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Identification of Translocon-associated Protein Delta as An Oncogene in Human Colorectal Cancer Cells

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Identifying the roles of genes in cancer is critical in discovering potential genetic therapies for cancer care. Translocon-associated protein delta (TRAPδ), also known as signal sequence receptor 4 (SSR4), is a constituent unit in the TRAP/SSR complex that resides in the endoplasmic reticulum and plays a key role in transporting newly synthesized proteins into the endoplasmic reticulumn. However, its biological role in disease development remains unknown to date. This is the first study to identify the role of TRAPδ/SSR4 in colorectal cancer cells in vitro. Upon successful transient knockdown of *TRAPδ/SSR4*, we observed significant reduction of cell viability in all colorectal cancer cell lines tested. Both HCT 116 and SW480 cell lines were significantly arrested at S and G1 phases, while DLD-1 cells were significantly apoptotic. Moreover, *TRAPδ/SSR4* stable knockdown HCT 116 and SW480 cells showed significantly lower viability, anchorage-independent growth, and increased S and G1 phase arrests. Overall, we conclude *TRAPδ/SSR4* is a potential oncogene in human colorectal cancer cells.

Key Words Translocon-associated protein delta, Signal sequence receptor 4, Oncogene, Endoplasmic reticulum, Colorectal cancer

INTRODUCTION

The endoplasmic reticulum (ER) is a dynamic, complex cellular organelle that exists in all eukaryotes. It is the largest organelle in animal cells and is composed of a highly convoluted, continuous membrane system characterized by two distinct structural domains, namely 1) the nuclear envelope and 2) the peripheral ER, which itself consists of a network of rough sheets and smooth, branched, dynamic tubules [1,2]. Cellular functions of the ER include protein synthesis, protein folding and modification, protein transport for secretion, protein degradation, lipid and steroid biosynthesis, carbohydrate metabolism, detoxification of harmful substances, establishment of contact with other cellular organelles, and the storage and regulated release of calcium [2]. Especially, the ER membrane is crucial in transporting proteins synthesized by the ribosomes to target destinations such as the Golgi apparatus, cell membrane, lysosomes, endosomes, and outside the cell; this process is termed protein translocation [2,3].

Protein translocation in the ER can occur in two modes: 1) post-translational and 2) co-translational [3]. Co-translational translocation occurs through the Sec61 membrane protein complex; during this process, the translocon is accompanied by cytosolic protein chaperones, auxiliary components, and modifying enzymes. One such auxiliary complex is the translocon-associated protein (TRAP) complex, also known as the signal sequence receptor (SSR) complex [3,4]. The TRAP complex is thought to be expressed in the ER membrane of most eukaryotes, with a notable apparent exception in *Saccharomyces cerevisiae*. Algae and plants feature a heterodimeric TRAP complex, the two subunits of which are TRAPα/SSR1 and TRAPβ/SSR2. Interestingly, the human ER contains a heterotetrameric TRAP complex with the four subunits TRAPα/SSR1, TRAPβ/SSR2, TRAPγ/SSR3, and TRAPδ/SSR4 [5]. In humans, TRAPα/SSR1, TRAPβ/SSR2, and TRAPδ/SSR4 singly span the ER membrane, whilst TRAPγ/SSR3 has four spans [6]. In addition to facilitating protein translocation, the TRAP complex is crucial in embryonic development and insulin biosynthesis [3,7]; however, its roles in diseases/disorders have not yet been fully elucidated.

Recent literature shows that pathologies such as cancer are closely tied to the ER and its functions. For example, cancer cells are often associated with extrinsic and intrinsic stresses (e.g., nutrient depletion, low pH, hypoxia, reactive

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oxygen species (ROS) production, and oncogene activation) that lead to excessive build-up of unfolded or misfolded proteins in the ER lumen, thereby causing ER stress [8,9]. In light of this, we hypothesized that ER-membrane-resident proteins can potentially have roles in cancer development. Interestingly, in 2022, a group of researchers showed that *TRAPδ/SSR4* messenger ribonucleic acid was highly expressed in colon adenocarcinoma, and this high expression was associated with metastasis [10]. However, no study to date has examined the biological activity of TRAPδ/SSR4 in any type of disease model. This study serves as the first and only so far to determine the role of TRAPδ/SSR4 in cancer, with the specific objective of identifying the role of TRAPδ/SSR4 in colorectal cancer cells in vitro.

MATERIALS AND METHODS

Materials

Human colorectal cancer cell lines (HCT 116, SW480, and DLD-1) were purchased from the American Type Culture Collection. Propidium iodide (PI)/ribonuclease A (RNAse A) staining buffer was purchased from BD Biosciences. The TACS™ annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was purchased from R&D Systems, Inc. The ROS-Glo™ hydrogen peroxide (H_2O_2) assay kit was purchased from Promega Corporation. Protease and phosphatase inhibitor cocktail was purchased from Sigma-Aldrich Inc. Primary antibodies for cyclin-dependent kinase 2 (CDK2-# 2546), CDK4 (# 12790S), Cyclin D1 (# 2978), Cyclin A2 (# 67955T), and β-actin (# 5125) were purchased from Cell Signaling Technology, Inc. The primary antibody for TRAPδ/SSR4 (# 11655-2-AP) was purchased from Proteintech Group, Inc. Anti-rabbit immunoglobulin G (# 7074) was purchased from Cell Signaling Technology, Inc. Control and *TRAPδ/SSR4* small interfering ribonucleic acids (siRNAs; control siRNA-A #sc-37007 and *TRAPδ/SSR4* #sc-63148, respectively) were purchased from Santa Cruz Biotechnology, Inc. Each siRNA was diluted to prepare a 10 mM stock solution according to the manufacturer's protocol, aliquoted, and stored at –20°C until use. Control and *TRAPδ/SSR4* short hairpin ribonucleic acid (shRNA) lentiviral particles (sc-108080 and sc-63148-V, respectively) and polybrene (i.e., infection reagent; sc-134220) were purchased from Santa Cruz Biotechnology, Inc. All cell culture and transfection reagents and other chemicals were purchased from Fisher Scientific International Inc. unless otherwise specified.

Cell culture and *TRAPδ/SSR4* transient transfection

Colorectal cancer cells were cultured using Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator (5% carbon dioxide and 37°C) and seeded at least 16 hours prior to each experiment. For transient transfection, cells were transfected with 100 nM of control and *TRAPδ/SSR4* siRNA using Lipofectamine™ 3000 as the transfection reagent according to the reagent manufacturer's protocol. Transfected cells were incubated at 37°C for 48 hours prior to further analysis.

