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Speedy/RINGO protein interacts with ERK/MAPK and PI3K/AKT pathways in SH-SY5Y neuroblastoma cells

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Abstract
Abnormal activity of ERK/MAPK and PI3K/AKT pathways is one of the most important factors for the development of many cancer types including neuroblastoma cancer. Apart from these two pathways, some cell cycle regulators such as Speedy/RINGO also contribute to neuroblastoma development. There is data reinforcing the possible communication of the components of ERK/MAPK and PI3K/AKT pathways in carcinogenic process. In addition to this, there are studies about the direct/indirect interaction of Speedy/RINGO with these pathways in different cell types other than neuroblastoma. However, there is not any study available showing the interaction of Speedy/RINGO with both pathways in neuroblastoma cells. Therefore, the aim of this study is to determine the possible effect of Speedy/RINGO on PI3K/AKT and ERK/MAPK pathways in SH-SY5Y neuroblastoma cells. For this aim, Speedy/RINGO was silenced by siRNA technique to analyze the effects of direct inhibition of Speedy/RINGO on these pathways. Results showed that Speedy/RINGO silencing caused a significant decrease in MEK1/2 expression and AKT phosphorylation. Afterward, MEK1/2 was inhibited using a specific inhibitor U0126. Data reveal a corresponding decrease in the Speedy/RINGO expression and AKT phosphorylation indicating a reciprocal interaction between ERK/MAPK and Speedy/RINGO. In addition, MTS analysis showed that both ERK/MAPK inhibition and Speedy/RINGO silencing significantly reduced the viability of SH-SY5Y cells. This study provides information about a possible interaction of Speedy/RINGO with PI3K/AKT and ERK/MAPK pathways in SH-SY5Y cells for the first time. It will not only help to better understand the cancer-prone interactions of these pathways but also enable us to identify the appropriate molecular targets for developing efficient treatment strategies.

Keywords Neuroblastoma · SH-SY5Y · PI3K/AKT · ERK/MAPK · Speedy/RINGO

Introduction

Neuroblastoma is the most common extracranial solid tumor among newborns and children [1]. One of the most important factors of the disease is the dysregulation of extracellular signal-regulated kinases/mitogen-activated protein kinase (ERK/MAPK) and phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathways [2, 3]. A recent study showed that ERK/MAPK signaling pathway is highly activated in neuroblastoma primary tumor cells [4], while another study indicated that the PI3K/AKT signaling pathway was significantly activated in neuroblastoma tissue samples [5].

ERK/MAPK signaling pathway regulates several proteins that have role in modulation of the cell cycle, migration, proliferation and apoptosis [6]. PI3K/AKT signaling pathway activates the cell cycle and apoptosis by regulating proteins located downstream in promoting cellular proliferation or apoptosis [7, 8].

Abnormal phosphorylation of the PI3K/AKT signaling pathway leads to increased cell survival, poor prognosis and chemotherapy resistance in patients with neuroblastoma cancer [9–11]. Therefore, targeting the PI3K/AKT signaling pathway with appropriate inhibitors appears to be a promising strategy especially for overcoming therapeutic resistance [12]. Similar to the PI3K/AKT signaling pathway, the ERK/MAPK is another signaling pathway that plays an essential role in tumor formation and developing resistance

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to anti-cancer therapies. Abnormalities in the ERK/MAPK signaling pathway may cause a wide range of cellular events from unregulated apoptosis to carcinogenesis [13, 14]. However, there is limited number of studies about the efficacy and function of the ERK/MAPK signaling pathway in neuroblastoma cancer. In one of these studies, it was pointed out that this signaling pathway plays a role in the transformation of neuroblastoma cells into transformed phenotype and gaining resistance to chemotherapy [15].

Besides abnormal interaction of the components of PI3K/AKT and ERK/MAPK signaling pathways in carcinogenic process [16], external effectors such as certain cell cycle regulator proteins may interact with these signaling pathways contributing carcinogenesis [17]. However, possible candidates of this interaction in neuroblastoma cells have not yet been elucidated. At this point, there is evidence indicating the possibility that one of the strongest candidate proteins effecting PI3K/AKT and ERK/MAPK signaling pathways may be the Speedy/RINGO protein, an unconventional cell cycle regulator [17].

