

TRAP1 regulates complement activation and mitochondrial stress adaptation to promote tumor cell resistance to complement-dependent cytotoxicity

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1 ***TRAP1 regulates complement activation and mitochondrial stress***
2 ***adaptation to promote tumor cell resistance to complement-***
3 ***dependent cytotoxicity***
4

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8
9 **Abstract**

10 Resistance of tumor cells to complement-dependent cytotoxicity (CDC) is a major obstacle to the
11 antitumor activity of therapeutic antibodies. The molecular network conferring resistance to
12 complement attack is incompletely understood. Mitochondrial chaperones coordinate cellular
13 adaptation to stress, but their contribution to complement resistance is only beginning to emerge.
14 The mitochondrial chaperone TNF receptor-associated protein 1 (TRAP1) protects cells against
15 oxidative and metabolic stress. As shown here, treatment of TRAP1 with the mitochondria-
16 targeted inhibitor Gamitrinib-TTP, as well as TRAP1 knockdown or knockout, markedly
17 sensitized cancer cells to CDC, whereas TRAP1 overexpression conferred resistance to CDC.
18 TRAP1 deficiency increased C3b deposition and membrane attack complex (MAC; C5b-9)
19 assembly without affecting antibody binding, C1q or C4b deposition, or the expression of the
20 membrane complement regulators CD46, CD55 and CD59. Interestingly, in the absence of TRAP1
21 recruitment of the soluble complement regulatory protein Factor H to the cell surface was reduced.
22 This may account for the increase in C3b binding. In addition, TRAP1 is shown to bind directly to
23 C9 and inhibit its polymerization, disclosing a second inhibitory mechanism against CDC.
24 Following complement attack, TRAP1 rapidly redistributed from mitochondria to the cell surface
25 and the extracellular compartment. Neutralization of extracellular TRAP1 by specific antibodies

enhanced MAC deposition and CDC, whereas extracellular recombinant TRAP1 reduced CDC. Beyond its effects on complement activation, TRAP1 is shown to protect mitochondria from complement-induced cell injury by limiting mitochondrial oxidative stress, preserving mitochondrial membrane potential and maintaining ATP production. Collectively, these findings identify TRAP1 as a multifunctional regulator that carries out extracellular complement regulation and intracellular mitochondrial stress adaptation, thereby determining tumor cell susceptibility to complement-mediated cell death. This work expands the emerging role of mitochondrial chaperones, like mortalin, in cell resistance to a complement stress and identifies TRAP1 as a promising therapeutic target for enhancing antibody-based cancer immunotherapy.

Keywords: TRAP1, Complement C3, C9, factor H, cell death.

Introduction

Cancer cells acquire adaptive mechanisms that enable them to withstand diverse stress-inducing, growth-inhibitory, and cytotoxic insults, thereby promoting survival under immune surveillance and anticancer therapy [1-3]. Despite considerable advances in cancer treatment, residual malignant cells frequently survive and give rise to recurrent disease that is often associated with increased resistance to therapy [4]. Therapeutic monoclonal antibodies have become an important component of cancer treatment; however, their efficacy is often limited by tumor-intrinsic resistance mechanisms that reduce antibody-mediated cytotoxicity [5, 6].

One of the principal effector mechanisms of therapeutic antibodies is activation of the classical complement pathway [7]. Antibody binding to the tumor cell surface initiates complement activation, culminating in assembly of the membrane attack complex (MAC), which perforates the plasma membrane and induces complement-dependent cytotoxicity (CDC) [8, 9]. In addition to membrane damage, MAC deposition triggers calcium influx, mitochondrial dysfunction, reactive oxygen species (ROS) generation, and activation of intracellular signaling pathways that ultimately determine whether cells recover or undergo cell death [10-12]. Tumor cells evade CDC through multiple extracellular and intracellular mechanisms [10, 13]. The best characterized extracellular mechanisms include overexpression of the membrane complement regulators CD46, CD55, and CD59, together with recruitment of the soluble inhibitor Factor H to the cell surface [14, 15]. Factor H binding depends largely on surface glycans, particularly sialic acids, thereby limiting C3b amplification and MAC formation. Disruption of complement regulators or inhibition of Factor H recruitment markedly enhances CDC [15-17]. In addition, intracellular signaling pathways, including activation of PKC and ERK, promote survival following complement attack [18, 19], indicating that resistance to CDC is regulated by coordinated extracellular and intracellular defense mechanisms.

Evidence is accumulating that molecular chaperones also contribute to resistance against complement-mediated attack. We previously demonstrated that the cytosolic chaperones Hsp70 and Hsp90, along with the mitochondrial Hsp70 chaperone mortalin (HSPA9), protect cancer cells from CDC [20, 21]. These observations suggest that proteostasis and mitochondrial homeostasis are important determinants of cellular responses to complement attack.

TRAP1 (TNF receptor-associated protein 1), the mitochondrial member of the Hsp90 family, is a central regulator of mitochondrial proteostasis, oxidative metabolism, and cellular adaptation to

stress [22-25]. TRAP1 preserves mitochondrial integrity by regulating protein folding, limiting ROS accumulation, maintaining oxidative phosphorylation, and preventing opening of the mitochondrial permeability transition pore [26-28]. In addition, TRAP1 promotes resistance to chemotherapy and apoptosis and is overexpressed in numerous human malignancies, where elevated expression is associated with poor clinical outcome [29-39]. Consequently, TRAP1 is emerging as a promising therapeutic target [40], and Gamitrinib, a mitochondria-targeted Hsp90 inhibitor, is currently undergoing clinical evaluation [41].

Despite extensive studies of TRAP1 in mitochondrial biology and cancer progression, its role in complement-mediated immune resistance has not been investigated. Here, we demonstrate that TRAP1 regulates tumor resistance to complement attack through several mechanisms. We show that TRAP1 enhances recruitment of Factor H to the tumor cell surface, thereby reducing C3b deposition and MAC assembly. In addition, TRAP1 directly interacts with C9 and inhibits its polymerization. Beyond its effects on complement activation, TRAP1 protects mitochondria from MAC-induced damage by limiting ROS-mediated injury and preserving cell viability. Collectively, these findings identify TRAP1 as a regulator of complement-mediated cell death that coordinates extracellular complement regulation with intracellular mitochondrial protection.

Materials and methods

Cells and reagents: Human erythroleukemia K562 and lymphoma Raji cells were cultured at 37 °C and 5% CO₂ in RPMI-1640 (Sartorius Biological Industries, Beit Haemek, Israel). Human Embryonic Kidney cells HEK 293T, TRAP1-knockout (KO) HEK-293T, human colorectal carcinoma cells HCT-116, TRAP1-KO HCT-116, human bladder cell carcinoma cells UMUC3 and TRAP1-KO UMUC3, and wild-type and TRAP1-KO mouse breast cancer cells (MBC) were cultured in DMEM (Sartorius). The TRAP1-KO cells were obtained from Dr. Len Necker

(National Cancer Institute (USA), National Institutes of Health, Bethesda) and Dr. Didier Picard (University of Geneva, Switzerland). Cell growth media were supplemented with 10% Fetal Calf Serum (Rhenium, Modi'in, Israel), 1% sodium pyruvate (Sartorius), 1% glutamine (Sartorius), and 0.2% antibiotic–antimycotic mixtures (Rhenium).

Normal human serum (NHS), obtained from 3H-Biomedical AB (Uppsala, Sweden) was used as a source for complement. Heat-inactivated NHS (HIS, heated for 30 min at 56⁰C) served as a negative control to NHS. Purified C9 and C3b and C8- and C9-depleted sera were purchased from Complement Technology (Tyler, Texas). Recombinant TRAP1 was purchased from GeneScript (Nanjing, China).