TRAPδ/SSR4 knockdown stable cell line establishment

Stable knockdown cells for *TRAPδ/SSR4* were established using shRNA lentiviral particles. HCT 116 and SW480 cells were infected according to the manufacturer's protocol with control and *TRAPδ/SSR4* shRNA lentiviral particles to produce wild-type and *TRAPδ/SSR4* stable cells, respectively. Infected cells were screened using 10 μg/μL of puromycin in cell culture media for 14 to 21 days. Wild-type and *TRAPδ/SSR4* stable cells were maintained in DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin, and kept free from mycoplasma (Figure S1).

Cell viability

Cell viability was determined using the MTT assay. After knocking down *TRAPδ/SSR4*, cells were treated with 100 μL of MTT solution diluted in DMEM (1:9 ratio) for two hours in 37°C. Following removal of the MTT mixture, 100 μL of dimethyl sulfoxide was added to each well, and the absorbance was measured at 540 nm using a microplate reader (Bio-Tek Instruments Inc.).

Cell cycle distribution

Cell cycle distributions were determined using flow cytometry. After knocking down *TRAPδ/SSR4*, cells were harvested by trypsinization and fixed with 70% ethanol in PBS. Next, the fixed cells were washed serially using the following reagents: 1) 50% ethanol in PBS, 2) 20% ethanol in PBS, and 3) PBS. After washing, the cells were stained with PI/RNAse staining buffer and the cell cycle distribution was analyzed using a BD LSRFortessa™ system (BD Biosciences).

Annexin V-FITC and PI staining

Apoptosis was assayed using the TACS™ annexin V-FITC apoptosis detection kit. After knocking down *TRAPδ/SSR4*, cells were harvested by trypsinization and washed twice with PBS. Washed cells were resuspended in Annexin V-FITC and PI staining buffer and incubated for 15 minutes according to the manufacturer's protocol. Next, the resuspended cells were diluted according to the manufacturer's protocol, and the live and dead cell populations were quantified using a BD LSRFortessa™ system.

ROS measurement

Cellular levels of ROS were measured using the ROS-Glo™ H_2O_2 assay kit according to the manufacturer's protocol. After knocking down *TRAPδ/SSR4*, cells were incubated for 48 hours. Approximately six hours prior to the completion of the

incubation, a derivatized luciferin substrate was added to the cells, allowing it to react with cellular H_2O_2. Once the incubation was complete, the detection solution was added to cells to produce a light signal, which was proportional to the H_2O_2 level. Luminescence readings were obtained using a microplate reader and were validated to reflect the H_2O_2 levels produced by live cells.

Protein lysate preparation and western blotting

After knocking down *TRAPδ/SSR4*, cells were washed twice with PBS and lysed using radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors. After collecting the protein lysate, the protein concentrations were determined using the bicinchoninic acid assay. Proteins were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane using the wet transfer method. Upon successful protein transfer, the membrane was blocked with 5% skim milk powder diluted in TBS tween-20 (TBST) for one hour at room temperature followed by thorough washing using TBST. Next, the membrane was incubated with the diluted primary antibody (according to the manufacturer's protocol) at 4°C overnight. Next, the membrane was washed thoroughly and incubated with the secondary antibody (made according to the manufacturer's protocol) for one hour at room temperature. After washing the membrane thoroughly, the target proteins were detected using enhanced chemiluminescence detection solution and H_2O_2 (6 mL:2 μL). Images were photographed using a Chemidoc MP Imaging System (Bio-Rad).

Anchorage-independent growth

Anchorage-independent growth in *TRAPδ/SSR4* knockdown stable cell lines was determined by the soft agar colony formation assay. A bottom layer containing 0.3% agar and cell culture medium was prepared and solidified in a six-well culture plate. Wild-type and *TRAPδ/SSR4* stable cells suspended in 0.3% agar and cell culture medium were added onto the bottom layer, solidified, and incubated in a humidified incubator (5% carbon dioxide and 37°C). Two hundred μL of cell culture medium was added to each well twice every week. Cells were cultured for 14 days, stained using 0.1% crystal violet in 10% ethanol for 30 minutes, and rinsed with water for removal of extra stain. Stained colonies were photographed using a Chemidoc MP Imaging System.

Statistical analysis

Statistical analysis was performed using Microsoft Excel software for Microsoft 365 (version 2405, Microsoft). All values were presented as means of three replicates along with the SD. Means were separated using Student's *t*-test, and significance differences were recorded as $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

RESULTS

Transient transfection led to significant knockdown of *TRAPδ/SSR4* in colorectal cancer cells

Western blotting data for the three colorectal cancer cell lines (HCT 116, SW480, and DLD-1) showed a remarkable decrease in TRAPδ/SSR4 protein post-transfection with *TRAPδ/SSR4* siRNA for 48 hours. Particularly, SW480 cells showed the highest inhibition of TRAPδ/SSR4 with 85% inhibition ($P < 0.001$; Fig. 1B), followed by HCT 116 cells with 79% inhibition ($P < 0.001$; Fig. 1A) and DLD-1 cells with 58% inhibition ($P < 0.01$; Fig. 1C). Thus, it was evident that the transfection was highly successful in silencing *TRAPδ/SSR4* leading to very little expression of TRAPδ/SSR4 protein. This observation confirmed the validity of using the commercial *TRAPδ/SSR4* siRNA to successfully knock down the *TRAPδ/SSR4* gene.

TRAPδ/SSR4 knockdown suppressed colorectal cancer cell viability

Interestingly, MTT results showed a highly significant cell viability suppression in all three colorectal cancer cells after knocking down *TRAPδ/SSR4*. SW480 cells showed the highest inhibition (i.e., 50%) for viability, with only 50% of cells being viable ($P < 0.001$) after silencing *TRAPδ/SSR4* (Fig. 2B). Meanwhile, approximately, 33% and 20% viability inhibitions were noted for HCT 116 ($P < 0.001$) and DLD-1 cells ($P < 0.05$), respectively (i.e., only 67% of HCT 116 and 80% DLD-1 cells were viable; Fig. 2A and 2C). This observation primarily implied the pro-tumorigenic role of TRAPδ/SSR4 in colorectal cancer cells.

