# Collapsin response mediator protein 4 enhances radio-sensitivity through calcium-mediated cell signaling

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# Abstract

**Background**: Radiation therapy, an effective treatment modality against various types of cancer including colorectal cancer, reduces local recurrence rate despite damaging both normal and cancer cells. However, the presence of cancer cells resistant to radiation therapy remains a major therapeutic obstacle; thus, understanding the mechanisms underlying radiation resistance is an important step toward achieving successful outcomes in cancer treatment. Hence, in the present study, radioresistant cell lines were established and the radiation-induced genetic changes associated with radiation resistance were examined.

**Methods**: We generated radioresistant colorectal cancer cell lines and subjected them to RNA sequencing. To know the relationship between CRMP4 downregulation and radiation resistance, western blotting and flow cytometry were used.

**Results**: *CRMP4* was identified as the candidate gene associated with radiation sensitivity. The intracellular $Ca^{2+}$ concentrations increased when cells were exposed to radiation, which in turn, initiated apoptosis. Decreased CRMP4 expression enhanced resistance to radiation and $Ca^{2+}$ ionophore A23187. Conversely, $Ca^{2+}$ deficiency by BAPTA-AM caused higher cell death in CRMP4-depleted cells than in CRMP4-expressing cells.

**Conclusion**: Our results indicated that CRMP4 influences $Ca^{2+}$ signaling pathways involved in apoptosis, and that CRMP4 is critical for radiation sensitivity in colorectal cancer as it can sensitize cancer cells to radiation therapy.


# Background

During cancer treatment, radiotherapy is used in conjunction with surgery to reduce the risk of recurrence and metastasis [1, 2]. Although radiotherapy may constitute a suitable treatment strategy for many cancer patients, the persistence of radiation-resistant tumor cells often poses a significant obstacle to an effective radiation-based therapy and leads to poor prognosis [3, 4]. Thus, understanding the mechanisms governing radiation resistance is a significant step toward enhancing the utility of radiotherapy.

Collapsin response mediator protein 4 (CRMP4), one of the five members of the cytosolic phosphoprotein family, is also known as dihydropyrimidinase-like protein 3 (DPYSL3) and shares 58% sequence homology with dihydropyrimidinase (DHPase) [5]. DHPase catalyzes the ring opening of 5,6-dihydrouracil to N-carbamyl-β-alanine and of 5,6-dihydrothymine to N-carbamyl-β-amino isobutyrate; however, whether CRMP4 demonstrates DHPase-like properties remains to be elucidated [6]. In contrast to the structure of DHPase, CRMPs have positively charged C-terminal that render them highly susceptible to proteolysis [7]. The C-terminal region of CRMP4 has been associated with neuronal cell injury and neurite damage [8]. Conversely, CRMP4 deletion *in vivo* exerts a neuroprotective effect against spinal cord injury owing to

decreased apoptotic cell death rate and suppressed inflammatory responses [9]. CRMP4 is thus considered an important therapeutic target for neuroregeneration. In addition, several studies have indicated that CRMP4 is involved in various types of cancers. For example, pancreatic and colorectal cancers (CRCs) show elevated CRMP4 expression, which strongly correlates with severe venous invasion, liver metastasis, and poor prognosis [10, 11]. Conversely, CRMP4 is regarded as a metastasis suppressor in prostate and breast cancer [12, 13]. These results indicate that a deeper analysis of CRMP4 function may offer new insights into potential cancer therapies.

The mitochondrial membrane potential (MMP) is the major component of the proton-motive force, which is the central intermediate of aerobic energy production and the driving force behind other physiological processes in the mitochondria, such as $Ca^{2+}$ uptake and antioxidant activity [14]. Cellular injury or stress stimulation directly elicits alterations in mitochondrial architecture, membrane potential, and oxidative capacity, which are associated with an irreversible loss of mitochondrial matrix contents and integral membrane protein constituents such as cytochrome c oxidase [15]. The release of cytochrome c from the mitochondria leads to the activation of caspase-3 and -9, resulting in apoptosis [16]. In turn, $Ca^{2+}$ ions serve as an important second messenger for multiple physiological processes [17]. Several studies have indicated that intracellular $Ca^{2+}$ levels are regulated by ionizing radiation [18]; moreover, the rise in intracellular $Ca^{2+}$ levels after exposure to radiation is crucial for a diverse array of signaling pathways that regulate critical cellular processes including apoptosis [19, 20]. $Ca^{2+}$ influx is facilitated by voltage- and ligand-gated $Ca^{2+}$ channels. Although CRMP4 has not been reported to be associated with $Ca^{2+}$ channels, CRMP2 was shown to interact with a novel N-type voltage-gated $Ca^{2+}$ channel [21, 22]; nevertheless, the functional role of $Ca^{2+}$ binding to CRMPs remains elusive.

In the present study, the RNA-seq technique was used to investigate radioresistant-associated genes in colorectal cancer. RNA-seq results showed *CRMP4* downregulation in the radiation-resistant cell lines, compared to the *CRMP4* expression level in their parental cell lines. Hence, the influence of CRMP4 on radiosensitivity was investigated. Additionally, the role of CRMP4 during the response of irradiated CRC cells to $Ca^{2+}$ influx stress remains unclear. In the present study, the relationship between CRMP4 and $Ca^{2+}$-mediated cell signaling was investigated to explain radiation sensitivity in CRC.

# Methods

### Cell culture

The CRC cell lines SW480 (KCLB No. 10228; colorectal adenocarcinoma), SW620 (KCLB No. 10227; colorectal carcinoma), HT-29 (KCLB No. 30038; colorectal adenocarcinoma), and RKO (ATCC CRL-2577; colorectal carcinoma) were obtained from the Korean Cell Line Bank (Seoul, Korea) and American Type Culture Collection (ATCC, USA). They were cultured in Dulbecco's modified Eagle medium (Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) and 100

μg/mL antibiotics (100 U/mL penicillin and 100 μg /mL streptomycin (Gibco) at 37 °C under an atmosphere of 5% $CO_2$ in a humidified incubator.

### Radiation and radioresistant cell line generation

Cells were seeded in 60-mm dishes and exposed to radiation from a $^{60}Co$ source (model 109 irradiator; JL Shepherd and Associates, San Fernando, CA, USA) at the indicated doses (0–5 Gy). Subsequently, cells were incubated at 37 °C under a humidified, 5% $CO_2$ / air atmosphere.

To generate radiation resistance CRC [23, 24] using SW620, RKO, SW480, and HT-29 cell lines, CRC cells were plated in 60-mm dishes at a density of $5 \times 10^3$ cells/dish and exposed to a 5-Gy dose of ionizing radiation, followed by a 15-day recovery period. This process was repeated for 24 treatment cycles totaling 120 Gy; finally, radioresistant IR-SW620, IR-RKO, IR-SW480, and IR-HT-29 cells lines were established.