Rabbit anti-cancer cells antibodies were prepared by immunizing rabbits subcutaneously with tumor cells mixed with adjuvant. The anti-human CD20 antibody, Rituximab, was purchased from Roche, Israel. Anti-human complement C1q, human C4, human C3 and human factor H were prepared in house in goats. Mouse anti-CD46, mouse anti-CD55, mouse anti-CD59, mouse monoclonal anti-C5b-9 (aE11 clone) and mouse anti- β actin antibodies were purchased from Santa Cruz Biotechnologies (Dallas, Texas). AF488-conjugated goat anti-mouse IgG antibody, FITC-labeled donkey anti-goat IgG antibody, AF594-conjugated goat anti-rabbit IgG antibody), HRP-conjugated goat anti-mouse IgG antibody, HRP-conjugated rabbit anti-mouse IgG antibody, HRP-conjugated rabbit anti-goat IgG antibody and HRP-conjugated goat anti-rabbit IgG antibody were purchased from Abcam (Cambridge, UK). Rabbit anti-TRAP1 antibodies were purchased from Cell Signaling Technologies (Danvers, MA) or BD Biosciences (San Jose, CA). Mouse anti-mortalin and mouse anti-TRAP1 were from Santa Cruz Biotechnologies. Cy3-conjugated donkey anti-goat IgG antibody, FITC-conjugated goat anti-human IgG antibody, Cy3-conjugated donkey anti-mouse IgG antibody, AF488-conjugated donkey anti-mouse IgG antibody, AF647-conjugated

goat anti-mouse IgG antibody, FITC-conjugated goat anti-rabbit IgG antibody, and FITC-conjugated Streptavidin were purchased from Jackson ImmunoResearch Laboratories (Baltimore, MD). Anti-His-tag antibody was purchased from Abcepta Inc (San Diego, CA). Mouse or goat IgG isotype controls, N-acetyl cysteine (NAC), 2',7'-dichlorodihydrofluorescein diacetate (DCFDA), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), and Ponceau solution were purchased from Sigma-Aldrich (St Louis, MO). The Mitochondrial Membrane Potential JC-1 kit was purchased from Abcam, whereas the LDH kit (G1780) and the ATP kit (CellTiter-Glo 2.0) were purchased from Promega (Madison, Wisconsin). Gamitrinib-TTP was from MedChemExpress (Monmouth Junction, NJ).

siRNA and Plasmid: Smart pool siRNAs targeting TRAP1 and scrambled siRNA were purchased from Dharmacon, Inc (Lafayette, CO, USA). Human TRAP1 gene cloned in pCDNA3.1 plasmid, attached or not to EGFP, was obtained from Drs. Len Neckers (NIH) and Didier Picard (University of Geneva) [42]. The plasmids were amplified in DH5-alpha *E. coli* overnight at 37°C in 2XYT media (Sigma-Aldrich, St Louis, MO) with Kanamycin selection (Sigma-Aldrich, St Louis, MO) and isolated by using the Nucleospin Plasmid kit (Macherey-Nagel, Düren, Germany). Sanger sequencing was performed to validate the sequence of the TRAP1 in the plasmid.

Transient transfection of siRNA and plasmids by electroporation: Cells (4×10^6) were resuspended in 400 μ l electroporation buffer (RPMI supplemented with PIPES, glutamate, calcium acetate and magnesium acetate) and mixed with siRNA (200 picomoles) or plasmid (1-2.5 μ g). They were kept for 10 min at room temperature and then subjected to electroporation in a 4-mm electroporation cuvette (Sigma-Aldrich, St Louis, MO) at 225 mV for 14 msec in an ECM-830 Electroporator (BTX Harvard Apparatus). Then, the cells were mixed with culture growth medium, plated and placed in the CO₂ incubator for 48-72 hrs.

Measurement of cell death and cell bound proteins by flow cytometry: For complement-dependent cytotoxicity (CDC), cancer cells were treated with antibodies diluted in HBSS buffer (30 min 4°C), followed by treatment with NHS (50%) as a source for complement (60 min, 37 °C). The required antibody dilution for the CDC was determined by antibody pre-titration on each cell type. Heat-inactivated NHS (HIS) was used as a negative control. The number of viable/dead cells was determined by propidium iodide (PI) or DAPI inclusion, quantified in a Cytoflex 4L Flow Cytometer using the CytExpert 2.5 software. Percentage of cell death was calculated by calculating the number of viable/dead cells in NHS versus HIS or with/without antibody. CDC of wild-type and TRAP1-KO mouse breast cancer cells (MBC) required no antibody pre-binding and was performed by treatment with NHS or HIS.

A two-step CDC was also performed, in which the cells were treated with rabbit antibodies (30 min, 4°C), followed by incubation with C8-deficient human serum for 15 min at 37 °C, and then, washed and treated with NHS containing 10mM EDTA. This allowed for buildup of the C5b-7 complex on the cells and then, on the second step, completion of the C5b-9 formation. Addition of recombinant TRAP1 or anti-TRAP1 antibodies only in the NHS-EDTA step enabled examination of their direct effect on the C8 and C9 binding steps.

To quantify the deposition of Complement components C1q, C4b, C3b, factor H and C5b-9 or of TRAP1 on the cell surface, the cells were treated with antibody and NHS (50%) under sublytic conditions (antibody dilution leading to 10-25% CDC) (15 min, 37 °C). Subsequently, the cells were labeled with goat anti-C1q (1:200), goat anti-C4 (1:200), goat anti-C3 (1:100) or goat anti-factor H (1:50) antibodies or with monoclonal mouse anti-C5b-9 (1:100) or mouse anti-TRAP1 (1:100) antibody. Next, the cells were treated with FITC-labeled secondary antibodies. The cells were analyzed in a Cytoflex Flow (4L) Cytometer as above. The mean fluorescence intensity

(MFI) was determined for each antibody and corrected by reducing the MFI obtained in cells treated with secondary antibodies but without the primary antibodies. In the same way, the level of expression of the membrane regulatory proteins CD46, CD55 and CD59 and of the bound anti-cells antibodies was quantified with secondary FITC-labeled antibodies.

Assessment of Mitochondrial damage: Cells were treated with antibody and NHS (50%) under sublytic and low-lytic conditions. Then, the mitochondrial membrane potential (MMP), the ATP levels and the intracellular ROS level were determined. To measure mitochondrial membrane potential, cells were stained with JC-1 (1 μ M) (Abcam) in a 96-well black plate. Similarly, the ATP levels were measured in a white plate by using the CellTiter-Glo 2.0 Kit. The results were quantified in a Synergy H1 microplate reader. To measure the intracellular ROS level, the cells were stained with 10 μ M DCFDA for 20 min at 37°C and were analyzed by using flow cytometry. To neutralize ROS activity, cells were treated with N-acetyl cysteine (NAC, 5mM, 30 min, 37°C) before complement activation.

Analysis of Cell lysates and extracellular proteins by Western blotting: To analyze cell lysates, cells treated with antibody and NHS (50%) under sublytic conditions were resuspended in SDS-PAGE lysis buffer (A2S Technologies, Israel) containing SDS (2%) and DTT (150 mM) and heated at 95°C for 5 min. To collect extracellular proteins, cells treated with antibody and NHS (50%) under sublytic conditions were incubated for 15 min in HBSS at 37°C. The cells were removed by centrifugation and the first supernatant, further sedimented at 13,000 x g and the second supernatant was analyzed for extracellular proteins. Cell lysates or supernatants were loaded on 10% polyacrylamide pre-cast gels (A2S Technologies, Israel) and subjected to electrophoresis at 100V. For protein staining, the gels were treated with the InstantBlue Coomassie Protein Stain (Abcam). For Western blotting, the proteins were transferred from the gel to a

nitrocellulose membrane (Whatman Portran BA-83) by electrophoresis (1 h, 200 mA). The membrane was blocked with 5% skimmed milk (BD Bioscience) in TBST for 60 min at room temperature (RT). For staining of TRAP1, the membranes were treated with anti-TRAP1 antibody in TBST (60 min, RT), washed and incubated with a secondary peroxidase-labeled antibody (60 min, RT). The protein bands were detected with an ECL substrate (Thermo-Fisher Scientific, Israel), and the optical density of the bands on a scanned gel was quantified by using the software ImageJ.

Measurement of protein-protein interaction, TRAP1 release and complement C9 polymerization by ELISA: To analyze TRAP1-C9 binding, recombinant TRAP1 or C9 was attached overnight at 4 °C to wells of a 96-well High-Affinity Binding Plate (Nunc, Thermo-Fisher Scientific, Israel) in carbonate-bicarbonate buffer. BSA was used as a negative control. Binding of the second protein, C9 or TRAP1, correspondingly, was quantified with specific and secondary HRP-labeled antibodies. The binding was quantified, after staining with TMB (ScyTek, UT, USA) and addition of a stop solution (1M HCl, Bio-Lab, Israel), at 450 nm in a Synergy H1 microplate reader (Biotek, Israel). In a competitive ELISA, the second protein was pre-mixed with the competing agent before its addition to the well.

To quantify the extracellular level of TRAP1, supernatant collected from cells subjected to a sublytic complement attack, as described above, were diluted in PBS and coated overnight in wells of high-affinity binding ELISA plates. Recombinant TRAP1 serially diluted in PBS was similarly coated in wells and used to generate a concentration standard curve. Next, the wells were washed, blocked and treated with mouse anti-TRAP1 antibodies (1 h, at room temperature) and HRP-labeled anti-mouse IgG antibodies. The quantity of TRAP1 was determined in a plate reader as described above. A four-parameter logistic regression model was used to generate the standard

207 TRAP1 curve, which had an R^2 of 0.98–0.99. Based on the standard curve, the concentration of
208 the extracellular TRAP1 was determined by interpolation.