TRAPδ/SSR4 knockdown induced growth arrest and apoptosis in colorectal cancer cells

Cell viability is usually suppressed by means of growth arrest in mitosis and/or cellular death; therefore, we investigated the cell cycle distribution and employed Annexin V-FITC and PI staining to determine which cellular events are associated with our observations of reduced cell viability. The cell cycle distribution assay showed significant induction of S-phase for HCT 116 cells ($P < 0.05$) and G1 phase for SW480 cells ($P < 0.05$) after knocking down *TRAPδ/SSR4*, with 1.24-fold induction in S phase for HCT 116 cells (Fig. 2D) and 1.13-fold induction in G1 phase for SW480 cells (Fig. 2E). Moreover, these observations were confirmed through expressions of growth phase-specific protein markers. Specifically, and as expected, we noted *TRAPδ/SSR4*-silenced HCT 116 cells to exhibit marked inhibitions decreased in the expression of CDK2 and Cyclin A2 proteins (52% and 88% inhibitions, respectively; Fig. 1A), which are responsible for moving cells from S phase to G2/M phase ($P < 0.001$ for both). Similarly, *TRAPδ/SSR4*-silenced SW480 cells exhibited a significant inhibitions reduction in CDK4 and Cyclin D1 proteins (30% and 35% inhibitions, respectively; Fig. 1B), which are responsible

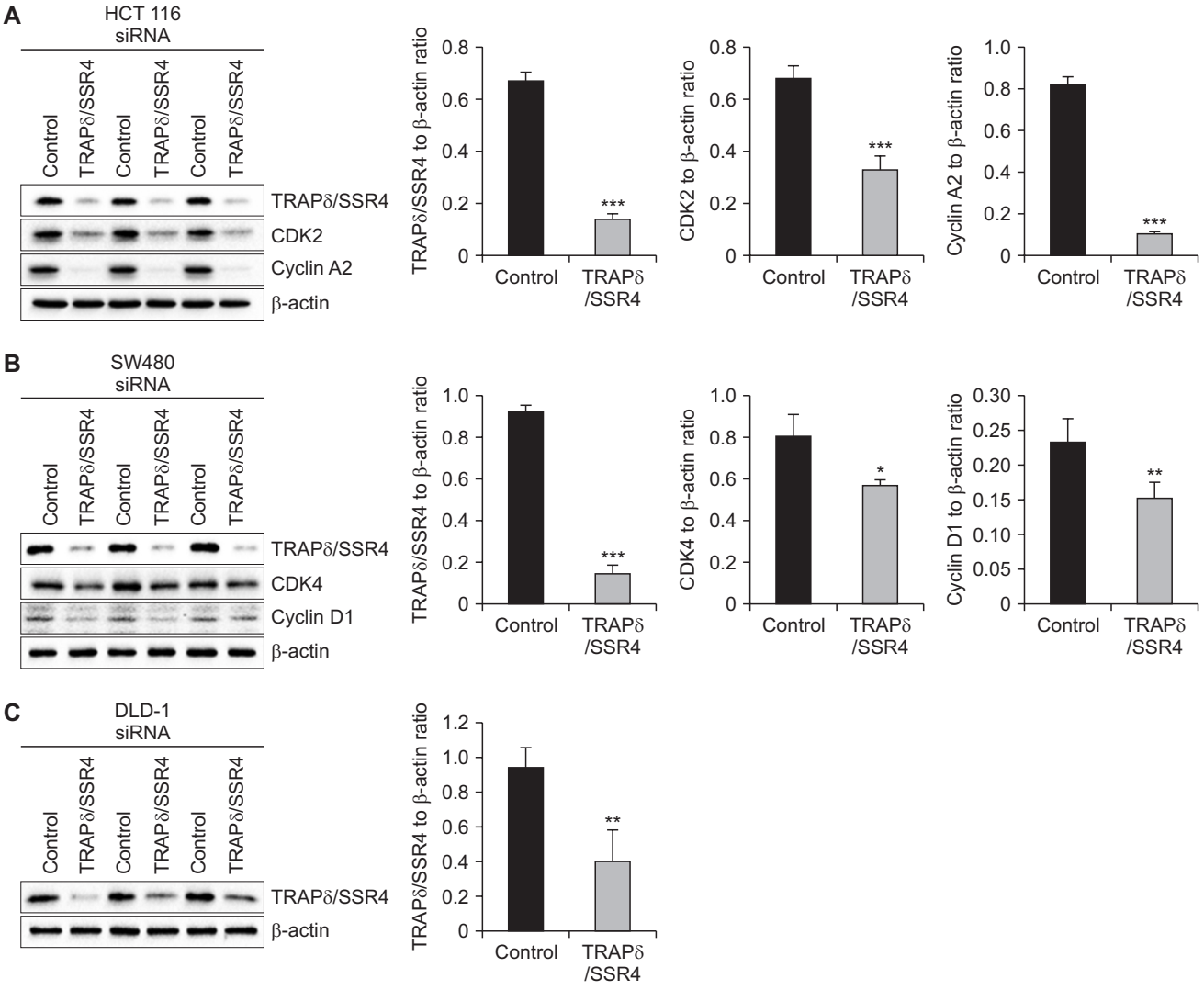

Figure 1. Effect of *TRAPδ/SSR4* siRNA knockdown on different protein expressions in colorectal cancer cells. Colorectal cancer cells were seeded on 6-well plates at least 16 hours prior to siRNA transfection. Cells were transfected with 100 nM of control and *TRAPδ/SSR4* siRNA using Lipofectamine™ transfection reagent (Fisher Scientific International Inc.) according to the reagent manufacturer's protocol. Cells were incubated for 48 hours at 37°C, harvested, and protein lysates were made. Western blotting data showed the expression of TRAPδ/SSR4 and cell cycle related protein markers in HCT 116 and SW480 cells (A, B) and the expression of TRAPδ/SSR4 in DLD-1 cells (C). Data are shown as means ± SD. TRAP, translocon-associated protein; SSR, signal sequence receptor; siRNA, small interfering ribonucleic acid; CDK, cyclin-dependent kinase. *P < 0.05, **P < 0.01, and ***P < 0.001.

for moving cells from G1 phase to S phase (P < 0.05 and P < 0.01, respectively).