### RNA sequencing analysis

The mRNAs of the established radioresistant IR-SW620, IR-SW480, IR-RKO, and IR-HT-29 cells were extracted using the Trizol (Sigma, St. Louis MO, USA) method. RNA was reverse-transcribed to cDNA followed by amplification and the resulting sequences were analyzed. Among the 25,207 genes, 70 genes were upregulated (>1.5 fold) and 45 genes were downregulated (<0.5 fold). These 115 genes were confirmed using RT-PCR analysis. Finally, the upregulated genes (*CXCR4*, *RAC2*, *HBE1*, *PTGDS*, and *LCN2*) and the downregulated genes (*SMO*, *RGS10*, *PRTFDC1,* and *CRMP4*) were identified as candidate genes associated with radiation resistance.

### RNA interference experiments

Small-interfering RNA (siRNA) duplexes of *CRMP4* were purchased from Bioneer (Daejeon, Korea). The specific target sequence of *CRMP4* siRNA was sense 5¢-GUG GAA GGA UUG UAG UCA UdTdT-3¢ and antisense 5¢-AUG ACU ACA AUC CUU CCA CdTdT-3¢. siRNA duplexes were transfected into cells using Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA). The short hairpin RNA (shRNA) targeting *CRMP4* was obtained from Origene (TL313373V; Rockville, MD, USA). The shRNA expression vector was transfected into the lentiviral packaging Lenti-X 293T cell line (Takara Bio USA, CA, USA). The culture supernatant containing virus particles was harvested 48-h post transfection. For the stable transduction of lentivirus, cells at 60 to 70% confluence were grown in 6-well plates. After 48 h, 1 μg/mL puromycin (Clontech, Mountain View, CA, USA) was added for selection.

### Western blot analysis

Cells were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP40, 0.25% sodium deoxycholate, 1 mM PMSF, protease inhibitor mixture (Sigma, St. Louis MO, USA), and 1 mM sodium orthovanadate). Proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene fluoride membrane, and

blocked with 5% skim milk/PBS-T buffer for 1 h. Subsequently, the membrane was incubated with the following primary antibodies: β-actin, CRMP4, cytochrome c (Santa Cruz Biotechnology, Dallas, TX, USA), and cleaved-PARP (cleaved-poly ADP-ribose polymerase) (Cell Signaling Technologies, Danvers, MA, USA). The bound antibodies were visualized with a horseradish peroxidase-conjugated secondary antibody using enhanced chemiluminescence (Clarity Western ECL; Bio-rad, Hercules, CA, USA) and the Ez-Capture MG system (Atto Corporation, Tokyo, Japan).

### Flow cytometry for the measurement of MMP, intracellular calcium levels, and apoptosis assay

The fluorescent probe Fluo 3-AM was used for the assessment of intracellular levels of $Ca^{2+}$ and the lipophilic cationic dye 3,3¢-dihexyloxacarbocyanine iodide (DiOC6(3)) was used for measuring disruption of the MMP (Δψm). Briefly, cells were exposed to a 5-Gy radiation dose and incubated for 72 h, then stained with 1 μM DiOC6(3) at 37 °C for 15 min in the dark, and analyzed using FACSverse flow cytometry (BD Biosciences, San Jose, CA, USA). For apoptosis analysis, cells were harvested and centrifuged at 800 rpm for 3 min following radiation treatment (48 h). Cells were carefully resuspended, then annexin-V (5 μL) and propidium iodide (PI) (5 μL) (BD Biosciences) were added and the cells incubated at 37 °C for 15 min in the dark. The stained cells were analyzed using FACSverse flow cytometry.

### Clonogenic assay

Cells were seeded into 60-mm dish plates at densities of 1, 2, 5, 8, and 10 × $10^3$ cells/plate. After 24 h, cells were radiated with the indicated dose of ionizing radiation. After 14 days, the colonies were subsequently fixed and stained with 0.1% crystal violet in 20% ethanol and were counted. Alternatively, the remaining attached cells were stained with crystal violet; after a wash step, the dye was solubilized and absorbance measurements at 590 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA) were taken.

### Cell-cycle analysis

Irradiated cells were trypsinized, washed in ice-cold PBS, and fixed with 70% ethanol on ice. Fixed cells were stained with PI (BD Biosciences) containing RNase (0.1 mg/mL) for 15 min at 37 °C, and cell population analysis was performed using a FACSCalibur flow cytometry.

### Measurement of cytochrome c release

The cytochrome c-releasing apoptosis assay kit (Abcam ab65311, Cambridge MA, USA) was used for detecting cytochrome c translocation from mitochondria into the cytosol. Irradiated or A23187-treated cells were lysed in a cytosolic extraction buffer, homogenized, the supernatant cytosol fraction separated by centrifugation, and the pellet resuspended in a mitochondrial extraction buffer, according to the manufacturer's instructions. Separated cytosolic and mitochondrial fractions were immunoblotted for the measurement of cytochrome c release.

### Cell-viability assay

Cell viability was assessed using the water-soluble tetrazolium salt (WST)-1 assay (Roche, Mannheim, Germany) according to the manufacturer's instructions. Briefly, 10 μL WST-1 reagent was added to each well of a 96-well plate ($1 \times 10^4$ cells/well). After incubation for 1 h, the conversion of WST-1 reagent into chromogenic formazan was evaluated using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

### Statistical analysis

All results were confirmed in at least three independent experiments; and data from one representative experiment is shown. All quantitative data are presented as the means ± standard deviation (SD); Student's t-tests and ANOVA were used for comparisons of means of quantitative data between groups; a value of $p < 0.05$ was considered statistically significant.

## Results

### CRMP4 expression was downregulated in radioresistant CRC cell lines

To identify genes associated with radiation-induced cell death, radioresistant IR-SW620, IR-RKO, IR-SW480, and IR-HT-29 cells lines were established as described above. CRC cells were exposed to ionizing radiation over several weeks for a total exposure of 120 Gy [23, 24]. Thereafter, the surviving cells were selected and characterized using radiation resistance that was lacking in parental cells. From the RNA sequencing analysis, a total of 25,207 genes were identified. Among them, 70 genes were upregulated (>1.5 fold), while 45 genes were downregulated (<0.5 fold). These 115 genes were confirmed using RT-PCR analysis. Finally, the upregulated genes (*CXCR4*, *RAC2*, *HBE1* [23], *PTGDS*, and *LCN2*) and downregulated genes (*SMO*, *RGS10*, *PRTFDC1*, and *CRMP4*) were identified as candidate genes associated with radiation resistance. It was determined that collapsin response mediator protein 4 (*CRMP4*) was significantly downregulated. Western blot analysis showed reduced CRMP4 expression in radiation-resistant cells (IR-SW480, IR-SW620, IR-RKO) compared with the expression level observed in parental cells (Fig. 1a). This result implied that *CRMP4* may act as a radiation sensitivity-associated gene.