209 To activate C9 polymerization, purified C9 was incubated with 42 μ M zinc chloride in Tris-HCl
210 buffer (Sigma-Aldrich). The quantity of the polymerized C9 was determined in a sandwich ELISA.
211 The microtiter plate wells were coated with the mouse anti-C5b-9 antibody aE11 or isotype control
212 IgG1 overnight at 4°C. Then, the polymerized C9 samples were added for 1h at 37°C, followed by
213 goat anti-C9 antibody and an HRP-labeled secondary antibody. The absorbance was quantified at
214 450 nm as described above.

215 **Confocal Microscopy:** Cells were treated with a sublytic dose of antibodies and NHS for 15 min
216 at 37 °C and then stained with Mito Tracker Far Red (Sigma-Aldrich) for 15 min at 37°C. The
217 cells were fixed with 2% paraformaldehyde (Sigma-Aldrich) for 15 min at room temperature.
218 Then, they were washed with buffer containing 0.1% saponin (Sigma-Aldrich) and 0.5% bovine
219 serum albumin (BSA). Next, the cells were treated with a blocking buffer containing saponin
220 (0.1%), FCS (10%), and BSA (0.5%). Then, the cells were stained with the anti-TRAP1 antibody
221 overnight before treatment with a secondary fluorophore-tagged (Cy3 or AF488) antibody for 60
222 min on ice. The cells were plated on a cover slip and imaged in a Leica TCS SP8 confocal
223 microscope. Linear brightness and contrast adjustments were done with identical settings for all
224 the treatments; no gamma changes were done. Images of the intracellular and cell surface staining
225 were analyzed by using ImageJ. To quantify the labeling at the cell surface, two concentric circles
226 were drawn, one below the cell surface and one around the cells. The cell surface intensity was
227 determined by subtracting the intensity of the intracellular staining (inner circle) from the whole
228 cell intensity (outer circle).

Statistical Analysis: The experiments were carried out in replicates, and the results were reproduced at least three times. A two-tailed, unpaired Student's t-test was used to establish statistical significance. One-way and two-way ANOVA were used for comparison of multiple groups and variables and analyzed by using the GraphPad Prism 11 software.

Results

TRAP1 protects cancer cells from CDC

To examine the involvement of TRAP1 in protection from CDC, K562 cells were treated with the TRAP1 inhibitor Gamitrinib-TTP (GTTP) dissolved in DMSO and then subjected to antibody and complement treatment. As shown in Figure 1A, CDC of GTTP-treated cells was significantly higher than that of cells treated with DMSO. Knockdown of K562 by transfection with TRAP1 siRNA (demonstrated in Fig. 1D and Supplementary Fig. S1) similarly sensitized the cells to CDC relative to cells transfected with Sc-siRNA (Fig. 1B). Next, K562 cells were transfected with a pEGFP-TRAP1 expression plasmid that led to TRAP1 overexpression (as demonstrated in Fig. 1E). As shown in Figure 1C, K562 cells overexpressing TRAP1 were protected from CDC relative to cells transfected with a control plasmid. These findings were confirmed in Raji cells. Knockdown of TRAP1 in CD20-bearing Raji cells enhanced their sensitivity to CDC by antibody (Rituximab, anti-CD20) and NHS (Fig. 1F). In contrast, overexpression of TRAP1 in Raji cells reduced their killing by Rituximab and NHS (Fig. 1G). Finally, the sensitivity to CDC was compared between wildtype and TRAP1 knockout cells [42]. As shown in Figure 1H, TRAP1 knockout conferred increased sensitivity to CDC of human UMUC-3, HEK-293T and HCT-116 cells as well as of the TRAP-knockout mouse breast cancer cells MBC. Re-expression of TRAP1

by plasmid transfection in HEK-293T TRAP1-KO cells protected the cells from CDC (Fig. 1I). The effect of GTTP on sensitivity of TRAP1-KO cells to CDC was tested. As expected, while HEK-293T-KO cells were more sensitive to CDC than wildtype cells, GTTP enhanced cell sensitivity to CDC in wildtype but not in TRAP1-KO cells (Fig. 1J). Thus, identifying TRAP1 as the major target of GTTP relevant to CDC.

TRAP1 down-regulates complement activation on cancer cells

The impact of TRAP1 on complement activation was tested in cells with knocked-down or knocked-out TRAP1. Key steps in complement activation on cells are C3b deposition and C5b-9 (MAC) membrane insertion [10]. C3b and MAC binding were tested under the same conditions (antibody and NHS) used for CDC measurement (Figure 1). TRAP1 knockdown in K562 cells led to enhanced C3b deposition (Fig. 2A) and MAC formation (Fig. 2B), as compared to control cells transfected with scrambled siRNA. In contrast, overexpression of TRAP1 in K562 cells by plasmid transfection resulted in reduced C3b deposition (Fig. 2D) and MAC level (Fig. 2E). Complement activation was tested also on TRAP1-KO HEK-293T cells. As shown, C3b deposition (Fig. 2G) and MAC deposition (Fig. 2H) were enhanced in the knockout cells relative to wildtype cells.

Factor H is a major soluble complement regulator which binds to C3b and inhibits its activation [43, 44]. Reduced factor H binding to C3b is expected to result in enhanced C3b deposition. The effect of TRAP1 on factor H-C3b binding was investigated. Treatment of K562 cells with antibody and complement resulted in binding of factor H, however, silencing of TRAP1 reduced factor H uptake on the cells (Figure 2C). Similarly, when comparing between wildtype HEK-293T and TRAP1-KO cells, lower level of factor H was measured on knockout cells than on wildtype cells (Fig. 2I). Complement activation on K562 cells treated with GTTP also showed reduced factor H uptake relative to control cells (Fig. 2F).

Additional factors affecting the extent and rate of complement activation include antibody binding level, C1q uptake level, C4b deposition level, and the level of expression of the membrane complement regulators CD46, CD55 and CD59 [15, 45]. Knockdown of TRAP1 had no effect on the level of antibody binding to K562 (Fig. 3A) and Raji (Fig. 3B) cells. Knockout of TRAP1 in HEK-293T cells had no effect on antibody binding level (Fig. 3C) and C1q uptake (Fig. 3D). Inhibition of K562 cells with GTTP or TRAP1 knockdown did not affect C1q uptake (Fig. 3E) and C4 binding level (Fig. 3F). No effect of TRAP1 on CD46, CD55 and CD59 expression levels was found in K562 cells subjected to TRAP1 knockdown (Fig. 3G) and in TRAP1-KO HEK-293T cells (Fig. 3H).

TRAP1 binds to C9 and inhibits C9 polymerization

CD59 and Mortalin have been shown to bind to C9 and inhibit C5b-9 formation [46, 47]. The direct interaction of recombinant TRAP1 with purified C9 was tested by ELISA, as described under Methods. Microtiter plate wells were coated with C9, C3b or BSA and TRAP1 binding was quantified. As shown, TRAP1 bound to C9 and not to C3b or BSA (Fig. 4A). Next, the wells were coated with increasing doses of TRAP1, and C9 binding was tested. As shown in Figure 4B, C9 bound in a dose-dependent manner to TRAP1. The capacity of TRAP1 to inhibit Zn^{++} -induced C9 polymerization [48], an activity expressed by HSP90 [20] and mortalin [47], was tested in an ELISA assay described under methods. TRAP1 significantly inhibited the process of C9 polymerization (Fig. 4C).

Next, the effect of GTTP and NaCl on the binding between TRAP1 and C9 was tested. The TRAP1 inhibitor GTTP partially reduced the binding of TRAP1 to C9 (Fig. 4D). Since GTTP is known to bind to the ATP pocket in the N-terminal of TRAP1 [49], this suggests that C9 and GTTP compete on the same active site at the N-terminal of TRAP1. As shown in Figure 4E, increasing salt

concentrations markedly reduced the interaction between TRAP1 and C9. The residual interaction observed at high salt concentration suggests that additional non-ionic forces contribute to TRAP1-C9 binding.

Complement induces relocation of TRAP1 to the cell surface and the supernatant

TRAP1 is primarily located in cancer cells in the mitochondria [50]. The location of TRAP1 in complement-activated cells was tested by confocal microscopy, as described under Methods. Representative images of K562 cells labeled after fixation with anti-TRAP1 antibodies (green fluorescence) are shown in Figure 5A. Most of the TRAP1 is seen clearly in the mitochondria, both in the NHS- and HIS-treated cells. However, only cells treated with NHS expressed TRAP1 also in the vicinity of the plasma membrane. The fluorescence intensity of the TRAP1 relocated to the plasma membrane plane was determined in 38 cells treated with HIS or NHS, as explained under Methods. As shown in Figure 5B, the quantity of the relocated TRAP1 was significantly higher in cells treated with NHS than with HIS.