Speedy/RINGO is a cell cycle regulatory protein that activates CDKs and controls the G1/S phase transition. For performing this function, Speedy/RINGO does not require activating phosphorylation by the CDK-activating kinase (CAK) and exhibits less sensitivity to cell cycle inhibitors such as p21Cip1 and p27Kip1. Due to these properties, Speedy/RINGO can inhibit apoptosis and maintain cancerous cell division by overriding many cell cycle control points [17, 18]. Indeed, in a study with SH-SY5Y neuroblastoma cells, Speedy/RINGO overexpression was observed to cause uncontrolled cell proliferation, while silencing Speedy/RINGO resulted in a decrease in tumor aggregates (tumorsphere) [17]. Furthermore, evidence from breast and neuroblastoma cancer studies strongly implicates that Speedy/RINGO overexpression has an important role in carcinogenesis by increasing CDK2 activity [19, 20]. Considering that Speedy/RINGO protein, ERK/MAPK and PI3K/AKT signaling pathways evidently confer a similar carcinogenic effect in neuroblastoma cancer, we asked the critical question "Could Speedy/RINGO have an interaction with PI3K/AKT and ERK/MAPK pathways in neuroblastoma?"

In a study examining tumor formation in breast tissue by Golipour et al., activation of the ERK/MAPK signaling pathway resulted in overexpression of Speedy/RINGO, and inhibition of the MEK1/2 enzyme in this signaling pathway by inhibitors such as PD98058 and U0126 decreased Speedy/RINGO expression [21]. On the other hand, studies on testis tissue have indicated an increase in the activity of the Speedy/RINGO-related proteins, Cyclin A2 and CDK2, as a result of Speedy/RINGO overexpression [22]. Mouse embryonic stem cell studies have also shown that the Cyclin A2-CDK2 complex has an important role in AKT overphosphorylation and activation [23] Analyzing the data of all these studies has strengthened our hypothesis that there may be an either direct or indirect interaction between Speedy/RINGO, ERK/MAPK and PI3K/AKT pathways.

From this point of view, we first used the STRING version 11.0 bioinformatics tool (https://string-db.org/) that shows the predicted functional associations between desired proteins by scanning all databases and literature. The diagram that emerged by the use of the STRING version 11.0 showed that Speedy/RINGO may have a regulatory effect on ERK/MAPK and PI3K/AKT signaling pathways further supporting our hypothesis (Fig. 1) [24]. However, there is no study available concerning a three-way interaction between these three players particularly in neuroblastoma cells.

Therefore, to analyze whether Speedy/RINGO affects ERK/MAPK and PI3K/AKT signaling pathways, Speedy/RINGO gene expression was inhibited in SH-SY5Y neuroblastoma cells using siRNA technique. Silencing Speedy/RINGO caused a significant decrease in MEK1/2 expression and AKT phosphorylation levels in SH-SY5Y cells. Then, we inhibited ERK/MAPK signaling pathway by specific MEK1/2 inhibitor U0126 to determine its effect on Speedy/RINGO expression in cultured SH-SY5Y neuroblastoma cells. Upon ERK/MAPK inhibition, Speedy/RINGO protein expression was found to be significantly decreased denoting a reciprocal interaction between ERK/MAPK and Speedy/RINGO. Concordantly, there was also a significant decrease in Cyclin A and CDK2 expression and AKT phosphorylation levels. All these data suggest that Speedy/RINGO interacts with ERK/MAPK and PI3K/AKT signaling pathways

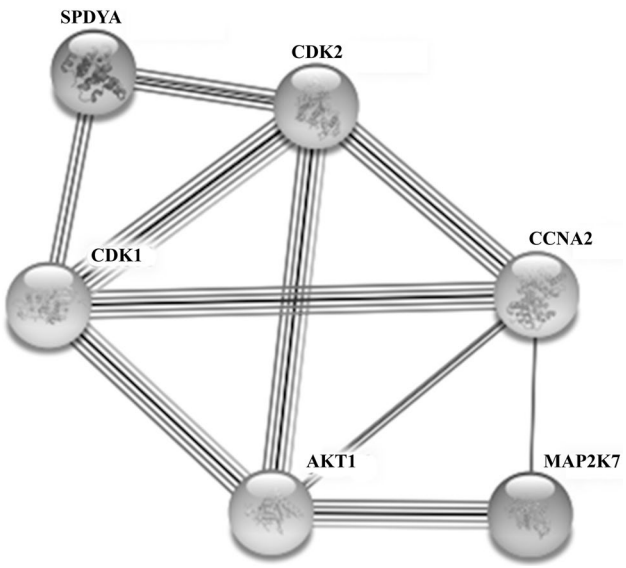

Fig. 1 Functional protein association networks analysis. Interacting protein prediction of Speedy/RINGO using STRING v11 database was shown. Protein names; SPDYA: Speedy/RINGO, CCNA2: Cyclin A, CDK1 and CDK2: Cyclin-dependent kinase 1 and 2, MAP2K7: Mitogen-activated protein kinase kinase, AKT1: Protein kinase B

in SH-SY5Y neuroblastoma cells. This study may guide for future studies targeting to reveal abnormal regulation of ERK/MAPK and PI3K/AKT signaling pathways, which plays a key role in the emergence of many cancers, especially neuroblastoma cancer.