In contrast, DLD-1 cells did not demonstrate any cell cycle arrest upon knocking down *TRAPδ/SSR4* via siRNA. However, there was a dramatic increase in the sub-G1 phase, which indicated that DLD-1 cells might undergo apoptosis when *TRAPδ/SSR4* is silenced. To test this hypothesis, we determined apoptosis via Annexin V-FITC and PI staining. The percentage of total apoptotic cells was three-folds higher in *TRAPδ/SSR4*-silenced cells compared to the control (P < 0.001; Fig. 2F). Upon closer examination, we noted that early apoptosis was increased by four-folds (P < 0.001) and late apoptosis by three-folds (P < 0.01).

HCT 116 cells with stable *TRAPδ/SSR4* knockdown exhibited suppressed viability, anchorage-independent growth, and induced S phase arrest

Western blotting data for *TRAPδ/SSR4* knockdown HCT 116 stable cells showed a significant inhibition in of TRAPδ/SSR4 protein expression (79%) at P < 0.001 (Fig. 3A). Upon confirming successful *TRAPδ/SSR4* knockdown, MTT and soft agar colony formation assays were carried out to determine the cell viability and anchorage independent growth. Viability of HCT 116 stable cells significantly decreased by 22% over five days (P < 0.001; Fig. 3B). Moreover, anchorage-independent growth was dramatically decreased, with both the

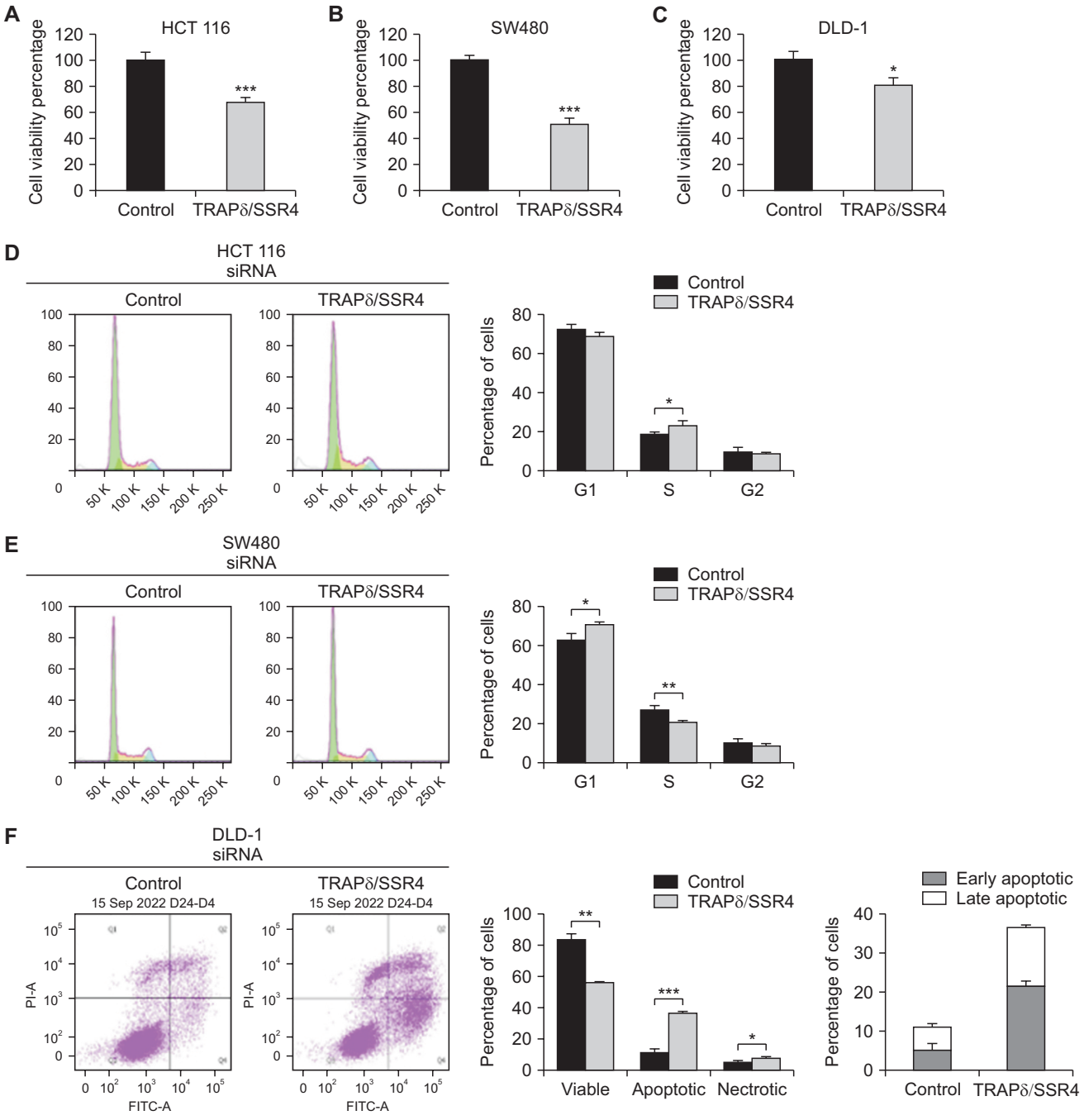

Figure 2. Effect of *TRAPδ/SSR4* siRNA knockdown on viability, cell cycle arrest, and apoptosis in colorectal cancer cells. Colorectal cancer cells were seeded on 96-well plates for viability assay and 6-well plates for cell cycle arrest and apoptosis assays at least 16 hours prior to transfection. Cells were transfected with 100 nM of control and *TRAPδ/SSR4* siRNA using Lipofectamine™ transfection reagent (Fisher Scientific International Inc.) according to the reagent manufacturer's protocol and incubated for 48 hours at 37°C. MTT assay was carried out to determine the viability percentage of HCT 116, SW480, and DLD-1 cells (A, B, C). Cell cycle distributions for HCT 116 and SW480 cells (D, E) were determined by flow cytometry and apoptosis in DLD-1 cells (F) was determined by annexin V-FITC and PI staining. Data are shown as means ± SD. TRAP, translocon-associated protein; SSR, signal sequence receptor; siRNA, small interfering ribonucleic acid; FITC, fluorescein isothiocyanate; PI, propidium iodide. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

colony number and the size drastically reduced. Compared to the wild type, stable HCT 116 cells with TRAPδ/SSR4 knockdown showed 92% significant inhibition of colony formation (Fig. 3C). In addition, HCT 116 stable cells exhibited a significant degree of S phase arrest at $P < 0.05$ (Fig. 3D) with 64% inhibition of Cyclin A2 expression ($P < 0.01$; Fig. 3A).