The effect of complement activation under sublytic conditions on the relocation of TRAP1 to the cell surface was investigated. K562, T47D, HCT-116 and UMUC-3 cells were subjected to antibodies and then to complement for 15 min. Next, the cells were washed and surface-bound TRAP1 was quantified by flow cytometry after labeling with anti-TRAP1 antibodies and secondary antibodies. As shown in Figure 5C, significantly more TRAP1 was found on cells treated with antibody and NHS than on cells treated with NHS but without antibody. Cell supernatants were also collected from the cells 15 min after treatment with NHS and analyzed by ELISA to determine how much TRAP1 was released out of the cells. Significantly more extracellular TRAP1 was found in the supernatant of K562 cells treated with antibody and NHS

than with NHS but without antibody (Fig. 5D). Percent cell death was also measured in the cells by PI inclusion (Fig. 5C) or LDH release (Fig. 5D).

To confirm that TRAP1 relocation is induced by the MAC, the effect of a C8-deficient serum (C8D) on the cells was tested. K562 cells were subjected to a sublytic dose of antibody and C8D or NHS. Then, the amount of TRAP1 on the cell surface (Fig 5E) and in the cell supernatant (Fig 5F) was measured as described above. Unlike NHS, C8D serum failed to trigger relocation and release of TRAP1.

Extracellular TRAP1 is protective against CDC

To assess the biological significance of extracellular TRAP1 relocation, the effect of anti-TRAP1 antibodies or soluble recombinant TRAP1 on complement-induced cell death was investigated. As shown in Figure 6A, anti-TRAP1 antibodies enhanced K562 cells sensitivity to CDC as compared to isotype control IgG (Fig 6A). The anti-TRAP1 antibodies enhanced CDC both by enhancing C3b deposition and MAC formation (Fig. 6B). To confirm the anti-TRAP1 antibodies enhance CDC by directly acting on the step of C5b-9 formation, rather than on C3b binding, a 2-step CDC assay was performed, as described under Methods. In principle, complement activation was first performed with antibodies and C8-deficient serum to activate C3b and C5b-7 binding. Then, the anti-TRAP1 antibodies were added together with NHS containing EDTA. As shown in Figure 6C, the anti-TRAP1 antibodies significantly enhanced CDC even when added at the final step of the MAC formation. This was confirmed by addition of recombinant TRAP1 instead of anti-TRAP1 antibodies. Recombinant TRAP1 was added, in a two-step assay, together with the NHS-EDTA, after the pre-treatment of the cells with antibody and C8D-NHS. As shown, recombinant TRAP1 significantly reduced both percent cell death and the level of C5b-9 formation (Fig. 6D). These

effects of the anti-TRAP1 antibodies and the recombinant TRAP1 point to a direct effect of extracellular TRAP1 on the MAC assembly.

TRAP1 protects the mitochondria from complement-induced damage

Inhibition of TRAP1 by GTTP or TRAP1 knockdown has been reported to reduce mitochondrial membrane potential and ATP level in cancer cells [51, 52]. In contrast, TRAP1 deficiency in knockout cells may enhance mitochondrial activity and ATP production, suggesting that, in some contexts, TRAP1 may also exert tumor-suppressive effects [53]. Early in the CDC process, mitochondria are targeted and damaged. We therefore investigated whether mitochondrial TRAP1 protects mitochondria against damage induced by membrane-bound complement C5b-9. Treatment of K562 cells with antibody and NHS reduced their MMP (Fig. 7A) and ATP levels (Fig. 7B). Pre-treatment of the cells with GTTP, before addition of antibody and NHS, reduced even further the MMP (Fig. 7A) and ATP levels (Fig. 7B) as well as increased cell death (Fig. 7C), demonstrating that TRAP1 protects the mitochondria from complement damage. Reducing the level of complement-induced reactive oxygen species (ROS) with NAC attenuated CDC in both GTTP-treated and untreated K562 cells (Fig. 7C). Treatment with NAC also reduced CDC of wildtype and TRAP1-KO HEK-293T (Fig. 7D). The level of complement-induced intracellular ROS was measured by cell staining with DCFDA. As shown in Figure 7E, higher level of ROS was found in TRAP1-KO HEK-293T cells than in wildtype cells. NAC pre-treatment reduced complement-induced ROS level in both wildtype and TRAP1-KO HEK-293T cells. Finally, the effect of NAC on MMP reduction induced by complement was tested. NAC treatment restored the mitochondrial membrane potential in both wildtype and TRAP1-KO HEK-293T cells exposed to antibody and complement (Fig. 7F). These results indicate that complement activation at the cell

surface triggers ROS production, leading to mitochondrial damage in cells regardless of the presence or absence of TRAP1.

Discussion

The present study identifies TRAP1 as a multifunctional regulator of complement resistance that links extracellular control of complement activation with intracellular adaptation to complement-induced stress. This dual mode of action substantially broadens the recognized biological repertoire of TRAP1 and supports a model in which tumor susceptibility to complement-dependent cytotoxicity (CDC) is determined not only by the extent of complement activation but also by the capacity of target cells to withstand the intracellular consequences of membrane attack complex (MAC) formation.

TRAP1 has been extensively studied as a mitochondrial chaperone that preserves mitochondrial function under conditions of metabolic and oxidative stress [50]. By regulating mitochondrial proteostasis, oxidative phosphorylation, redox balance and permeability transition, TRAP1 promotes tumor cell survival and contributes to resistance against chemotherapy, radiotherapy and targeted therapies [25, 54-56]. Our findings extend this concept by demonstrating that the same stress-response machinery also contributes to resistance against immune-mediated injury. Thus, complement attack should be viewed as another form of cellular stress that converges on mitochondrial pathways governing bioenergetics, redox homeostasis and cell fate.

Our finding that TRAP1-deficient cells exhibit increased susceptibility to complement-mediated killing (Fig. 1) identifies TRAP1 as a previously unrecognized determinant of complement resistance. The first major finding is that TRAP1 regulates complement activation independently of the classical membrane complement regulators (Figs 2, 3). TRAP1 deficiency is enhancing C3b

385 deposition and MAC formation without affecting antibody binding, C1q or C4 activation, or
386 expression of CD46, CD55 and CD59. These observations place TRAP1 downstream of classical
387 pathway initiation but upstream of alternative and terminal complement activation processes.
388 Because C3 amplification largely determines the amount of MAC generated on cells bearing
389 surface-bound antibodies, even modest changes in C3b deposition can profoundly influence CDC.
390 Our data indicate that one mechanism underlying this regulation involves enhanced recruitment of
391 Factor H, the principal soluble inhibitor of the alternative pathway. Factor H limits complement
392 amplification by accelerating decay of the alternative pathway C3 convertase and promoting
393 Factor I-mediated cleavage of C3b [57, 58]. Tumor cells frequently exploit this pathway by
394 increasing surface sialylation and thereby enhancing Factor H recruitment, enabling efficient
395 suppression of complement activation [15]. Although the molecular basis by which TRAP1
396 promotes Factor H binding remains unresolved, the present findings identify TRAP1 as an
397 additional component of this protective network. Future studies should determine whether TRAP1
398 directly affects Factor H or instead modifies the local cell-surface environment that supports Factor
399 H binding.

400 TRAP1 also regulates a second checkpoint within the terminal complement pathway through direct
401 interaction with C9 (Fig. 4). Recombinant TRAP1 binds C9, but not C3b, and inhibits C9
402 polymerization, indicating a selective role in regulation of MAC assembly. We previously
403 demonstrated that the cytosolic chaperone Hsp90 and the mitochondrial chaperone mortalin can
404 also target complement C9 and limit complement-dependent cell damage [20, 59]. The
405 identification of a second mitochondrial chaperone capable of directly modulating C9
406 polymerization substantially expands the repertoire of endogenous regulators of the terminal
407 complement pathway. More broadly, these findings suggest that proteins classically associated

with intracellular protein homeostasis and stress adaptation may also function directly in extracellular immune regulation.