Materials and methods

Functional protein association analysis using STRING v 11.0

For a preliminary interpretation of the interaction of Speedy/RINGO protein with PI3K/AKT and ERK/MAPK pathways, STRING v 11.0 server was used. This server gives information about interaction between two proteins that have role in a particular biological function by searching the available data in the literature [24].

Cell culture

SH-SY5Y cell line were supplied by Assoc. Prof. Emin Ilker Medine from Ege University Institute of Nuclear Sciences and cultured in Dulbecco's Modified Eagle Medium/high glucose (DMEM) (Sigma) containing 10% Fetal Bovine Serum (FBS) (Sigma), 100 U/mL penicillin-100 mg/mL streptomycin (Sigma), and 1 mmol/L L-glutamine (Sigma) at 37 °C, 5% CO_2. Cultures with sufficient confluency were washed with sterile Phosphate Buffered Saline (Thermo Fisher). Subsequently, cells were detached using 2.5% sterile Trypsin-Ethylene Diamine Tetraacetic Acid (EDTA) (Sigma).

In vitro silencing of Speedy/RINGO with siRNA method

The siRNA silencing method was optimized using the Santa Cruz Biotechnology siRNA silencing system protocol. First, 2×10^5 SH-SY5Y cells in each well were inoculated with growth medium without any antibiotics. Then, 6 µL of Speedy A siRNA (sc-153738, Santa Cruz Biotechnology) was mixed with 100 µL of transfection solution (sc-36868, Santa Cruz Biotechnology). On the other hand, 6 µL of transfection agent (sc-29528, Santa Cruz Biotechnology) was also mixed with 100 µL of transfection solution. The siRNA mixture was combined with the transfection agent mixture and incubated for 15–45 min at room temperature. At the end of the incubation period, 0.8 mL of transfection solution was added to the tube containing the total mixture and the total mixture was transferred to inoculated SH-SY5Y cells and incubated for 48 h at 37 °C, 5% CO_2. As negative control, control siRNA molecules (scrambled, sc-37007, Santa Cruz Biotechnology) were used. Scrambled siRNA molecules were prepared in the same way as Speedy A siRNA mixture and transferred to SH-SY5Y cells.

ERK/MAPK inhibition assay

For the inhibition of ERK/MAPK, the specific MEK1/2 inhibitor U0126 (Chemcruz) was used. Firstly, 1×10^6 SH-SY5Y cells were cultured in 25 cm^2 flasks containing growth medium at 37 °C, 5% CO_2 for 24 h. Then, 10 mM U0126 (Chemcruz) diluted in dimethyl sulfoxide (DMSO) was added to the cells and 0.1% DMSO was added to the control cells and cultured at 37 °C, 5% CO_2 for 48 h.

Western blotting

To analyze the expression levels of desired proteins upon treatment with MEK1/2 inhibitor U0126 and Speedy/RINGO siRNA, total protein was isolated from the cells using the Whole Cell Extraction Kit (2910, Millipore). Protein concentrations were measured with the Qubit™ Protein Assay Kit (Q33211, Thermo Fisher Scientific) using the Qubit™ 3.0 Fluorometer. After separating proteins with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred to the nitrocellulose membrane (sc-3724, Santa Cruz Biotechnology) using the Trans-Blot Turbo Transfer System (BioRad). After completing the transfer, membranes were blocked in 5% BSA/Tris-Buffered Saline, 0.1% Tween 20 (TBST) for 1 h at room temperature. At the end of this period, the membranes were incubated overnight at + 4 °C with primary antibodies diluted in 5% BSA/TBST. The following primary antibodies were used at 1:1000 dilutions; MEK1/2 mouse monoclonal antibody (sc-81504, Santa Cruz Biotechnology), Cdk2 mouse monoclonal antibody (sc-6248, Santa Cruz Biotechnology), Cyclin A Mouse monoclonal antibody (sc-239, Santa Cruz Biotechnology), p-Akt 1/2/3 mouse monoclonal antibody (sc-514032, Santa Cruz Biotechnology), p-Akt 1/2/3 mouse monoclonal antibody (sc-271966, Santa Cruz Biotechnology), Calnexin mouse monoclonal antibody (sc-80645, Santa Cruz Biotechnology) and GAPDH mouse monoclonal antibody (sc-47724, Santa Cruz Biotechnology) and SPDYA polyclonal antibody (PA1-16959, Thermo Fisher Scientific) at 1: 500 concentration. After incubating with appropriate secondary antibodies, blots were visualized using the Chemiluminescent Clarity Western ECL Substrate Kit (170-5061, BioRad). Blots were scanned and the densities of the specific protein bands were quantified and normalized with Calnexin and GAPDH as internal loading controls using ChemiDoc™ Imaging Systems (BioRad) and Lab 4.0 Software.