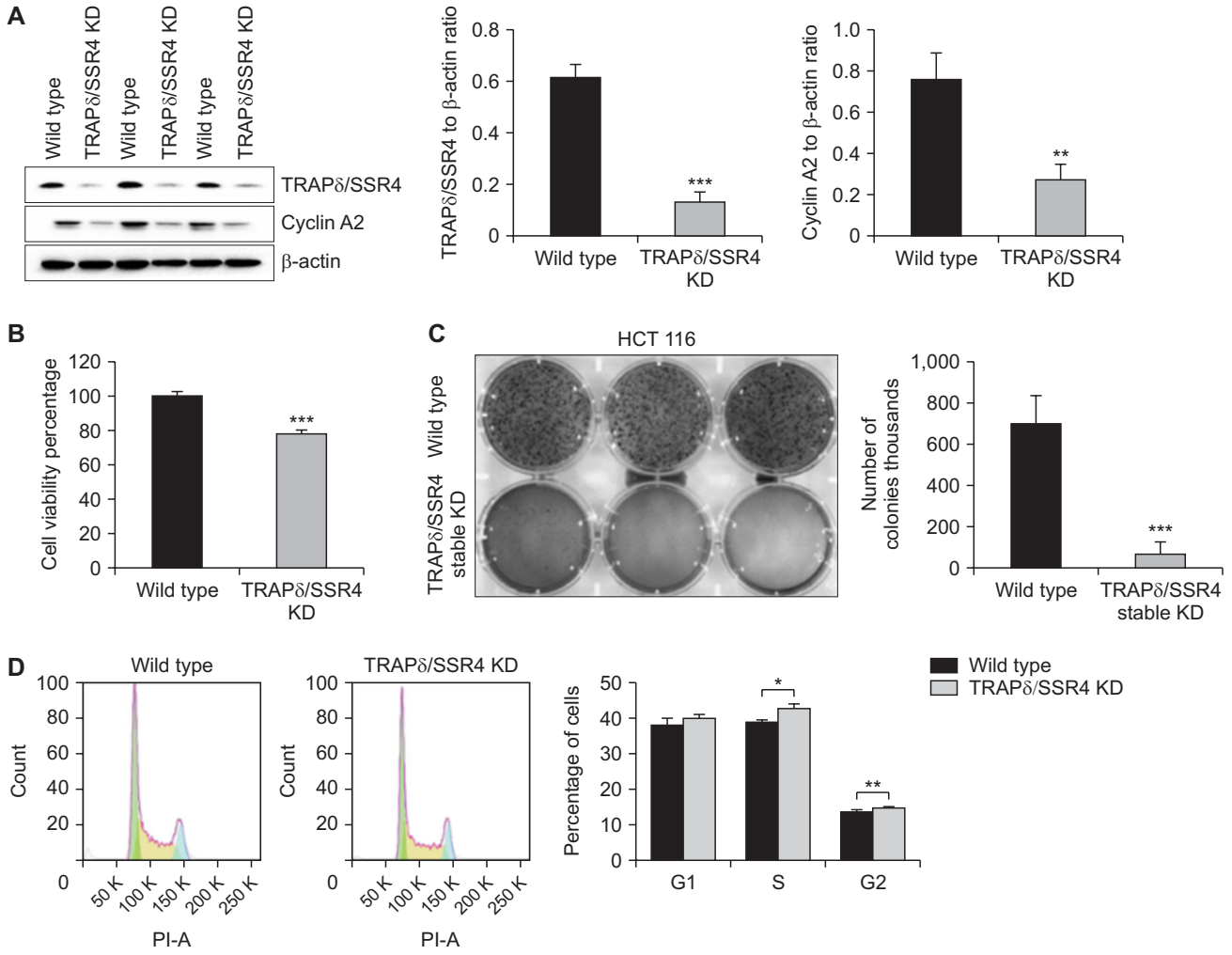

Figure 3. Protein expressions, viability, colony formation, and cell cycle arrest in *TRAPδ/SSR4* stable HCT 116 cells. Wild-type and *TRAPδ/SSR4* knockdown stable HCT 116 cells were seeded on 96-well plates for viability assay and 6-well plates for protein expression, soft agar colony formation, and cell cycle distribution assays. Western blotting images demonstrated the expression of TRAPδ/SSR4 and cell cycle related protein markers (A). MTT assay was conducted to determine the viability percentage (B), soft agar colony formation assay to determine anchorage independent growth (C), and flow cytometry to determine cell cycle arrest (D) for *TRAPδ/SSR4* stable HCT 116 cells. Data are shown as means ± SD. TRAP, translocon-associated protein; SSR, signal sequence receptor. PI, propidium iodide. *P < 0.05, **P < 0.01, and ***P < 0.001.

SW480 cells with stable *TRAPδ/SSR4* knockdown exhibited suppressed viability, anchorage-independent growth, and induced G1 phase arrest

Western blotting data for *TRAPδ/SSR4* knockdown SW480 stable cells showed a significant (P < 0.05) inhibition of TRAPδ/SSR4 protein (48%; Fig. 4A). Upon confirming *TRAPδ/SSR4* knockdown, MTT and soft agar colony formation assays were conducted to determine the cell viability and anchorage independent growth. Viability of SW480 stable cells significantly decreased by 24% over five days (P < 0.001; Fig. 4B). In addition, anchorage-independent growth was decreased, with reductions in both the colony number and the size. Stable SW480 cells with TRAPδ/SSR4 knockdown showed 95% significant inhibition of colony formation compared to the wild type (Fig. 4C). As expected, SW480 stable cells exhibited a significant degree of G1 phase arrest at P < 0.05 (Fig. 4D) with 28% inhibition of CDK4 (P < 0.05; Fig. 4A). We noted an apparent inhibition of cyclin D1 expression in TRAPδ/SSR4 knockdown SW480 stable cells; however, upon statistical analysis, it was not significantly different from wild type (Fig. 4A).