An equally unexpected finding was the rapid redistribution of TRAP1 from mitochondria to the cell surface and extracellular compartment following complement attack (Fig. 5). Although predominantly mitochondrial, TRAP1 has also been detected in other compartments, including the endoplasmic reticulum [60], the mitochondria-lysosome interface [61], and the surface of endothelial cells [62]. Stress-induced extracellular localization has likewise been reported for several intracellular chaperones, including Hsp70, Hsp90 and mortalin, although the trafficking mechanisms involved remain incompletely understood [63-65]. Complement-induced membrane repair, extracellular vesicle release, and unconventional protein secretion represent plausible mechanisms underlying the rapid surface and extracellular appearance of TRAP1 and warrant future investigation. Importantly, extracellular TRAP1 appears to retain biological activity (Fig. 6). Neutralization of extracellular TRAP1 increased C5b-9 deposition and CDC, whereas recombinant TRAP1 reduced complement-mediated killing. These findings indicate that extracellular TRAP1 may actively regulate the terminal complement pathway rather than merely representing passive release of intracellular contents following complement-mediated membrane injury.

Mitochondria are among the earliest intracellular targets of membrane-inserted MAC complexes. Complement attack induces Ca^{2+} influx, mitochondrial depolarization, increased production of reactive oxygen species (ROS), and ATP depletion-interconnected events that can amplify one another and ultimately determine whether cells restore homeostasis or undergo dysregulated cell death (10, 11, 66-68). Inhibition of TRAP1 markedly exacerbated these responses, whereas scavenging ROS with N-acetyl-L-cysteine largely restored mitochondrial membrane potential and

ATP production and significantly reduced CDC (Fig. 7). These findings identify mitochondrial oxidative stress as a critical mediator of complement-induced cell death and suggest that preservation of mitochondrial integrity is an essential component of TRAP1-dependent protection.

The integration of extracellular complement regulation with intracellular mitochondrial protection has important therapeutic implications. Current strategies to enhance CDC largely focus on increasing complement activation, either by improving antibody-mediated complement fixation or by targeting individual complement regulators such as CD55, CD59, or factor H. Although these approaches can enhance complement activation, they do not address the intrinsic capacity of tumor cells to withstand and recover from complement-induced injury. In contrast, TRAP1 inhibition may simultaneously disrupt extracellular and intracellular defense mechanisms by enhancing complement activation while reducing mitochondrial tolerance to complement-induced oxidative stress. Such a multifaceted mode of action could provide greater therapeutic benefit than targeting individual components of the complement cascade alone. These findings are particularly relevant to therapeutic monoclonal antibodies whose clinical activity depends, at least in part, on complement activation, including rituximab, ofatumumab, alemtuzumab, and daratumumab (7, 69). Our results suggest that combining TRAP1 inhibitors, such as Gamitrinib, with complement-activating antibodies could enhance therapeutic efficacy through complementary mechanisms: increasing MAC formation while simultaneously lowering the threshold for complement-induced cell death. Because TRAP1 inhibition is already being explored as an anticancer strategy (41), the present findings broaden the therapeutic rationale for targeting TRAP1 to include the potentiation of antibody-mediated immunotherapy.

Collectively, our findings strongly suggest that effective resistance to complement-dependent cytotoxicity (CDC) depends not only on restricting complement activation at the cell surface but also on the capacity of tumor cells to withstand the intracellular consequences of membrane attack complex (MAC) formation. This concept is consistent with earlier evidence that complement resistance of human carcinoma cells is determined by multiple membrane-associated and intracellular mechanisms, including complement-regulatory proteins, protein kinases, and sialic acid[15]. Our findings extend this framework by identifying TRAP1 as a multifunctional regulator that coordinates these complementary levels of defense. TRAP1 promotes Factor H-dependent regulation of complement amplification and inhibits C9 polymerization required for the MAC assembly, whereas intracellularly, it preserves mitochondrial integrity and enhances cellular adaptation to complement-induced stress. These coordinated activities identify mitochondrial adaptation as an integral determinant of complement resistance and broaden the emerging role of mitochondrial chaperones in complement biology. More generally, our findings support a model in which tumor susceptibility to CDC is determined by the combined efficacy of extracellular complement regulation and intracellular stress adaptation, providing a conceptual framework for the development of combination therapies that simultaneously target both processes.

Figure Legends

Figure 1. TRAP1 confers protection against CDC. Cancer cells were treated with activating antibody or HBSS (Ctrl) and then with NHS or HIS as described under Methods and percent cell death was measured by using PI exclusion. (A-C) Prior to CDC, K562 cells were treated with the TRAP1 inhibitor GTTP at 100 μ M or with DMSO as control for 60 min at 37°C (A), or were

transfected with 400 picomoles of TRAP1 or scrambled siRNA for 48 hr (B), or transfected with a TRAP1 expression EGFP plasmid or an empty plasmid or tested untransfected (UT) (C). Knockdown of TRAP1 after transfection with TRAP1 siRNA was confirmed by Western Blotting (D and Fig. S1). Overexpression of TRAP1 after transfection with a TRAP1-expression EGFP plasmid was confirmed by FACS analysis of the level of EGFP in the cells (E). CDC of Raji cells by Rituximab and NHS was tested on cells transfected with TRAP1-siRNA or scrambled siRNA (F), or transfected with TRAP1 expression plasmid or empty vector (G). CDC of TRAP1-knockout and wildtype human UMUC-3, HEK-293T, HCT-116 cells and mouse breast cancer (MBC) cells was also tested (H). Next, the impact of TRAP1-re-expression in TRAP1 knockout HEK-293T cells on CDC was tested (I). The effect of GTTP on sensitivity of TRAP1-knockout and wildtype cells to CDC was also tested (J). All experiments were repeated three times. ***, $p < 0.001$, **, $P < 0.01$, two-way ANOVA (C) or Student t-test.

Figure 2. TRAP1 inhibits Complement activation and C5b-9 deposition. K562 cells were either transfected with TRAP1-siRNA or scrambled-siRNA (Sc-siRNA) (A-C) or with a TRAP1 plasmid or an empty plasmid (D, E) or were treated with GTTP (100 μ M) or DMSO (F) for 60 min at 37°C. TRAP1-KO and -wildtype HEK-293T cells were also tested (G-I). Cells were treated with a sublytic dose of antibody or with buffer (Ctrl) and then with NHS for 10 min at 37°C. After washing, the cells were stained with anti-C3 antibodies (A, D, G), anti-C5b-9 antibodies (B, E, H) or anti-factor H antibodies (C, F, I), followed by fluorescently-labelled secondary antibodies, and analyzed by FACS. Mean fluorescent intensity (MFI) was calculated. **, $P < 0.01$; ***, $p < 0.001$ in Student t-test.

Figure 3. TRAP1 does not regulate antibody binding, C1q and C4b uptake and expression of CD46, CD55 and CD59. (A-C) The level of antibody binding was determined by FACS in K562 (A) or Raji (B) cells transfected with TRAP1-siRNA or scrambled siRNA and in TRAP1-KO and wildtype HEK293 cells (C). Ctrl, represents cells treated with buffer instead of antibodies. (D, F) C1q binding was tested in K562 cells treated for 60 min at 37°C with GTTP or control DMSO (D) or TRAP1-KO and wildtype HEK293 cells (E) after antibody binding and treatment for 15 min at 37°C with NHS or HIS. (F) C4b binding was tested in K562 cells transfected with TRAP1-siRNA or scrambled siRNA after antibody binding and treatment for 15 min at 37°C with NHS. (G, H) The expression level of CD46, CD55 and CD59 was measured by FACS in K562 cells transfected with TRAP1-siRNA or scrambled siRNA (G) and TRAP1-KO and wildtype HEK293 cells (F). ns, non-significance, Student t-test.

Figure 4. TRAP1 binds to C9 and inhibits C9 polymerization. (A, B, D, E) The binding of recombinant TRAP1 and purified C9 was tested by ELISA. The wells were either coated with C9 (A, D, E) or TRAP1 (B) and the binding level of TRAP1 and C9 was detected with anti-TRAP1 or anti-C9 antibody, accordingly, and a peroxidase-labeled secondary antibody. (A) TRAP1 significantly bound to C9 but not to C3b and BSA. (B) C9 bound to TRAP1 in a dose-dependent mode. (D) TRAP1-C9 binding was partially reduced by addition of GTTP. (E) TRAP1-C9 binding was abrogated by increasing concentrations of NaCl. (C) Zinc-induced C9 polymerization level was measured by ELISA. C9 mixed with TRAP1, mortalin or buffer control was treated with 42μM ZnCl₂ or buffer (Con.) for 60 min at 37°C. Then, it was added to wells pre-coated with monoclonal anti-C5b-9 aE11 antibodies or isotype control IgG. The level of C5b-9 in the wells

was quantified with a polyclonal anti-C9 antibody and a peroxidase-labeled secondary antibody.
***, $p < 0.001$, Student t-test (A, C) or one-way ANOVA (B, D E).

