Next, we found that S6K and S6 phosphorylation decreased significantly when PRAS40 was silenced (Figures 2, 4-6) and increased notably when PRAS40 was overexpressed (Figures 3 and 5B). Apoptosis inducers lead to the dephosphorylation of S6K and 4E-BP1 [34, 35]. S6K is responsible for BAD phosphorylation, which inactivates this proapoptotic molecule [27, 36]. In addition, treatment of cells with mTOR inhibitors may lead to reduced synthesis of RPL11 and thereby destabilization of p53 [37]. Thus, the repression of mTOR activation could explain the mechanism by which silencing of PRAS40 induces upregulation of p53 in a manner that is dependent on RPL11 [20]. Therefore, activation of the mTOR pathway may be essential for PRAS40-mediated apoptosis deregulation. These results are consistent with the trials using mTOR inhibitors that demonstrated effects on ESFTs [29, 38].

The relationship between PRAS40 and mTOR signaling is controversial. PRAS40 was initially identified as an inhibitor of mTOR signaling. This inhibition could be relieved upon phosphorylation by Akt in HEK293 cells [14], T cells [39], HepG2 cells [13], 3T3-L1 cells [34] and cardiomyocytes [40]. PRAS40 silencing results
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in increased mTOR signaling in mice and liver cancer cells [13, 41]. However, other reports argue against this proposed function of PRAS40 in mTOR activation [11, 13, 41, 42]. Gene transfer of PRAS40 in rats promotes mTOR pathway activation, and PRAS40 knockout reduces mTOR pathway activation in the brain [43]. We also found significant decreases in S6K and S6 phosphorylation in response to PRAS40 silencing (Figures 2, 4–6), and an increase in S6

Figure 6. Tumorigenesis in vivo is inhibited by PRAS40 knockdown. A673 cells were transfected with or without (-) scrambled (con) or PRAS40 (#1 and #2) siRNAs. After 48 hours, 5×10⁶ cells were subcutaneously injected into nude mice. Eight injections were performed for each cell type. A. (-) and (#2), (con) and (#1) were inoculated in the two posterior flanks of the same mouse. Representative images are presented. B. Tumor volumes were recorded every two days. **, p<0.01. C. On day 17, tumors were obtained and dissected. Representative images are presented. D. Tumor weights were measured. *, p<0.05. E. Immunohistochemical analysis of the p-IR and p-S6 levels and hematoxylin and eosin (HE) staining in tumor samples. Scale bars, 50 µm.
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phosphorylation in response to ectopic expression of PRAS40 in A673 cells (Figures 3 and 5). The finding that PRAS40 expression also increased S6 phosphorylation (Figures 3 and 5) indicates a novel relationship among Akt, PRAS40 and S6. The serine 183 phosphorylation of PRAS40 was not suppressed even upon treatment with rapamycin, an inhibitor of mTOR (Figure 5B), indicating that serine 183 of PRAS40 could also be phosphorylated by factors other than mTOR. Therefore, the influence of PRAS40 on mTOR pathway signaling may differ in different cell types, tissues or stimulations. Regarding the issue that PRAS40 regulates mTOR signaling in ESFTs, we are planning to further analyze the details of this mechanism in the future.

Upon further investigation, IR autophosphorylation was found to be significantly downregulated (Figures 5A and 6E) by PRAS40 deletion and was notably upregulated by PRAS40 overexpression (Figure 5B). Although details regarding the enhancement of IR autophosphorylation caused by PRAS40 must be determined, these results provide an appropriate interpretation for the facilitation of both Akt and mTOR signaling pathways by PRAS40 simultaneously and suggest a positive feedback control of IR activation by PRAS40, which potentially plays an important role in apoptosis deregulation by PRAS40. These results could provide novel insights, given that the combination of mTOR inhibitors and IGF-1R antibodies are much more effective in ESFTs than are single-agent treatments [44].

Thus, PRAS40 deregulates apoptosis in ESFTs through enhancing IR phosphorylation to activate Akt and mTOR pathways. Investigating the mechanisms of PRAS40 in the promotion of IR autophosphorylation will further help to determine the function of PRAS40 and thus provide strong evidence for PRAS40 as a candidate target for ESFT treatment.

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Disclosure of conflict of interest

None.

Authors’ contribution

D.L., J.L., L.G., K.M. and L.H. designed the study and analyzed the data; L.H. wrote the manuscript; D.L., J.L., L.G., D.W. and L.H. conducted the experiments.

Abbreviations

PRAS40, Proline-rich Akt substrate of 40kDa; mTOR, mammalian target of rapamycin; ESFTs, Ewing sarcoma family tumors; IR, Insulin receptor; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling; PI3K, phosphoinositide 3-kinase; S6K, S6 kinase; 4E-BP1, eIF4E-binding protein 1; BAD, Bcl-2-associated death promoter; RPL11, Ribosomal proteins 11.

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