TRAPδ/SSR4 knockdown increased ROS release

Interestingly, all *TRAPδ/SSR4* silenced cell lines generated significantly high levels of ROS in the form of H_2O_2. Of transiently transfected cells, HCT 116 generated three times more H_2O_2 than the control (P < 0.01), while SW480 and DLD-1 cells generated around twice more H_2O_2 (P < 0.01 and P < 0.001, respectively; Fig. 5A, 5B, and 5C). Similarly, both stable cell lines generated twice as much as those from

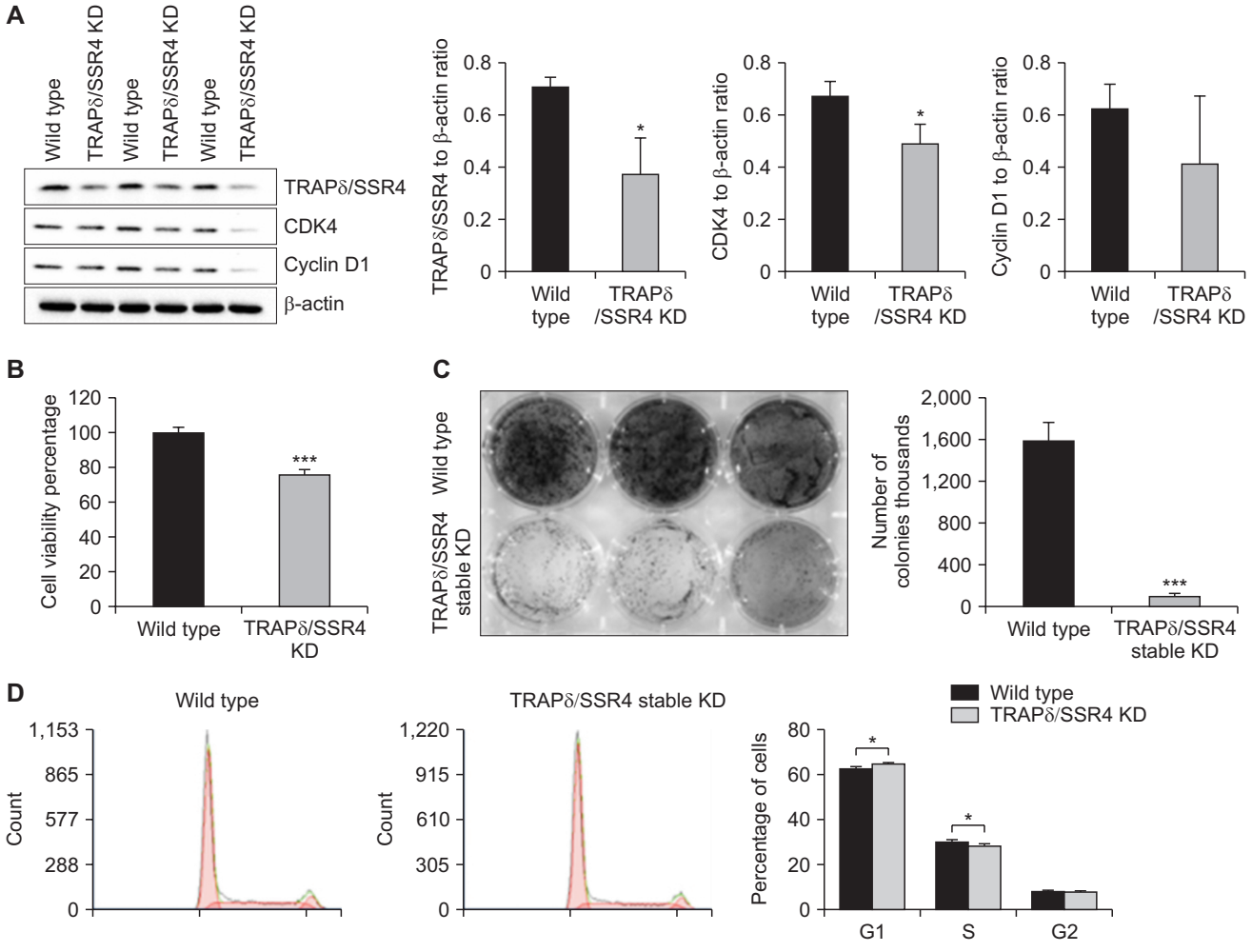

Figure 4. Protein expressions, viability, colony formation, and cell cycle arrest in *TRAPδ/SSR4* stable SW480 cells. Wild-type and *TRAPδ/SSR4* knockdown stable SW480 cells were seeded on 96-well plates for viability assay and 6-well plates for protein expression, soft agar colony formation, and cell cycle distribution assays. Western blotting images demonstrated the expression of TRAPδ/SSR4 and cell cycle related protein markers (A). MTT assay was conducted to determine viability percentage (B), soft agar colony formation assay to determine anchorage independent growth (C), and flow cytometry to determine cell cycle arrest (D) for *TRAPδ/SSR4* stable SW480 cells. Data are shown as means ± SD. TRAP, translocon-associated protein; SSR, signal sequence receptor; CDK, cyclin-dependent kinase. **P* < 0.05 and ****P* < 0.001.

wild type cells (*P* < 0.001; Fig. 5D and 5E). However, we noted that this event was not associated with mitochondrial dysfunction, because the cyanine dye JC-1 staining showed that *TRAPδ/SSR4* knockdown did not affect the mitochondrial membrane potential (ΔΨm; Figure S2).

DISCUSSION

The association of the TRAP/SSR complex with chronic diseases is intriguing, and hence is becoming an emerging subject of research, with a very limited number of studies investigating its involvement in disease development over the past decade. However, to date, the biological role of the TRAPδ/SSR4 subunit specifically in chronic disease models remains understudied. Indeed, our present study serves as is the first to define the function of TRAPδ/SSR4 in cancer, thereby becoming the only study so far that shows an association with a disease model.

Of the other TRAP/SSR subunits, TRAPβ/SSR2 has been studied quite extensively, especially for its implications in human melanoma and hepatocellular carcinoma. Clinical studies have demonstrated a negative correlation between *TRAPβ/SSR2* transcript levels and the survival of primary melanoma and hepatocellular carcinoma patients. Moreover, due to the cell proliferative, colony formation, and anti-apoptotic functions of TRAPβ/SSR2 protein, the *TRAPβ/SSR2* gene has been identified to promote tumorigenesis in human melanoma and hepatocellular carcinoma [11,12].