Figure 5. Complement-induced TRAP1 relocation to the cell membrane and its release from the cells. (A) K562 cells treated with sublytic antibody and NHS or HIS control were fixed, permeabilized and then TRAP1 location was detected by immunofluorescence in a Confocal Microscope. Representative images of TRAP1-labeled cells after HIS (control) or NHS treatment and brightfield images of the cells are shown. (B) TRAP1 level at the plasma membrane plane was quantified in 38 representative images of HIS or NHS-treated cells with ImageJ, as described in Methods. The intensity of the fluorescence (AU, arbitrary units) at the cells' plasma membrane (PM) is shown. (C-D) Cancer cells (HCT-116, UMUC-3, K562, and T47D) were treated with a sublytic dose of antibody and NHS or with NHS without antibody as a control. (C) The level of TRAP1 on the cell surface (mean fluorescence intensity, MFI) was measured by FACS. (D) The level of TRAP1 released into the supernatant was determined by collecting cell supernatant, clearing it by centrifugation at 13,000 g, and performing a TRAP1 ELISA. Recombinant TRAP1 was used to design a standard concentration curve to quantify in ng the amount of TRAP1 in the released fraction. (E, F) The amount of bound and released TRAP1 in K562 cells subjected to sublytic complement activation for 15 min at 37°C was determined. (E) Bound TRAP1 after treatment with antibody and NHS or C8- deficient NHS (C8D) or with HIS is shown. (F) Amount of extracellular TRAP1 released from cells activated by NHS, C8D or HIS is shown. The level of cell death was determined by PI inclusion (C, E, F) or LDH release (D).
***, $p < 0.001$, Student t-test.

Figure 6. Extracellular protective effects of TRAP1: (A) K562 cells were treated with anti-K562 antibodies, then with anti-TRAP1 antibody or isotype control IgG (4°C) and finally with

NHS or HIS (37°C). Percent cell death was determined by PI inclusion (A). The level of C3b and MAC deposition was measured by FACS as described above (B). (C) K562 cells were treated with anti-K562 antibodies (30 min at 4°C), followed by C8D-NHS for 15 min at 37°C. The C5b-7-bearing cells were washed and mixed with anti-TRAP1 antibody or isotype control IgG. Then, they were treated with NHS containing 10 mM EDTA (60 min at 37°C). Percent cell death was determined by PI inclusion. (D) K562 cells were treated with antibodies and C8D-NHS as above. Then, recombinant TRAP1 or buffer was added followed by EDTA-NHS. Cell death and MAC deposition were measured as above. ***, $p < 0.001$, Student t-test.

Figure 7. TRAP1 protects mitochondria from complement-induced stress: MMP, ATP and ROS. Cancer cells were treated with antibody or buffer and NHS, and cell death and the level of mitochondrial membrane polarization (MMP), ATP and ROS were determined as described under Methods. (A-C) K562 cells were pre-treated with GTTP (100 μ M) or DMSO as control (60 min at 37°C), then with complement and the level of MMP (A) and ATP (B), as well as percent cell death (C) were determined. (D-F) TRAP1-KO and wildtype HEK-293T cells were treated with NAC (5mM) or HBSS (for 30 min at 37°C) and then treated with complement and the percent cell death (D) and the level of oxygen radicals (DCFDA staining) (E) and MMP (F) were determined. **, $p < 0.01$, ***, $p < 0.001$, Student t-test.

Supplementary Fig. S1. Selective knockdown of TRAP1 after transfection with TRAP1-siRNA was confirmed by Western Blotting. Shown is the uncropped Western Blotting image of K562 cell lysates prepared after transfection (400 picomoles, followed by 48 h cell culture) with TRAP-1 siRNA, Mortalin-siRNA or scrambled siRNA. The blotted membrane was developed with mouse anti-TRAP1 mixed with mouse anti-actin antibodies (loading control), followed by a secondary

rabbit anti-mouse IgG antibody. The rectangle indicates the region from which the bands shown in Figure 1D were extracted.

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Ethics

None

Conflict of interest:

The authors have no conflict of interest to disclose.

Authors Contribution:

Conceptualization: ZF. Methodology: RB, YG and LZ. Formal analysis: RB, YG and LZ. Investigation: RB, YG and LZ. Figures preparation: RB. Resources: ZF. Writing – Original Draft: RB and ZF. Writing – Reviewing & Editing: RB and ZF.

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Data Availability:

Data and materials will be available upon reasonable request.