MTS cell viability assay

Cell viability analyses were performed using MTS Cell Viability Assay Kit (ab197010, Abcam) to observe the effect of the treatments using U0126 chemical and siRNA technology on the vital activities of the cells. U0126 and siRNA-treated 1×10^4 cells/well were seeded in a 96-well plate. After 48 h, control SH-SY5Y cells and U0126- and siRNA-treated SH-SY5Y cells were added 20 µL of MTS solution and incubated for 3.5 h in a 37 °C 5% CO_2 incubator. After incubation, a spectrophotometric analysis was performed by measuring absorbance at 490 nm wavelength using SpectraMax i3 (Molecular Devices). Data were obtained in triplicate by three independent experiments.

Integrative pixel analysis

Photoshop CS6 Software version 13.0 was used to analyze the relative density of protein bands in Western blot images. The relative expression value was normalized to "1" in the control cells for each protein analyzed, and then relative protein expression fold changes of the experimental groups were calculated.

Statistical analysis

Statistical Package for Social Sciences (SPSS) version 22.0 program was used and the results were statistically analyzed according to paired 2-tailed Student's *t*-test. A *p* value of less than 0.05 was considered significant and a *p* value of less than 0.001 was considered extremely significant for all statistical analyses. For Western blot and MTS cell viability analysis, error bars in the graphs were generated using ± standard deviation (SD).

Results

STRING functional protein association analysis indicated that Speedy/RINGO is a strong interacting candidate for ERK/MAPK and PI3K/AKT signaling pathways

The main question of our hypothesis is "Can Speedy/RINGO have a communication with ERK/MAPK and PI3K/AKT signaling pathways in neuroblastoma cells?" To gain an insight into our hypothesis, STRING protein–protein interaction network (version 11.0) was used. The results showed that the expression of Speedy/RINGO and related proteins (CCNA2: Cyclin A, CDK1 and CDK2: Cyclin-dependent kinase 1 and 2) have close interaction with the members of ERK/MAPK and PI3K/AKT signaling pathways (MAP2K7: Mitogen-activated protein kinase kinase, AKT1: Protein kinase B) (Fig. 1). STRING data provided a preliminary insight supporting our hypothesis.

Speedy/RINGO inhibition by siRNA gene silencing decreased CDK2 and Cyclin A protein expressions in SH-SY5Y neuroblastoma cells

To analyze the effect of Speedy/RINGO on ERK/MAPK and PI3K/AKT pathways, we silenced Speedy/RINGO by siRNA method and Western blotting results showed that there was a statistically significant 65% decrease in Speedy/RINGO protein level in siRNA-treated SH-SY5Y neuroblastoma cells compared to control cells (Fig. 2a, $p = 0.00003$ $^{***}p < 0.001$). Speedy/RINGO, which contains a highly conserved specific region that enables the binding and activation of CDKs, activates them by directly binding to CDK2 which is also the partner of Cyclin A. In this context, upon inhibition of Speedy/RINGO, the levels of Speedy/RINGO-related proteins CDK2 and Cyclin A were analyzed. Western blotting results showed that Speedy/RINGO inhibition significantly reduced CDK2 and Cyclin A protein expression levels by 45% and %33, respectively (Fig. 2c, $p = 0.0000002$ $^{***}p < 0.001$; Fig. 2d, $p = 0.00001$ $^{***}p < 0.001$). These data indicated that Speedy/RINGO inhibition causes a reduction in the protein levels of Speedy/RINGO partners, CDK2 and Cyclin A, as expected.

Speedy/RINGO inhibition by siRNA gene silencing decreased AKT phosphorylation in SH-SY5Y neuroblastoma cells

Referring to the results of the above-mentioned study with mouse embryonic stem cells showing the Cyclin A-CDK2-dependent phosphorylation and activation of AKT, we analyzed AKT phosphorylation levels to determine whether PI3K/AKT signaling was affected by the decrease in Speedy/RINGO, CDK2 and Cyclin A levels. AKT phosphorylation on its two functionally essential phosphorylation sites, Ser473 and Thr308 was found to be significantly reduced by 59% and 39%, respectively (Fig. 2e, $p = 0.0000003$ $^{***}p < 0.001$; Fig. 2f, $p = 0.000473$ $^{***}p < 0.001$).

MEK1/2 expression level was downregulated upon Speedy/RINGO inhibition by siRNA gene silencing in SH-SY5Y cells

To analyze the effect of Speedy/RINGO on ERK/MAPK signaling pathway, the expression level of MEK1/2, one of the key components of ERK/MAPK pathway was analyzed upon Speedy/RINGO silencing. Western blotting results showed that Speedy/RINGO inhibition significantly reduced the MEK1/2 protein expression by 69% (Fig. 2b, $p = 0.0000001$ $^{***}p < 0.001$) which is a strong indicator of