Since CRMP4 expression is significantly elevated in the SW620 and RKO cell lines, these cells were chosen as our experimental model. IR-resistant cell lines were established with acquired resistance to radiation. A clonogenic assay was performed to confirm the establishment of IR-SW620 and IR-RKO cell lines (Fig. 1b). The degree of apoptosis was monitored following radiation. Cells irradiated with a 5-Gy radiation dose and then stained with annexin V and PI were analyzed using a FACSverse flow cytometer. Apoptosis was reduced in the IR-SW620 and IR-RKO cells when compared with the degree of apoptosis seen in parental cells (Fig. 1c). To investigate the effect of radiation on the cell cycle, PI staining was employed. It was found that the cells were arrested at the G2/M phase following radiation, probably due to DNA damage. The radiation-induced G2/M arrest was reduced in both IR-SW620 and IR-RKO cells

compared to the corresponding rate of cell-cycle arrest in parental cells (Fig.1d). These cell lines were thus determined to be well suited to radiation resistance mechanistic studies.

### *CRMP4* knockdown by siRNA augmented radiation resistance

Loss-of-function experiments using siRNAs were performed on CRC cell lines to determine whether CRMP4 reduction was involved in radiation resistance. First, CRC cell lines SW480, Caco-2, and KM12C, all of which expressed CRMP4, were selected and transfected with *CRMP4* siRNA. The clonogenic assays revealed that *CRMP4* depletion was associated with resistance to radiation in these cells (Fig. 2a). Next, it was examined whether increased clonogenic survival by *CRMP4* knockdown was associated with reduced apoptosis. Annexin V staining was used to investigate the degree of apoptosis induced by radiation in *CRMP4* knockdown cells and mock cells. The number of apoptotic cells following radiation in *CRMP4* knockdown cells was less than that found in mock cells transfected with negative-control siRNA (Fig. 2b). This implied that CRMP4 depletion was associated with greater resistance to radiation. In the FACS cell-cycle analysis, CRMP4 depletion decreased the extent of G2/M arrest associated with radiation (Fig. 2c). Collectively, these findings indicated that the suppression of CRMP4 significantly contributes to the development of radiation resistance in CRC cells.

### CRMP4 depletion attenuated radiation-induced cytochrome c release from mitochondria

Irradiation has been shown to induce various cellular and molecular damage outcomes, including apoptosis, in which cytochrome c release from mitochondria constitutes a critical event [25]. The amount of cytochrome c released from the mitochondria of irradiated cells was measured using a mitochondria/cytosol fractionation kit. Irradiated cells induced cytochrome c release in a time-dependent manner. The maximum amount of cytochrome c release was attained at 72 h of exposure to 5 Gy of radiation. Fig. 3a shows that decreasing amounts of cytochrome c were detected in the cytosol of the radiation-resistant IR-SW620 cells compared to the level in the cytosol of parent cells. These differences affect cell susceptibility to radiation.

To investigate CRMP4 action in CRC cells, CRMP4 expression was stably knocked down in SW620 and RKO cells using lentiviral vectors. Next, the effect of CRMP4 on cytochrome c release was evaluated following radiation exposure. Fig. 3b shows that the loss of CRMP4 expression was associated with reduced cytochrome c release and PARP cleavage. Previous studies have indicated that the release of cytochrome c from mitochondria during apoptosis is associated with low MMP ($\Delta\Psi m$). The lipophilic cationic dye 3DiOC6(3) was used to monitor MMP and determine whether CRMP4 reduction was associated with MMP loss. Cells were irradiated, and MMP was measured over time. IR-resistant cells showed decreased mitochondrial depolarization compared to the level of depolarization seen in parental cells (Fig. 3c). Likewise, CRMP4 absence was associated with a reduction in the radiation-induced MMP collapse when compared with mock cells (Fig. 3d). These results indicated that CRMP4 absence can mitigate MMP collapse following exposure to radiation.

### CRMP4 depletion attenuated calcium-mediated cell death signaling

Consequent to its role as an incredibly versatile signaling ion, uncontrolled cytosolic $Ca^{2+}$ influx induces mitochondrial dysfunction and cell death pathways [4, 20]. Intracellular $Ca^{2+}$ concentrations were measured using the cell-permeable fluorescent $Ca^{2+}$ indicator, Fluo 3-AM, in flow cytometry to determine whether CRPM4 expression could modulate $Ca^{2+}$ influx. Increased intracellular $Ca^{2+}$ concentrations were observed in all irradiated cells irrespective of CRMP4 expression. (Fig. 4a). These data indicated that CRMP4 is not involved in intracellular $Ca^{2+}$ influx regulation. Nonetheless, the influx of intracellular $Ca^{2+}$ by radiation is associated with apoptosis. Excessive intracellular $Ca^{2+}$ concentrations can also cause the mitochondrial permeability transition (MPT) pore to open. The MPT pore is interrupted by cyclosporin A (CsA). When clonogenic assays were performed, CsA prevented radiation-induced apoptosis (Fig. 4b). Based on these results, it was proposed herein that $Ca^{2+}$ is critical to radiation-induced cell death.

It was further analyzed whether CRMP4 is required for $Ca^{2+}$-mediated apoptosis signaling using the $Ca^{2+}$ ionophore A23187, which boosts intracellular $Ca^{2+}$ levels. The WST-1 assay results revealed that CRMP4-depleted cells and IR-resistant cells significantly reduced A23187-induced cell death in a dose-dependent manner compared to parent cells (Fig. 4c). Thereafter, the mitochondrial release of cytochrome c into the cytosol was analyzed following A23187 treatment; it was determined that the levels of cytosolic cytochrome c following treatment with A23187 decreased in CRMP4-depleted cells compared with the corresponding levels in mock cells (Fig. 4d). Likewise, under the same experimental conditions, decreased mitochondrial depolarization was observed in CRMP4-depleted cells. (Fig. 4e). It was theorized that these differences in the degree of cytochrome c release and mitochondrial depolarization may be associated with CRMP4 expression in $Ca^{2+}$-mediated cell death signaling.

### $Ca^{2+}$ inhibitor reduced the CRMP4 level and enhanced radioresistance

To evaluate the role of $Ca^{2+}$ signaling in radiation-induced apoptosis, 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM), a cell-permeant intracellular $Ca^{2+}$ chelator that is used in manipulating intracellular $Ca^{2+}$ levels, was employed. In previous experiments, the inhibition of MPT by CsA reduced CRMP4 expression. Therefore, it was confirmed that CRMP4 expression was altered by intracellular $Ca^{2+}$ levels. Interestingly, CRMP4 expression in CRC cell lines treated with BAPTA-AM was also decreased in a dose-dependent manner (Fig. 5a). However, the reduction in CRMP4 expression by BAPTA-AM warrants further investigation.

BAPTA-AM is commonly used to form $Ca^{2+}$ buffers with well-defined $Ca^{2+}$ concentrations. Thus, BAPTA-AM maintains intracellular $Ca^{2+}$ homeostasis. The buffering of intracellular $Ca^{2+}$ concentrations by BAPTA-AM also reduced A23187-induced release of cytochrome c in SW620 cells (Fig. 5b). Western blot results showed that cleaved-PARP was augmented following radiation exposure; conversely, its level was diminished following treatment with radiation and the $Ca^{2+}$ chelator BAPTA-AM (Fig. 5c). Likewise, BAPTA-AM prevented radiation-induced mitochondrial depolarization in both mock and CRMP4-depleted cells (Fig. 5d). These data indicated that treatment with the cytosolic $Ca^{2+}$ chelator BAPTA-AM can

alleviate A23187- and radiation-induced cell damage. This data suggest that radiation-induced cell death is mediated by cytosolic $Ca^{2+}$ signaling and $Ca^{2+}$ homeostasis, which are both essential for cell survival.