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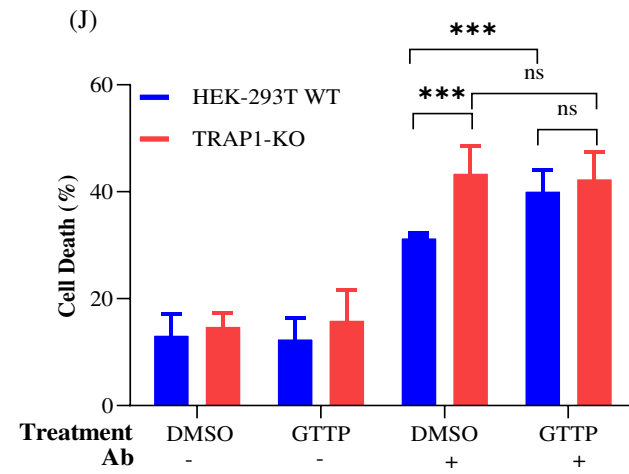
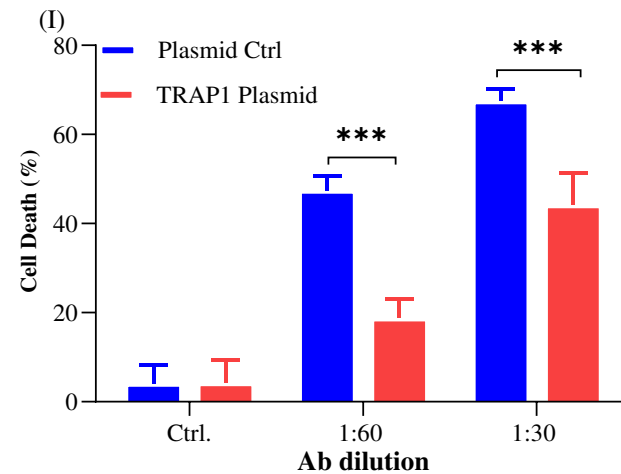
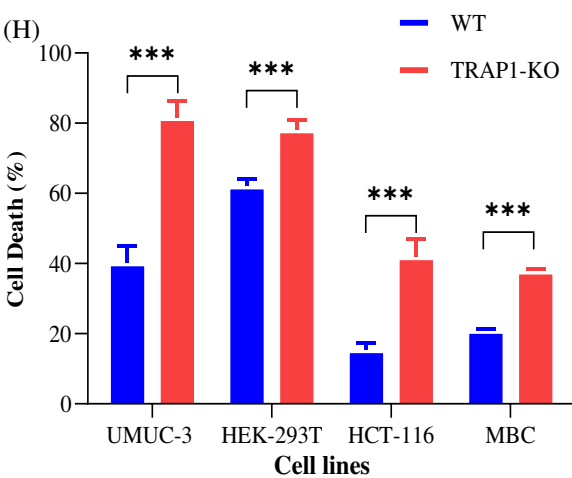
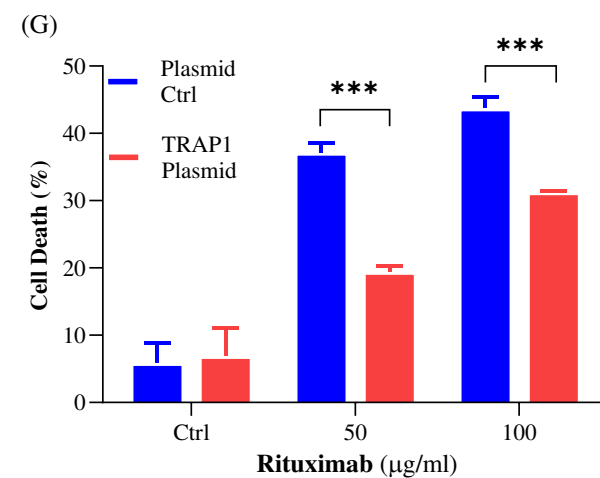
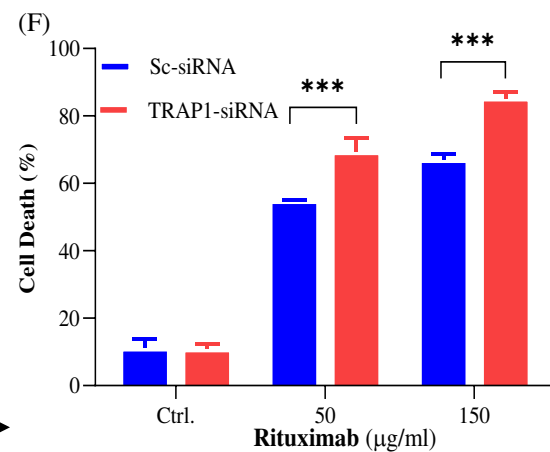
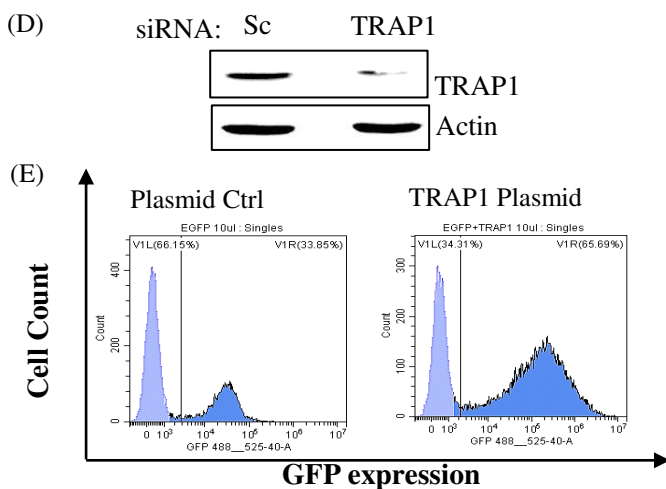
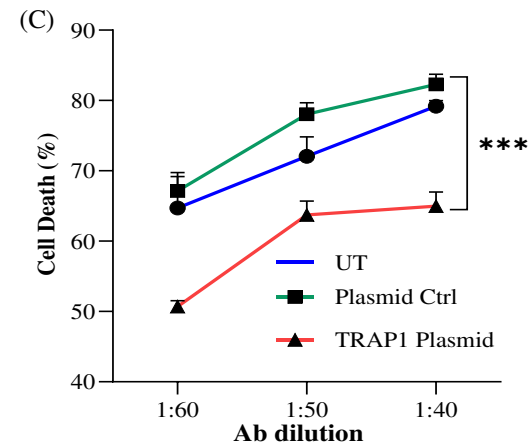
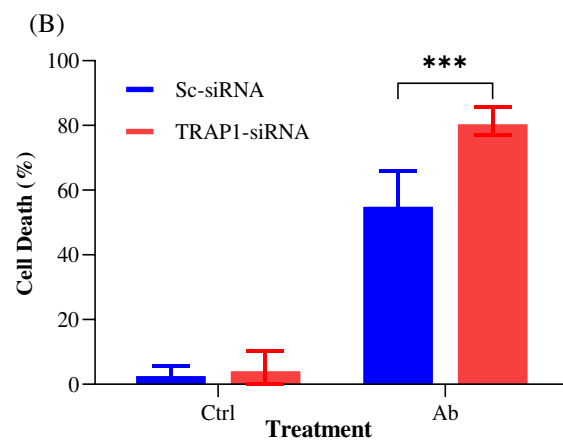
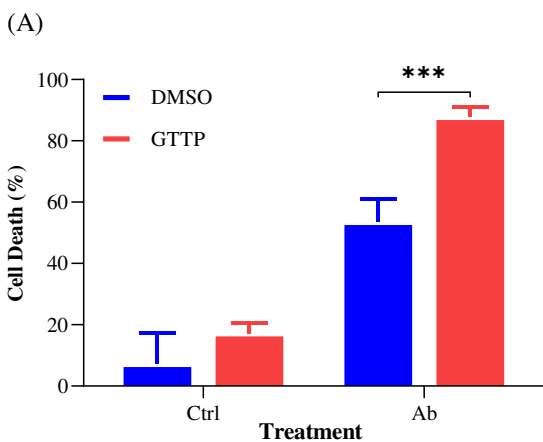
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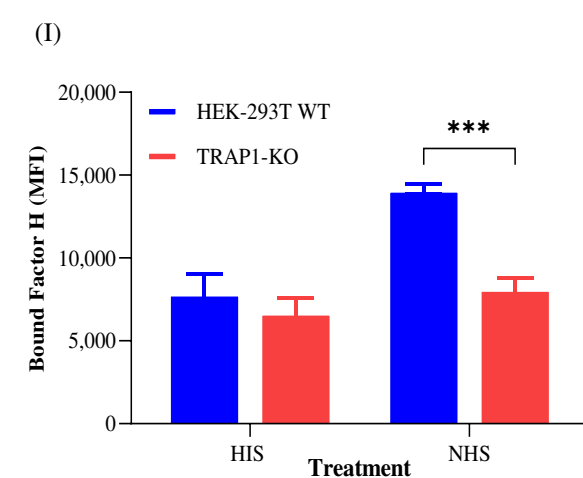
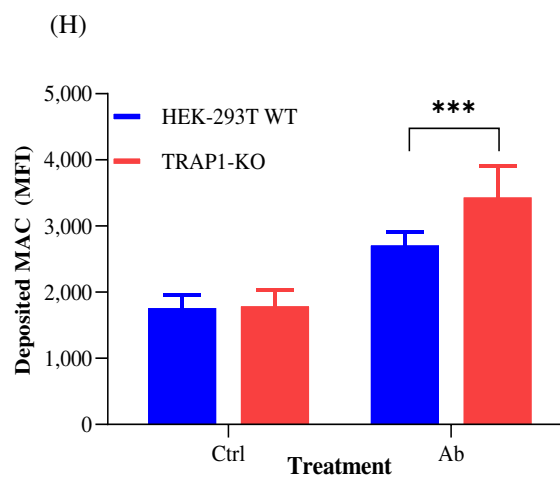