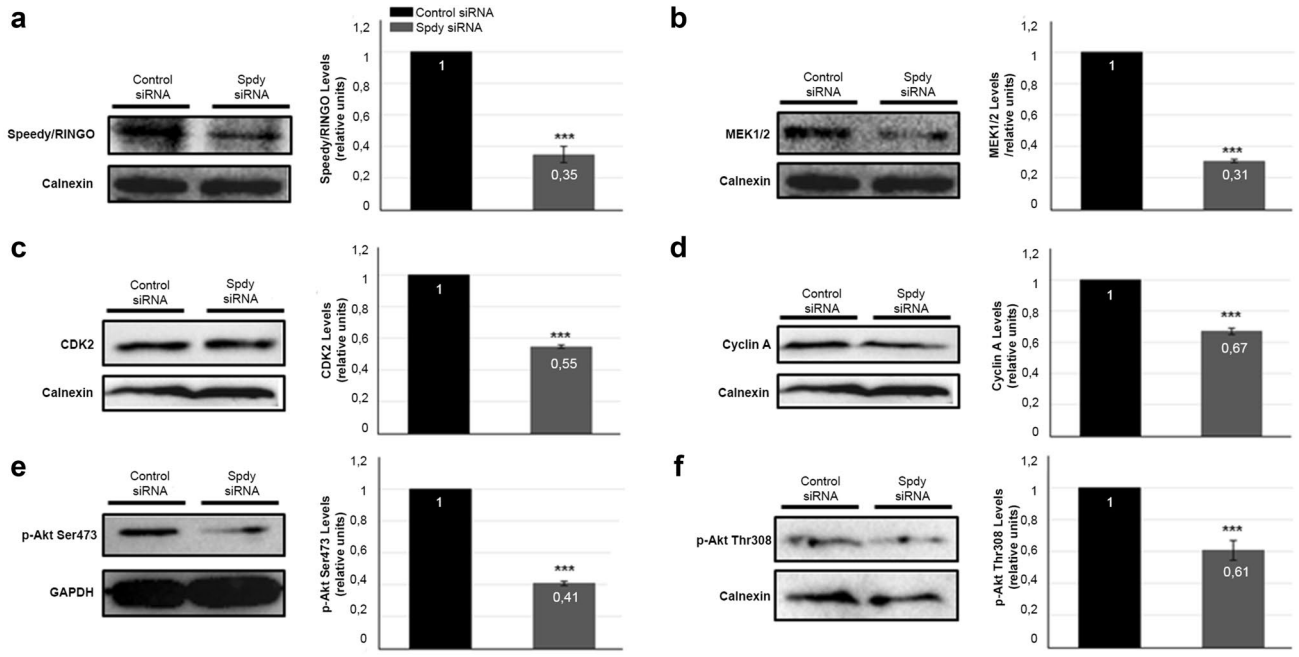

Fig. 2 Effect of Speedy/RINGO-targeting small interfering siRNA (Speedy A) gene silencing method in SH-SY5Y cells on SPDYA, MEK1/2, CDK2, Cyclin A, phospho-AKT Ser473 and phospho-AKT Thr308 protein expressions were analyzed by Western blot analysis (**a**, **b**, **c**, **d**, **e**, **f**). Calnexin and GAPDH antibody used as an internal control. Graph indicating quantitative analysis of signals in blots. Results were represented as fold changes. Bar represents mean values ± SD (***$p < 0.001$)

the interaction between Speedy/RINGO and ERK/MAPK signaling pathway.

Expression level of Speedy/RINGO was downregulated upon ERK/MAPK inhibition by U0126 in SH-SY5Y cells

Considering the results of breast tissue tumorigenesis study showing the effect of ERK/MAPK inhibition on Speedy/RINGO expression, to examine the same effect in SH-SY5Y neuroblastoma cells, ERK/MAPK signaling pathway was inhibited by the administration of 10 mM U0126 (Chemcruz), a MEK1/2 inhibitor, and as shown in Fig. 3a, the MEK1/2 level was very significantly decreased by 49% ($p = 0.000013$ ***$p < 0.001$).

The effect of inhibition of the ERK/MAPK signaling pathway on the level of Speedy/RINGO expression was then analyzed by Western blot and the results showed that ERK/MAPK pathway inhibition caused a significant 35% decrease in the Speedy/RINGO expression level (Fig. 3b, $p = 0.0000086$ ***$p < 0.001$). These data showed that the interaction between Speedy/RINGO and ERK/MAPK signaling pathway is mutual.

Expression levels of CDK2 and Cyclin A proteins reduced upon decrease in Speedy/RINGO expression in U0126-treated SH-SY5Y neuroblastoma cells

The effect of Speedy/RINGO downregulation on CDK2 and Cyclin A expression levels due to ERK/MAPK inhibition were analyzed and as expected, Western blot results showed a significant 16% and 21% reduction in CDK2 and Cyclin A expression levels, respectively, in SH-SY5Y cells (Fig. 3c, $p = 0.001541$ **$p < 0.001$; Fig. 3d, $p = 0.0000034$ ***$p < 0.001$). These data showed that ERK/MAPK signaling pathway inhibition also affects the expression of Speedy/RINGO-related proteins.