Our approach in this study to determine the role of TRAPδ/SSR4 on in the development and progression of colorectal cancer cells is more or less similar to the above-mentioned studies on TRAPβ/SSR2 in human melanoma and hepato-

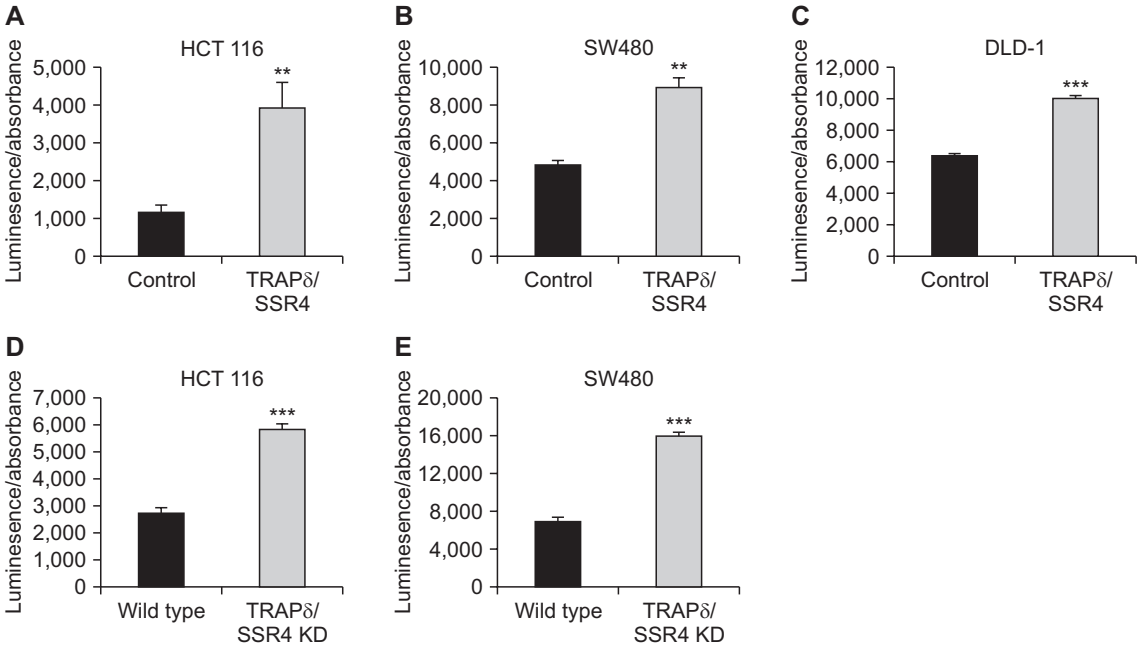

Figure 5. ROS production after silencing *TRAPδ/SSR4* in colorectal cancer cells. Upon silencing *TRAPδ/SSR4* either transiently or stably, ROS production was determined using ROS-Glo™ H_2O_2 assay kit (Promega Corporation) according to the manufacturer's protocol. ROS production 48 hours post-transfection of *TRAPδ/SSR4* siRNA in HCT 116, SW480, and DLD-1 (A, B, C). ROS production in *TRAPδ/SSR4* stable HCT 116 and SW480 cell lines (D, E). Data are shown as means ± SD. TRAP, translocon-associated protein; SSR, signal sequence receptor; ROS, reactive oxygen species. $^{**}P < 0.01$ and $^{***}P < 0.001$.

cellular carcinoma; however, there are distinct differences. Based on the key findings of He et al. [10], we hypothesized that *TRAPδ/SSR4* may possess oncogenic properties at the cellular level in colorectal cancer cells. Thus, our first step was to observe the basal expression of TRAPδ/SSR4 protein in colorectal cancer and normal colon cells. We screened the following eight cell lines and noted that they all had high basal expression of TRAPδ/SSR4 protein: HCT 116, SW480, SW620, HT-29, HCT-15, LoVo, Caco-2, and CCD-18Co (Figure S3). The basal expression of TRAPδ/SSR4 was not different across the above cell lines. Thus, all eight cell lines are likely candidates for cell models of *TRAPδ/SSR4* knockdown. This data is comparable to the study by He et al. [10] showing that *TRAPδ/SSR4* mRNA expression was significantly higher in colorectal adenocarcinoma tissues compared to normal tissues, and particularly high in tumor infiltrating lymphocytes in colorectal adenocarcinoma patients, suggesting that this might be associated with lymph node metastasis.

Next, we designed successive experiments to silence *TRAPδ/SSR4* temporarily (via siRNA transfection) or permanently (via establishing knockdown stable cell lines using shRNA lentiviral particle transduction). We selected three cell lines for siRNA transfection (i.e., HCT 116, SW480, and DLD-1) and two for establishing mycoplasma-free, *TRAPδ/SSR4* knockdown stable cells (HCT 116 and SW480). All these cell lines showed a remarkable decrease in cell viability following *TRAPδ/SSR4* knockdown, suggesting that this phenotype is a common cellular event across colorectal cancer cells, and hence *TRAPδ/SSR4* might potentially play an oncogenic role in colorectal cancer. However, further experiments remain required to cement this conclusion.

Cell viability is primarily determined by two mechanisms: 1) cellular growth and division and 2) cellular death. Cell growth and division is a cyclic process with four phases: 1) Gap 1 (G1 phase), 2) Synthesis (S phase), 3) Gap 2 (G2 phase), and 4) Mitosis (M phase). Upon aberrant activity or manipulation, cell cycle arrest can occur at any phase, and the cell no longer continues growth and division. In the present study, we noted that HCT 116 and SW480 cells respectively undergo S and G1 phase arrest upon knocking down of *TRAPδ/SSR4*. During G1 phase, a cell grows and prepares for DNA synthesis, while during S phase, it duplicates its DNA; thus, TRAPδ/SSR4 knockdown may hinder the overall DNA synthesis process, leading to inhibition of cell growth. Moreover, the G1 phase is dependent on CDK4 and Cyclin D1 (i.e., proteins that initiate cell preparation for DNA synthesis and regulate G1 phase exit), whereas the S phase is dependent on CDK2 and Cyclin A1 (i.e., proteins that initiate DNA synthesis and regulate S phase exit). Interestingly, results of this study imply that proteins associated with G1 and S phases may be regulated by TRAPδ/SSR4, thereby causing growth arrest in colorectal cancer cells. In addition, our results clearly showed that TRAPδ/SSR4 dependent cell cycle arrest is cell specific. For example, regardless of the TRAPδ/SSR4 silencing method, we observed that HCT 116 cells were arrested at the S phase, whilst SW480 cells were arrested at the G1

phase. Though this observation appears to be unique, it is not uncommon. For example, Wang et al. [13] observed that silencing coatomer protein complex subunit β2 (i.e., a protein that assists formation of intra-cellular transport vesicles) arrested RKO cells at G1 phase, whereas HCT 116 cells were arrested at the S phase.