### $Ca^{2+}$ deficiency-induced cell death was inhibited via CRMP4

The functional significance of CRMP4 in $Ca^{2+}$ signaling was investigated. Several studies have demonstrated the extensive use of BAPTA-AM for buffering $Ca^{2+}$, however, high concentrations of BAPTA-AM have been found to deplete intracellular $Ca^{2+}$ stores [11, 26]. Cells were treated with a high concentration of BAPTA-AM to assess the $Ca^{2+}$ deficiency-induced cell death rate; the surviving cells were stained with crystal violet dye (Fig. 6a). Also, WST-1 assays were performed for cell-viability measurement, as shown in Fig. 6b. These results showed that BAPTA-AM-induced $Ca^{2+}$ deficiency leads to cell death in both IR-resistant and CRMP4-depleted cells in a dose-dependent manner, but no significant effect was observed in parental cells. This indicated that $Ca^{2+}$ deficiency-induced apoptosis depends on the CRMP4 expression levels. As anticipated, cleaved-PARP was elevated in both IR-resistant and CRMP4-depleted cells treated with BAPTA-AM in a dose-dependent manner, but not in parental cells (Fig. 6c). Based on these results, it was hypothesized that CRMP4 is important for survival during cellular stress responses to BAPTA-AM-induced $Ca^{2+}$ deficiency, and that it is involved in $Ca^{2+}$ signaling.

## Discussion

Previously, γ-irradiation-resistant CRC cell lines were established and novel candidate molecules implicated in radioresistance were identified using RNA sequencing analysis [23]. Among the genes significantly downregulated in radioresistant cell lines than in their parent cells, *CRMP4* was further examined in the present study with regard to the association between tumor growth and radioresistance. CRMPs influence various intracellular signal transduction pathways including VEGF, RhoA, GSK3β, and sema3A [27, 28]; moreover, CRMP4 is involved in neurodevelopmental disorders such as schizophrenia, neurological disorders such as Alzheimer's disease [29], and various types of cancers such as breast, prostate, gastric, and hepatocellular carcinomas [12, 30-33].

$Ca^{2+}$ constitutes a ubiquitous diffusible intracellular second messenger that is released inside cells upon ligand interaction with membrane receptors; it is especially associated with diverse cellular functions related to cell growth but can induce apoptosis. A primary cause of $Ca^{2+}$-induced mitochondrial damage is the nonspecific pore opening of the mitochondrial membrane leading to the activation of MPT, which causes loss of MMP, rupture of the outer mitochondrial membrane, and leakage of intermembrane proteins, such as cytochrome c, to the cytoplasm [34-36]. Therefore, the disruption of $Ca^{2+}$ homeostasis in cells affects various signaling pathways including those associated with proliferation and apoptosis [16, 26]. It was recently reported that patients with Hodgkin's lymphoma who receive a total dose of more than 30 Gy of radiotherapy displayed significantly higher $Ca^{2+}$ scores than other patients, putting them at a higher risk of coronary artery disease [37]. Nonetheless, our understanding of the correlation between radiation and $Ca^{2+}$ homeostasis remains insufficient.

Our results showed that radiation-induced intracellular $Ca^{2+}$ influx increased similarly in both mock and CRMP4-depleted cell lines. However, CRMP4 enhances sensitivity to radiation. Likewise, A23187-induced cell death is decreased in CRMP4-depleted cells compared with the cell death rate in mock cells. Based on these results, it can be inferred that CRMP4 plays a role in $Ca^{2+}$ signaling pathways involving apoptosis in CRC cells. In addition, the cellular effects of BAPTA-AM, a cell-permeant intracellular $Ca^{2+}$ chelator that acts as an intracellular $Ca^{2+}$ buffer, on CRC cells were investigated. Western blot results showed that BAPTA-AM was associated with increased radiation resistance in cancer cells. Cleaved-PARP levels in radiosensitive parental cells were significantly reduced upon BAPTA-AM treatment (5 μM). These results implied that $Ca^{2+}$ significantly influences radiation-induced apoptosis. It was also revealed that the depletion of intracellular $Ca^{2+}$ induced by high concentrations of BAPTA-AM (10-20 μM) was associated with a lower cell survival rate in CRMP4-depleted cells, but not in CRMP4-expressing cells. According to previous studies, several intracellular protein transport systems can be affected by the chelation of $Ca^{2+}$ with BAPTA-AM [38], potentially including vesicle formation. It was therefore predicted herein that CRMP4 could be involved in $Ca^{2+}$ signaling, since CRMP4 activity would be essential for cell survival during intracellular $Ca^{2+}$ deficiency.

Our results also indicated that CRMP4 expression levels were altered by several factors, including CsA and BAPTA-AM, implying that CRMP4 may be an adjustable target protein. It has been reported that CRMP4 expression levels are regulated by miRNAs. miR-130a upregulation has been reported to target the 3'UTR region of CRMP4 in gastric cancer cells and promote tumor progression via CRMP4 inhibition [39]. Conversely, VEGF enhances CRMP4 expression levels in gastric cancer cells, which is inhibited by the MAPK inhibitor, PD98059, and the PI3K inhibitor, LY294002. In mice, CRMP4 overexpression facilitates tumor growth and metastasis [40]. Taken together, these results implied that CRMP4 can be controlled and that the modulation of CRMP4 expression at the cellular level may affect cancer-cell radiation sensitivity.

# Conclusion

In conclusion, it was confirmed herein that the CRMP4-mediated $Ca^{2+}$ signaling pathway is critical for radiation sensitivity in colorectal cancer. CRMP4 thus serves as a potential target for the sensitization of colorectal cancer cells to radiotherapy. Additionally, it was confirmed that intracellular $Ca^{2+}$ levels are an influential factor in colorectal cancer radiation therapy.

# Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

All the authors agree to the publication clause.

### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Competing interests

The authors declare that they have no competing interests.


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### Authors' contributions

SYP, JTK, BYK, and HGL designed the study; SYP, JTK, YSH, ESP, HRY, and KEB performed the experiments; SYP, HJ, SRY, and HJC contributed essential reagents or tools; SYP, JTK, HJC, BYK, and HGL analyzed the data; SYP, JTK, and HGL wrote the manuscript. All authors critically revised the manuscript and approved the final version of the manuscript.


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None.