AKT phosphorylation reduced upon decrease in Speedy/RINGO expression in U0126-treated SH-SY5Y neuroblastoma cell line

Downregulation of Speedy/RINGO due to inhibition of ERK/MAPK signaling pathway was followed by a decrease in CDK2 and Cyclin A expressions. To analyze the effect of these decreases on AKT phosphorylation, we performed immunoblotting and results indicated that phosphorylation of AKT on both Ser473 and Thr308 phosphorylation sites

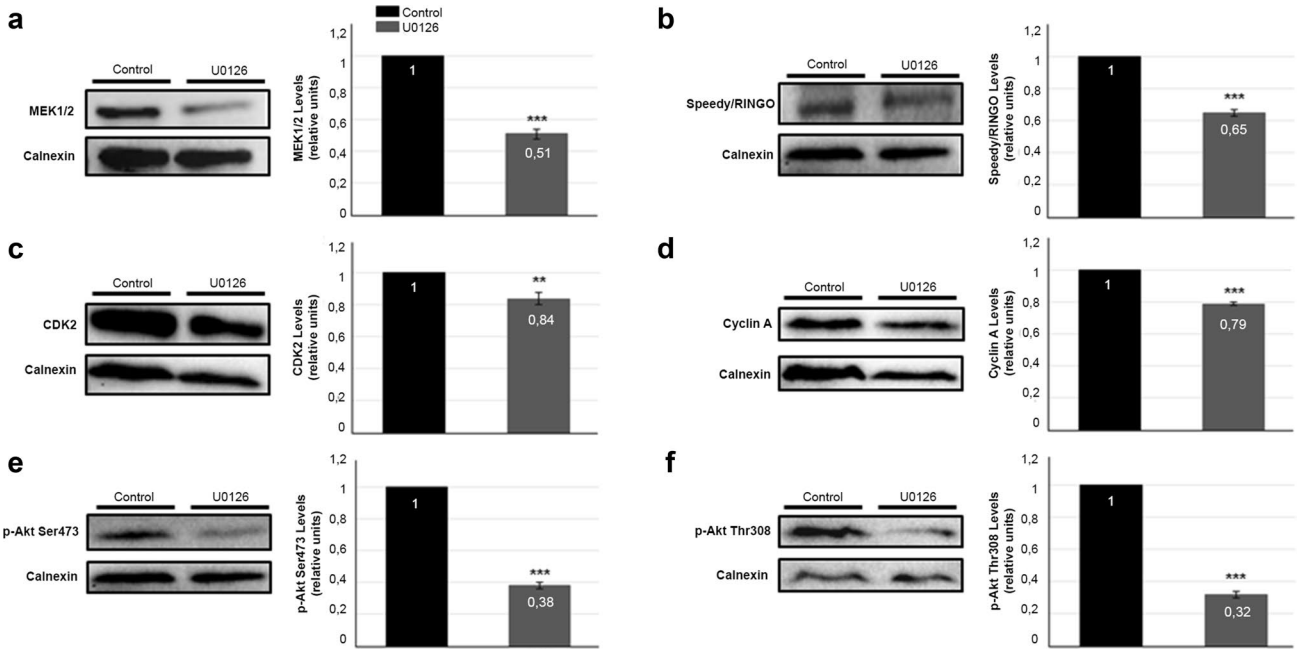

Fig. 3 Effect of inhibition ERK/MAPK signaling pathway on MEK1/2, SPDYA, CDK2, Cyclin A, phospho-AKT Ser473 and phospho-AKT Thr308 protein expressions were analyzed by Western blot analysis (**a**, **b**, **c**, **d**, **e**, **f**). SH-SY5Y cells were treated by 10 mM U0126 for 48 h. Calnexin antibody used as an internal control. Graph indicating quantitative analysis of signals in blots. Results were represented as fold changes. Bar represents mean values ± SD (**$p < 0.01$, ***$p < 0.001$)

decreased very significantly by 62% and 68%, respectively (Fig. 3e, $p = 0.0000007$ ***$p < 0.001$; Fig. 3f, $p = 0.0000005$ ***$p < 0.001$).