The cell cycle distribution assay for DLD-1 cells did not show arrest at any phase; however, the sub-G1 phase was highly induced (Figure S4). Sub-G1 phase is a representation of loss of DNA (which could be a result of apoptosis and other cell death mechanisms such as pyroptosis [14]) preceding G1 phase. Thus, the significantly low number of cells entering G1 phase might have not been effective enough to demonstrate any cell cycle arrest. In addition, other researchers have also observed varying extent of cellular death among HCT 116, SW480, and DLD-1 cells. For example, Li et al. [15] observed DLD-1 cells yielded higher cellular death (caused by apoptosis, pyroptosis, necroptosis) compared to HCT 116 and SW480 cells in the presence of inflammatory cytokines. The main reason for these observations could potentially be the diversity of a cellular genetic profile. Of these three cell lines, HCT 116 cells express wild type adenomatous polyposis coli (APC), tumor suppressor protein p53 (TP53), and B-Raf proto-oncogene, serine/threonine kinase (BRAF). However, DLD-1 cells express mutated APC and TP53 and wild type BRAF while SW480 cells have mutations in all APC, TP53, and BRAF [16].

Another probable mechanism for the decreased cell viability in the present study could be the increases in early and late apoptosis; however, this mechanism needs to be further elucidated since we did not observe significant changes in protein markers related to apoptosis upon knocking down *TRAPδ/SSR4*. We measured several apoptotic proteins (such as Bax, Bak, and PARP) using Western blot, and observed that TRAPδ/SSR4 knockdown tends to increase the expression of Bax apoptotic protein marker without statistical significance (Figure S5). A key cellular event that distinguishes benign and malignant tumors is anchorage-independent growth in the latter. This allows cancer cells to proliferate even without extracellular matrix proteins to adhere to [17]. We demonstrated decreased anchorage-independent growth in *TRAPδ/SSR4* knockdown stable cells through soft agar colony formation assay. This cellular event was in accordance with the decreased cell viability and cellular growth arrest, demonstrating that TRAPδ/SSR4 is an ER-resident protein possessing oncogenic properties.

The ER is one of the major organelles producing intracellular H_2O_2, a non-radical form of ROS. It contributes to 45% of intracellular H_2O_2, followed by peroxisomes (35%) and mitochondria (15%) [18]. We found that all cell lines showed increased ROS release upon silencing *TRAPδ/SSR4*. We speculate that an increase of ROS might be associated with the increase of cell cycle arrest and apoptosis, because many studies claimed that both cell cycle arrest and apoptosis are induced by ROS generation in colorectal cancer cells [19,20]. In parallel with this, we measured the ΔΨm to see if TRAPδ/SSR4 knockdown-induced ROS release is associated with mitochondrial dysfunction; however, we observed no difference (Figure S2).

Since the ER is a major organelle producing intracellular H_2O_2, a non-radical form of ROS, which contributes to 45% of intracellular H_2O_2, followed by peroxisomes, and mitochondria [18], we propose that the ROS released by TRAPδ/SSR4 knockdown could be majorly from the ER. The main contributor for ER H_2O_2 is oxygen utilizing enzymes at the ER membrane or lumen [21]. These non-radical forms are scavenged by glutathione, a molecule that is transported from the cytosol to the ER through Sec61 mediated pathway—a key protein complex closely associated with TRAP/SSR complex during translocation [22]. Based on these observations, we speculate that TRAPδ/SSR4 knockdown might hinder the Sec61-mediated glutathione recruitment into the ER, thereby enhancing the ROS accumulation. If true, this could be one of the mechanisms by which *TRAPδ/SSR4* silenced cells trigger growth arrest or undergo apoptosis. According to previous research, TRAPδ/SSR4 has been identified as one of the subunits in the TRAP/SSR complex that might possess an isoform-specific function. Phoomak et al. [23] knocked out TRAPδ/SSR4 in A549 (a lung cancer cell line) and HEK-293–Halo1N-Cas9 (epithelial like cells from kidneys) cell lines to examine the possibility that the loss of TRAPδ/SSR4 could induce the dissolution and degradation of other TRAP/SSR subunits. However, they noted that knocking out TRAPδ/SSR4 did not eliminate the other TRAP/SSR subunits in both cell lines. Since the TRAP/SSR complex is ubiquitously expressed in eukaryotic cells, it is safe to assume that knocking down TRAPδ/SSR4 does not dissociate or degrade the other TRAP/SSR subunits in colorectal cancer cells.

To see if reintroduction of TRAPδ/SSR4 reverses the effects of TRAPδ/SSR4 knockdown, we cloned the coding sequence of TRAPδ/SSR4 into pcDNA™3.1/V5-His TOPO™ TA expression vector (Thermo Fisher Scientific; Figure S6), overexpressed it in TRAPδ/SSR4 knockdown stable cell lines, and compared the cell viability. The results indicated that the overexpression of TRAPδ/SSR4 did not reverse the decreased cell viability in HCT116 and SW480 TRAPδ/SSR4 knockdown stable cells (Figure S7). Moreover, Western blotting images showed that reintroduction of TRAPδ/SSR4 did not restore the TRAPδ/SSR4 to its original extent in wild-type HCT 116 and SW480 cells (the reintroduction of TRAPδ/SSR4 was verified through V5—the tag protein; Figure S7). Due to the very low level of exogenous TRAPδ/SSR4 (compared to the endogenous level of TRAPδ/SSR4), we speculate that it was not able to at least partially reverse the phenotypes observed in the TRAPδ/SSR4 knockdown conditions (e.g., decreased cell viability).

In summary, our study is the first to discover the role of TRAPδ/SSR4 related to cancer. Decreased expression of

this protein suppresses viability and anchorage independent growth, whilst causing cell cycle arrest or apoptosis in colorectal cancer cells. Thus, we are confident that TRAPδ/SSR4 is an oncogenic ER-resident protein.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

SUPPLEMENTARY MATERIALS

Supplementary materials can be found via https://doi.org/10.15430/JCP.24.014.

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