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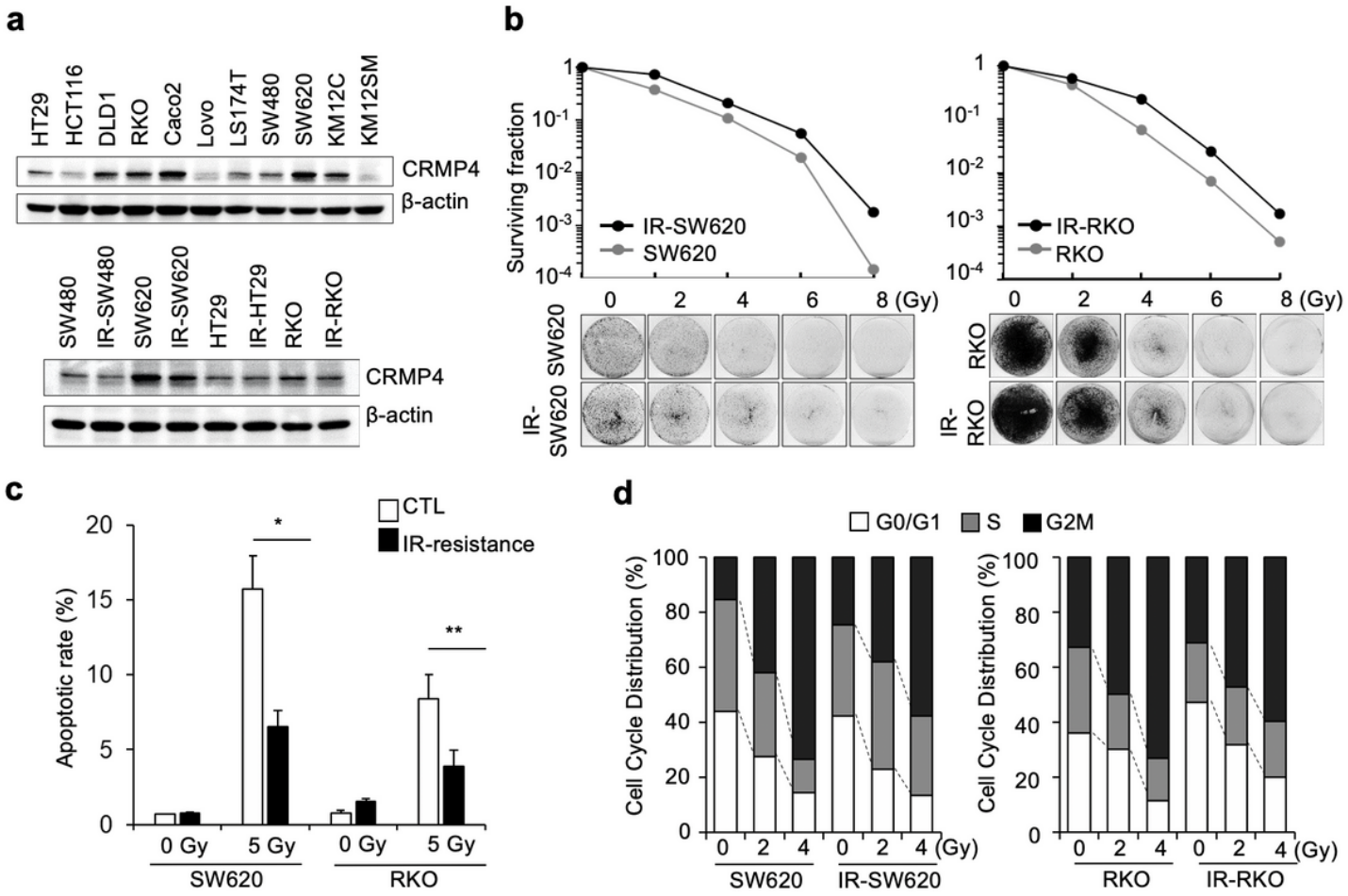
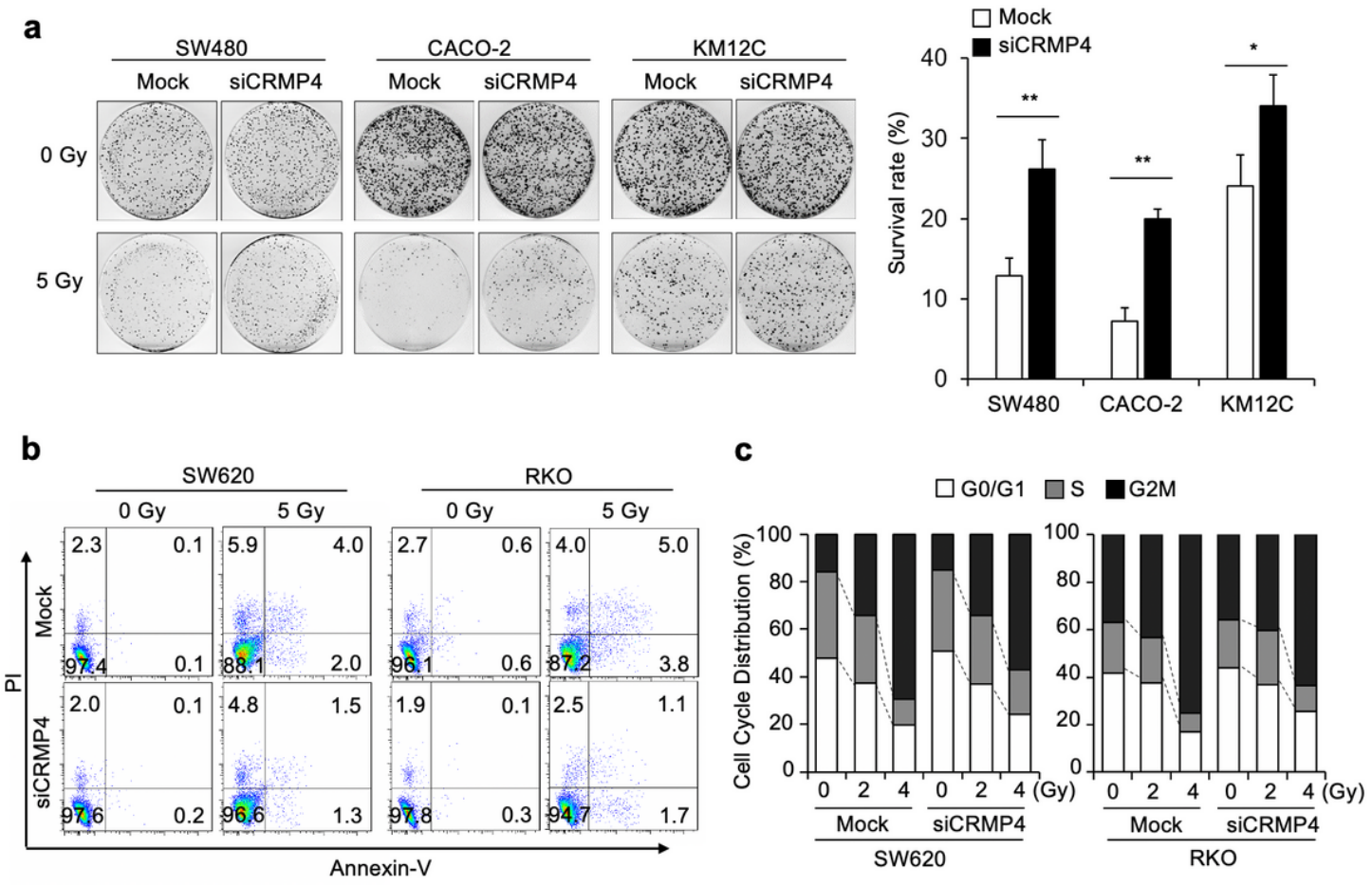
# Figures