U0126 inhibition of ERK/MAPK signaling and siRNA silencing of Speedy/RINGO both reduced the viability of SH-SY5Y cells

MTS analysis was performed to observe the effect of ERK/MAPK inhibition and Speedy/RINGO silencing on the vital activities of SH-SY5Y neuroblastoma cells. 36.28% viability rate of U0126-treated SH-SY5Y neuroblastoma cells (Fig. 4a, $p = 0.0000011$ ***$p < 0.001$) and 86.81%

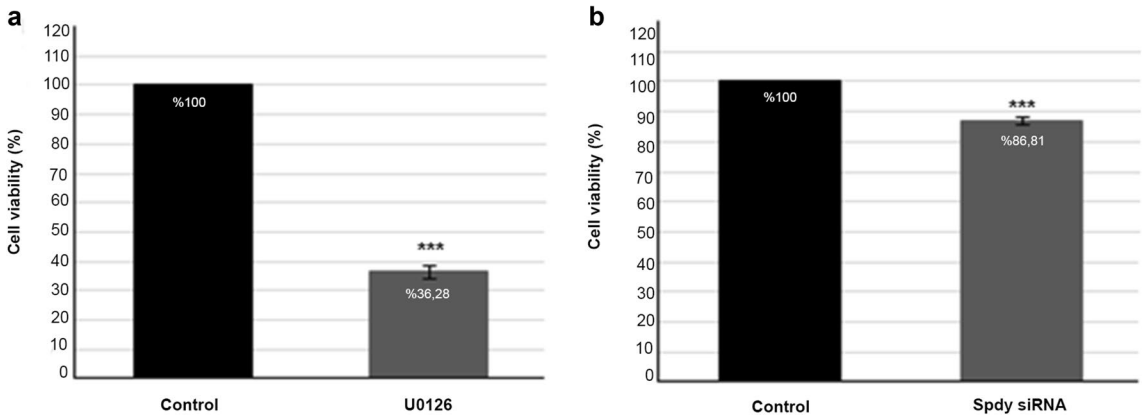

Fig. 4 Results of MTS cell viability assay. **a** Data are given as cell viability (%) in U0126-treated SH-SY5Y cells and in negative control SH-SY5Y cells for 48 h. Bar represents mean values ± SD. **b** Data are given as cell viability (%) in Speedy/RINGO-specific siRNA-treated SH-SY5Y cells and in negative control SH-SY5Y cells for 48 h. Bar represents mean values ± SD (***$p < 0.001$)

viability rate of siRNA-treated SH-SY5Y neuroblastoma cells (Fig. 4b, $p = 0.00004$, $***p < 0.001$) were obtained and results showed that both treatments significantly reduced the viability of SH-SY5Y cells to different extents.

Discussion

The growth and survival of many cancer cells, including neuroblastoma cells, are known to be primarily due to the aberrant signaling of ERK/MAPK and PI3K/AKT pathways, as well as the overexpression of oncogenic protein Speedy/RINGO. Moreover, their abnormal activation becomes extremely important especially for neuroblastoma cancer since it leads to chemotherapy resistance and reduced therapeutic efficacy [16]. At this point, considering the almost identical role of Speedy/RINGO, ERK/MAPK and PI3K/AKT pathways in carcinogenesis, as well as the controllable nature of these pathways by effector molecules, it is worthwhile to explore the potential of Speedy/RINGO as an effector molecule for ERK/MAPK and PI3K/AKT pathways. Moreover, there are suggestive studies implying that various cell cycle regulators such as p53 and Speedy/RINGO which is known to be overexpressed in many tumors may have effect on ERK/MAPK and PI3K/AKT pathways [25].

To analyze the possible interaction between Speedy/RINGO, ERK/MAPK and PI3K/AKT pathways, we successfully silenced Speedy/RINGO gene expression using siRNA method in SH-SY5Y neuroblastoma cells (Fig. 2a). Silencing Speedy/RINGO gene caused a significant decrease in MEK1/2 expression level in SH-SY5Y cells (Fig. 2b). This result is a first-time indication of the interaction between Speedy/RINGO and the ERK/MAPK signaling pathway in SH-SY5Y neuroblastoma cells. Although this result shows the presence of an interaction between them, the molecular basis of this interaction needs to be further explored to reveal whether there occurs a direct or indirect interaction between Speedy/RINGO and the components of ERK/MAPK signaling.

On the other hand, levels of Speedy/RINGO-related proteins, CDK2 and Cyclin A were shown to decrease subsequent to the inhibition of Speedy/RINGO (Fig. 2c, d) which is in concordance with the data that Speedy/RINGO promotes cell proliferation through the activation of CDK2 [26] in association with Cyclin A in the G1/S phase transition in different cell types including testis cells [20, 27].

Furthermore, considering that Cyclin A and CDK2 play an important role in the over-phosphorylation of AKT [23], we analyzed the amounts of AKT phosphorylation in SH-SY5Y cells with the expectation of a decrease. Immunoblotting results showed that AKT phosphorylation levels both on Ser473 and Thr308 sites were decreased (Fig. 2e, f). This result is remarkable in that it shows an indirect interaction between Speedy/RINGO and PI3K/AKT pathway via Cyclin A and CDK2 for the first time.

After examining the effects of Speedy/RINGO on ERK/MAPK and PI3K/AKT signaling pathways, we also examined the effect of ERK/MAPK inhibition on Speedy/RINGO expression in SH-SY5Y cells to get insight about the nature of interaction between Speedy/RINGO protein and the ERK/MAPK signaling in neuroblastoma cells. Our results showed that Speedy/RINGO was downregulated in SH-SY5Y neuroblastoma cells upon treatment with the MEK1/2 inhibitor U0126 (Fig. 3a, b) indicating a mutual interaction between Speedy/RINGO and ERK/MAPK pathway in SH-SY5Y neuroblastoma cells. With regard to this decrease in Speedy/RINGO expression, we determined a decrease in both CDK2 and Cyclin A protein levels in U0126-treated SH-SY5Y cells (Fig. 3c, d). These results showed for the first time that ERK/MAPK inhibition in SH-SY5Y neuroblastoma cells is effective on the expression levels of both Speedy/RINGO protein and Speedy/RINGO-related proteins, Cyclin A and CDK2.

Based on the decrease in Cyclin A and CDK2 levels, Ser473 and Thr308 phosphorylation levels of AKT were shown to be decreased significantly in U0126-treated SH-SY5Y cells (Fig. 3e, f). This result may also give a preliminary insight about the presence of a potential cascade beginning with MEK1/2 inhibition, continuing with the decrease in Speedy/RINGO, CDK2 and Cyclin A expressions, ending up with the decrease in AKT phosphorylation. Thus, the observed downstream cascade of MEK1/2 inhibition not only supports our hypothesis that Speedy/RINGO may have an effect on the ERK/MAPK and the PI3K/AKT signaling pathways but also takes it a step further by pointing out the possibility of a triple interaction between Speedy/RINGO and the two signaling pathways. However, this is yet a preliminary prediction, and further experiments should be conducted to obtain functional data to be precise about the existence of such a cascade.

Finally, MTS cell viability analysis indicated that U0126-treated SH-SY5Y neuroblastoma cells showed a 60% decrease in viability, while Speedy/RINGO-specific siRNA-treated SH-SY5Y neuroblastoma cells showed a 15% decrease in viability (Fig. 4a, b). The observed difference in the degree of the decrease in cell viability upon ERK/MAPK inhibition compared to Speedy/RINGO inhibition may be attributed to the degree of the resulting decrease in the phosphorylation levels of AKT. We determined that MAPK inhibition by U0126 caused a higher decrease in p-AKT levels compared to the decrease observed upon siRNA silencing of Speedy/RINGO which indicates a more active PI3K/AKT signaling in Speedy/RINGO-silenced cells. Considering that PI3K/AKT pathway is the primary survival pathway for the cells, it is reasonable to have a relatively low level of decrease in cell viability of Speedy/RINGO-silenced

neuroblastoma cells compared to both control and ERK/MAPK-inhibited cells.

In summary, our findings provide first-time evidence that anti-apoptotic mitotic regulator Speedy/RINGO interacts with ERK/MAPK and PI3K/AKT signaling pathways in SH-SY5Y neuroblastoma cells. Moreover, observed successive effects of MEK1/2 inhibition on Speedy/RINGO and PI3K/AKT signaling encouraged us to suggest a potential triple interaction between them which is also supported by the data obtained from STRING database (Fig. 1). However, it strongly requires further and detailed exploration.

Revealing the effectors of ERK/MAPK and PI3K/AKT signaling pathways which are known to be dysregulated in many cancer types, including neuroblastoma, is important to thoroughly understand the molecular basis of neuroblastoma, thereby choosing the strong potential candidates as therapeutic targets such as Speedy/RINGO.

Conclusion

In this study, we showed for the first time that anti-apoptotic mitotic regulator Speedy/RINGO is in relationship with ERK/MAPK and PI3K/AKT signaling pathways in SH-SY5Y neuroblastoma cells. In addition, it was determined that the interaction between Speedy/RINGO and ERK/MAPK pathway is reciprocal in SH-SY5Y cells. Moreover, the results of this study give the first insights suggesting a triple interaction network between Speedy/RINGO, ERK/MAPK and PI3K/AKT. These data provide significant implications on the utility of versatile molecules such as the Speedy/RINGO protein for the development of effective and solution-oriented treatment methods, especially for neuroblastoma. Therefore, this study will pave the way for future studies underlying the molecular events responsible for the dysregulation of ERK/MAPK and PI3K/AKT signaling pathways, which play a key role in the emergence of many cancers, especially neuroblastoma cancer.

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Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [AY], [YK] and [SK]. The first draft of the manuscript was written by [AY] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The authors confirm that the data supporting the findings of this study are available within the article.

Code availability In this study, no content that will require code availability.

Compliance with ethical standards

Conflicts of interest The authors declare that they there are no competing interests.

Ethical approval Ethic approval is not required for this study.

Consent to participate In this study, no content that will require consent to participate.

Consent for publication In this study, no content that will require consent for publication.

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