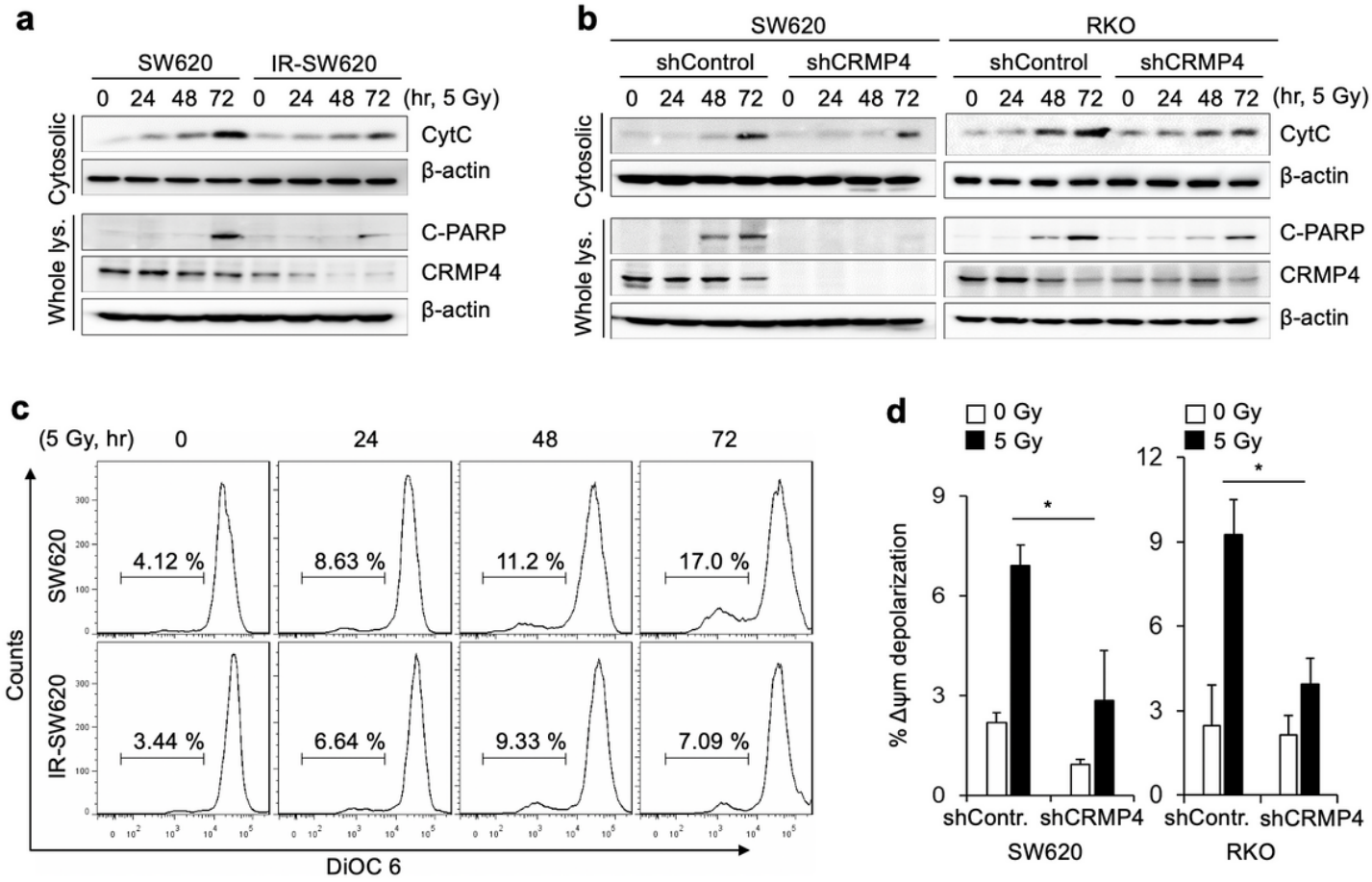

## Figure 1

The ionizing radiation-resistant cell lines demonstrated an increased survival rate compared with the survival rate of the parental cells. (a) CRMP4 expression in various colorectal cancer cell lines. (b) Cells were irradiated to the indicated doses of radiation and allowed to grow for 2 weeks. Thereafter, cells were stained with crystal violet and scored for the colony-forming capacity of irradiated cells. (c) Cells were treated with 5 Gy of radiation. After 48 h, cells were stained with annexin V and PI (propidium iodide), following which apoptotic cells were analyzed by flow cytometry. (d) Cells were treated with the indicated doses of radiation. After 24 h, cells were analyzed by flow cytometry. A quantitative graph showed the percentage of cell-cycle distribution in parental cells and IR-resistant cells. * $P < 0.05$, ** $P < 0.01$ compared with control cells.



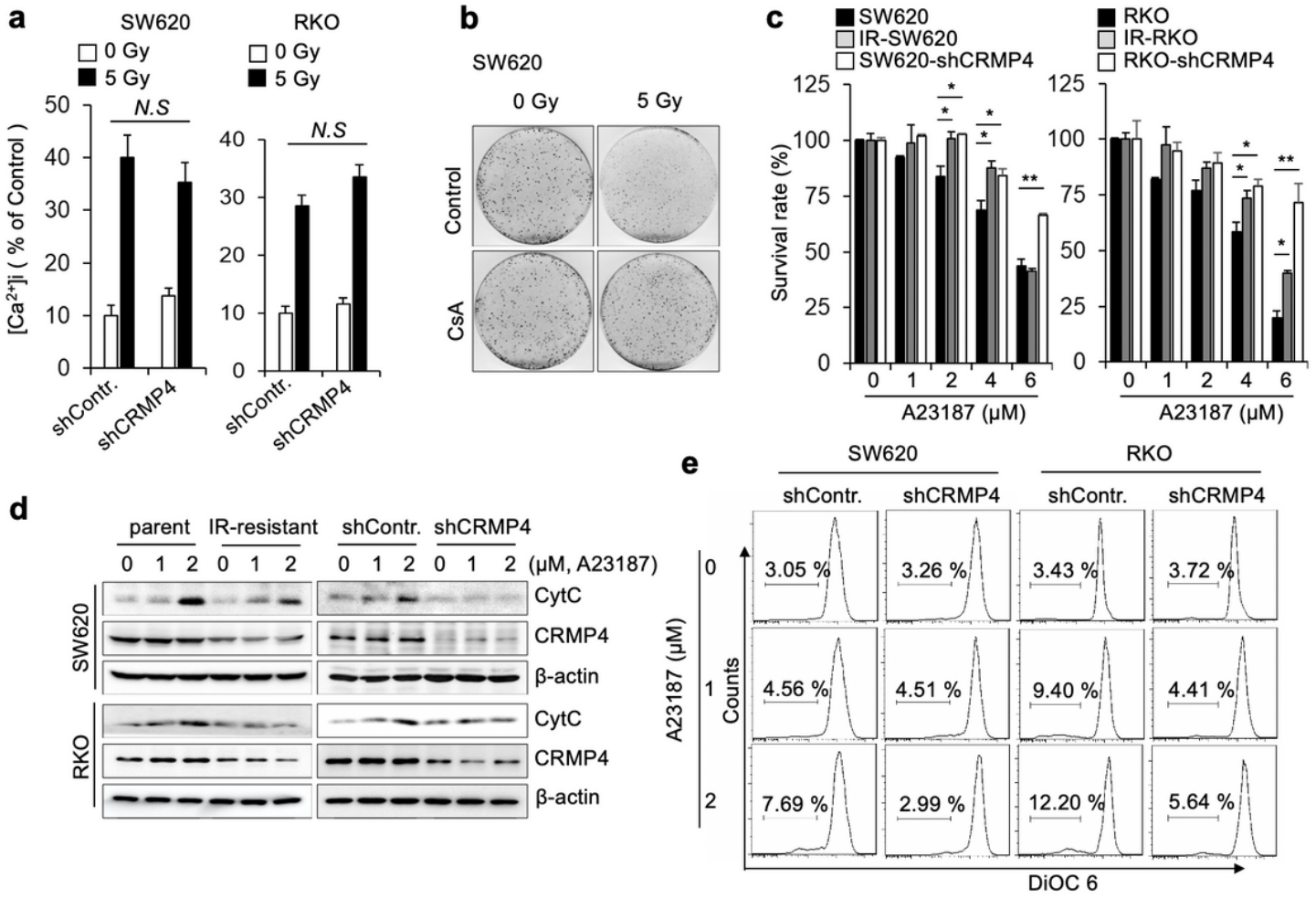

## Figure 2

CRMP4 deficiency enhanced resistance to radiation. (a) After transiently transfecting cells with control siRNA or CRMP4 siRNA, cells were analyzed using clonogenic assays. The colony-formation ability of CRMP4 knockdown cells was significantly increased, in comparison with mock cells, following radiation. Representative images are shown in the left panel and a quantitative graph is shown in the right panel. * $P < 0.05$, ** $P < 0.01$ versus the control cells. (b) Cells were treated with a 5-Gy radiation dose. After 48 h, the percentages of apoptotic cells were measured by flow cytometry. (c) After 24 h post-radiation, cells were stained with PI (propidium iodide) and cell-cycle distribution was measured by flow cytometry.



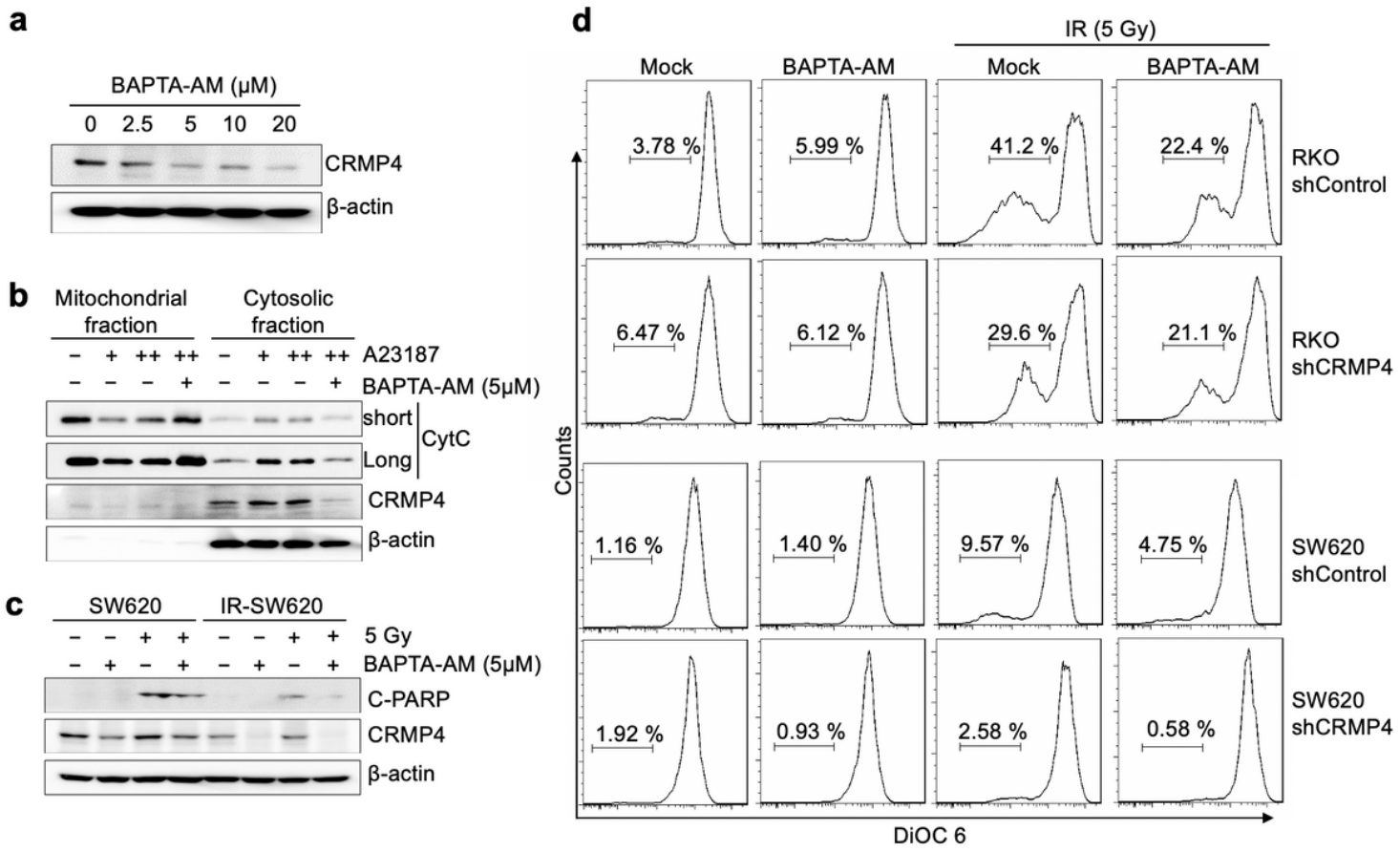

## Figure 3

Mitochondrial depolarization decreased via CRMP4 deficiency. (a) To detect cytochrome c release, the cytosolic fractions of SW620 and IR-SW620 cells were separated, as mentioned in the Materials and Methods section, at different time points (0, 24, 48, and 72 h) following a 5-Gy radiation dose. The cytosolic fraction and whole lysate were analyzed by western blot analysis. (b) SW620 and RKO cells were infected with lentivirus encoding shRNA against CRMP4 or nonspecific sequence. Cytosolic fractions and whole lysates of infected cells were harvested at indicated times following radiation and analyzed by western blot analysis. (c) Mitochondrial depolarization was measured by DIOC6(3) (3,3'-dihexyloxacarbocyanine iodide) fluorescence in SW620 and IR-SW620 cells for various periods of time following a 5-Gy radiation dose. (d) Mock cells and CRMP4-depleted cells were treated with 0 or 5 Gy of radiation. After 72 h, mitochondrial membrane potential was analyzed by a flow cytometry. * $P < 0.05$ compared with control cells.




## Figure 4

CRMP4 plays a role in the Ca2+ -mediated cell death signaling pathway. (a) Mock cells and CRMP4-depleted cells were treated with 0 or 5 Gy of radiation. After 74 h, cells were loaded with Fluo 3-AM and the intracellular Ca2+ level was measured by flow cytometry. N.S, not significant, compared with control cells. (b) SW620 cells were exposed to a 5-Gy radiation dose in the presence/absence of CsA (cyclosporin A) (5 μM, 1 h before). After 14 days, cells were stained with 0.1% crystal violet. Representative images showed surviving cells (c) Cells were treated with an increasing dose of A23187. After 24 h, cells were incubated with WST-1 (water-soluble tetrazolium salt-1) reagent and the absorbance was measured with a microplate reader. Cell viability is expressed as the percentage of control cells. *P < 0.05, **P < 0.01. (d) Cells were incubated with the indicated doses of A23187 for 12 h. The cytosolic fractions were isolated and the contents of cytochrome c and CRMP4 were examined by western blot analysis. (e) Mock cells and CRMP4-depleted cells were treated with the indicated doses of A23187. After 24 h, mitochondrial membrane potential was analyzed by flow cytometry




## Figure 5

Blocking the rise of intracellular Ca2+ enhances radioresistance. (a) SW620 cells were treated with either indicated doses of BAPTA-AM or A23187 for 24 h and CRMP4 expression was analyzed by western blot analysis. (b) SW620 cells were treated with A23187 (+, 1 μM; ++, 2 μM) in the presence/absence of 5 μM BAPTA-AM for 12 h. Mitochondrial and cytosolic fractions were extracted for western blot analysis. (c) SW620 and IR-SW620 cells were treated with (or without) 5 μM BAPTA-AM before a 5-Gy radiation dose. After 72 h, the levels of C-PARP (cleaved-poly ADP-ribose polymerase) and CRMP4 were detected by western blot analysis. (d) Cells were exposed to 5 Gy of radiation in the presence/absence of 5 μM BAPTA-AM. After 72 h, mitochondrial membrane potential was analyzed by flow cytometry

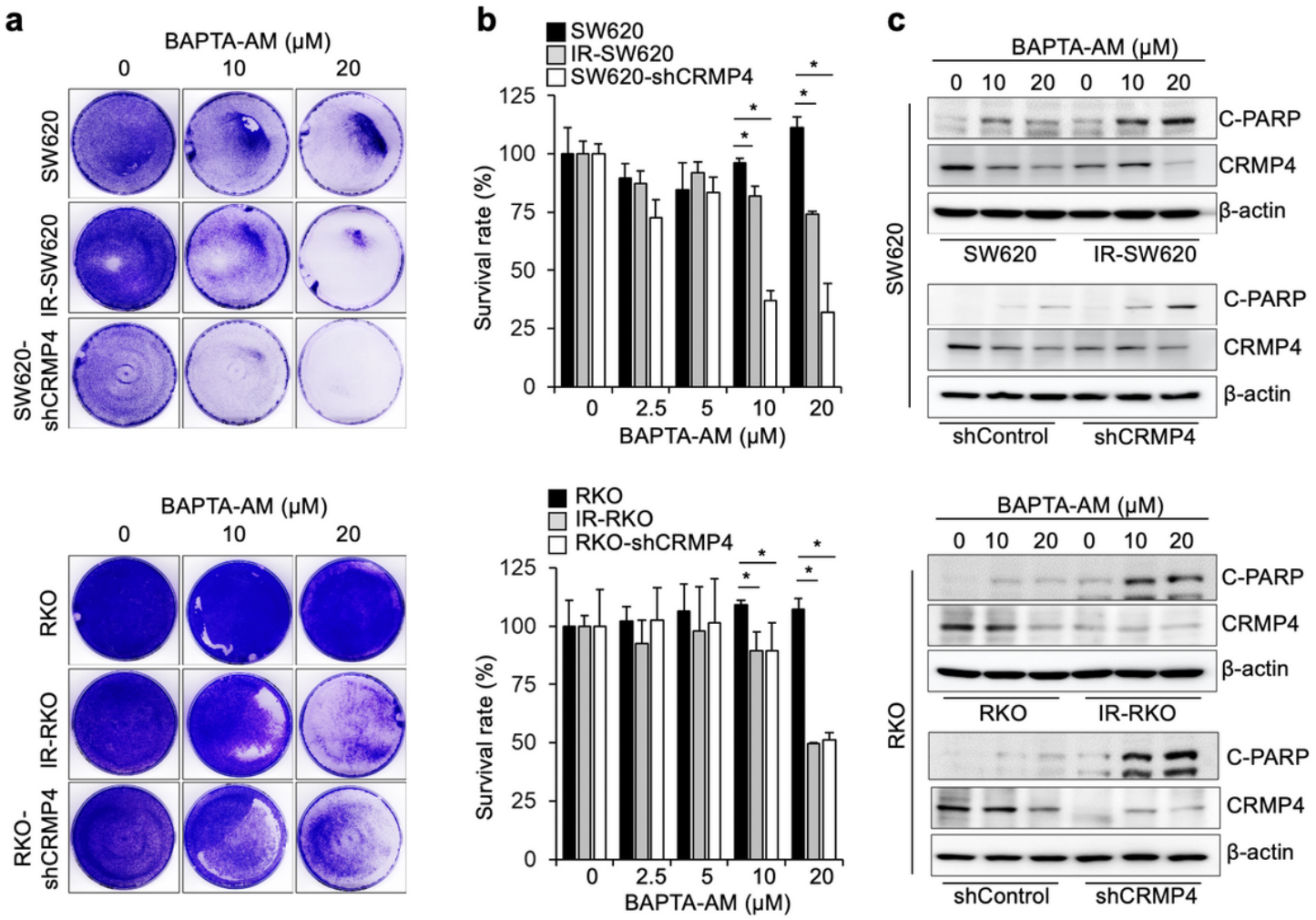



**Figure 6**

BAPTA-AM enhances cell death in CRMP4-depleted cells. (a) Cells were treated with the indicated doses of BAPTA-AM and incubated for 72 h. Cells were stained with 0.1% crystal violet. Representative crystal violet images showing surviving cells. (b) Cells were treated with the indicated doses of BAPTA-AM. After 48 h, WST-1 (water-soluble tetrazolium salt-1) reagent was added to each well and incubated for an additional 4 h. The absorbance was measured by a microplate reader. Cell viability is expressed as the percentage of control cells. **P < 0.01 (c) Cells were treated with various concentrations of BAPTA-AM for 48 h. Cell lysates were subjected to western blot analysis with C-PARP (cleaved-poly ADP-ribose polymerase) and CRMP4 antibodies.

# Supplementary Files

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- crmp4figsuppl200901.pptx