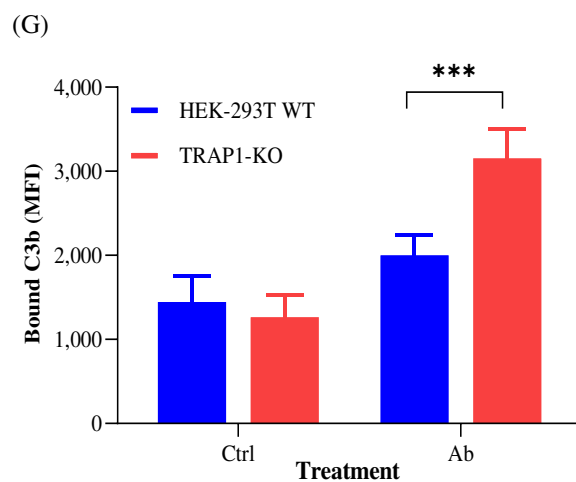
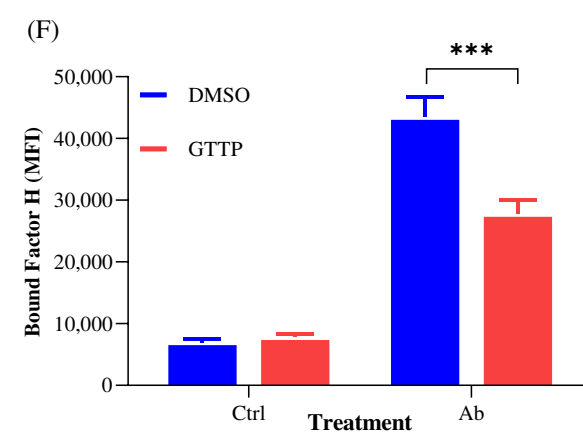
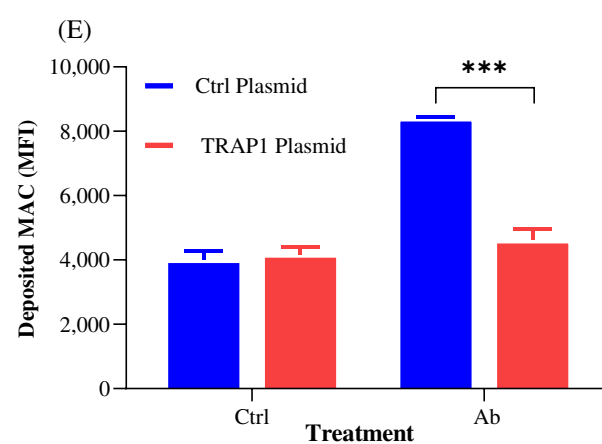
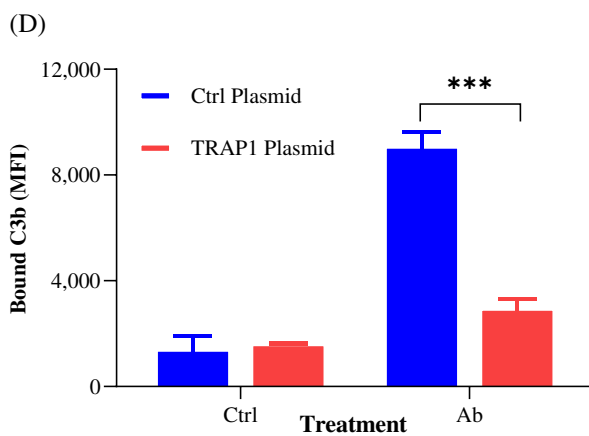
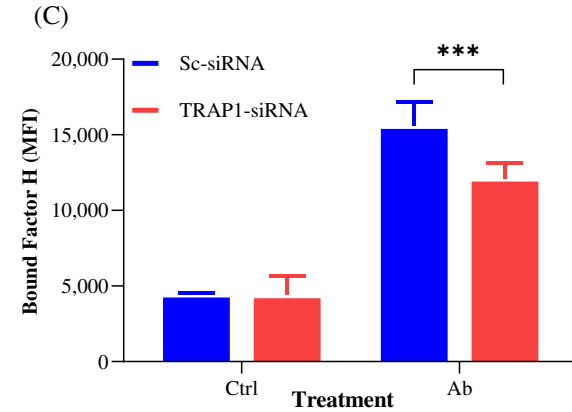
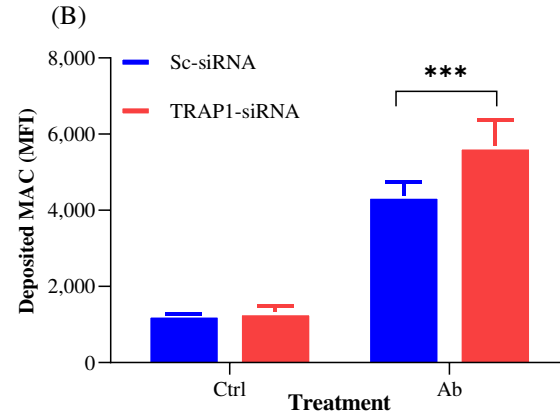
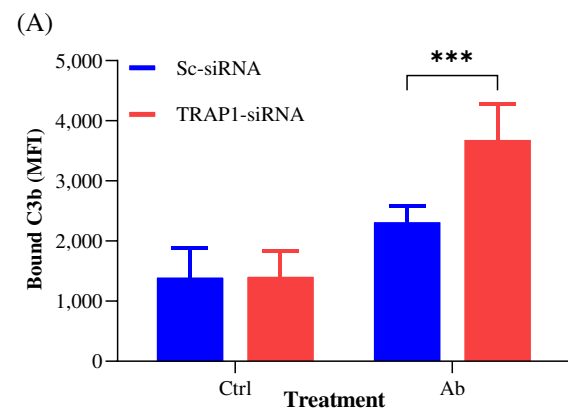
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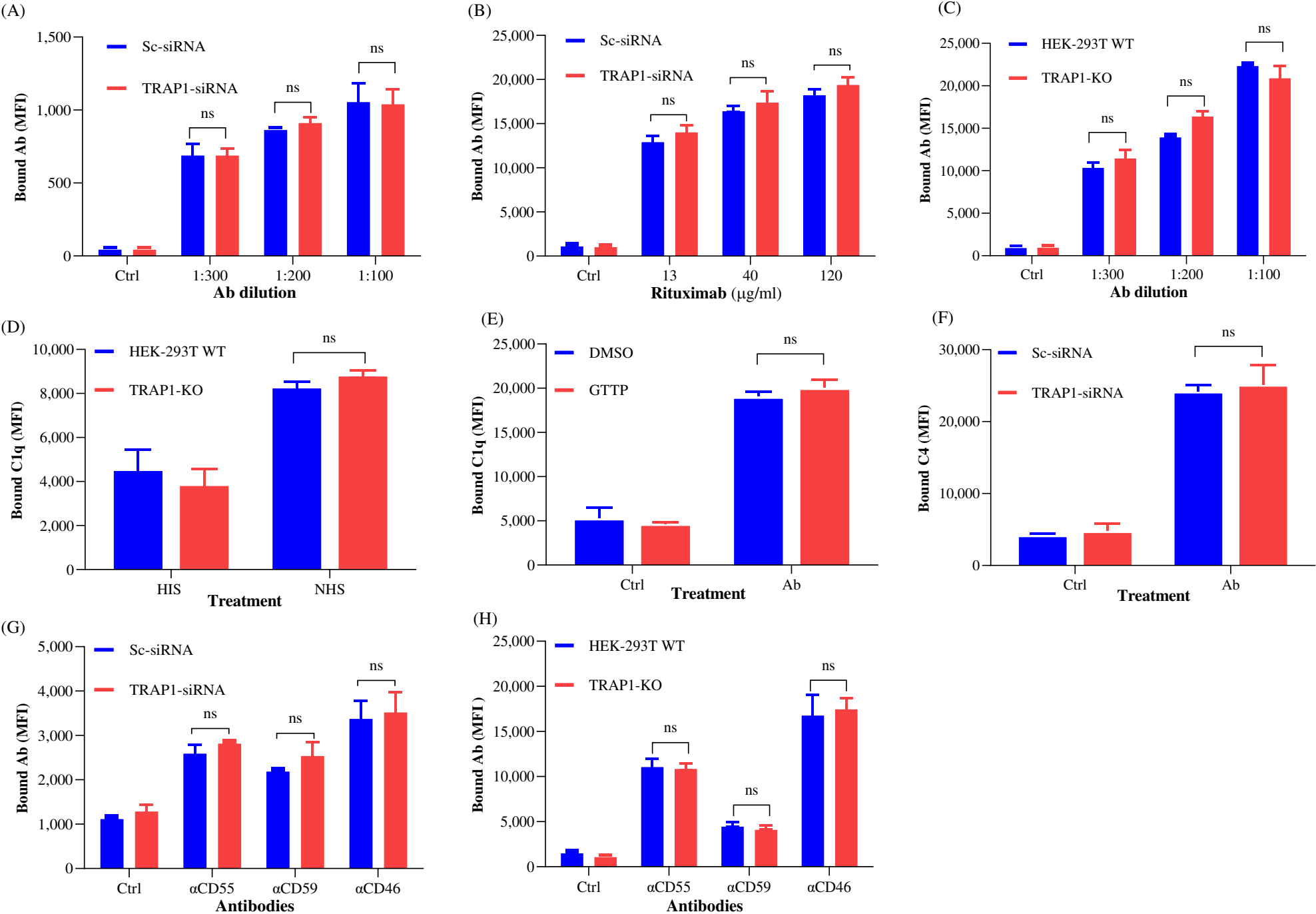
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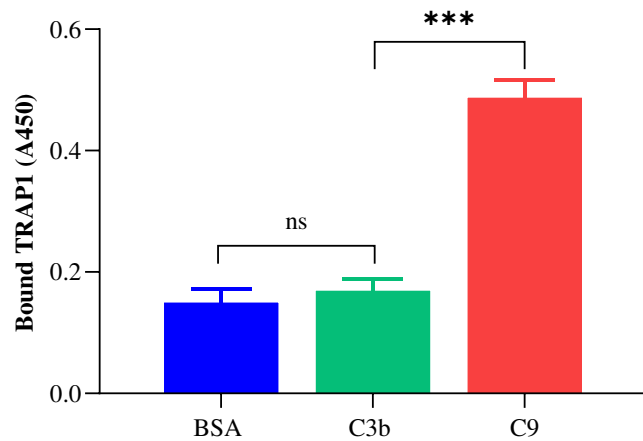
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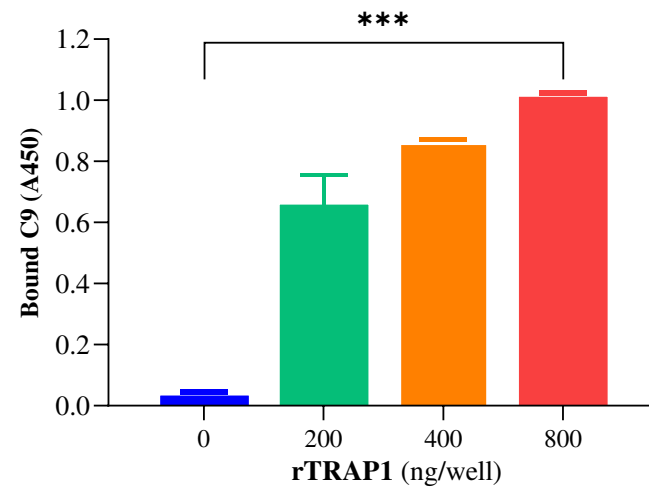




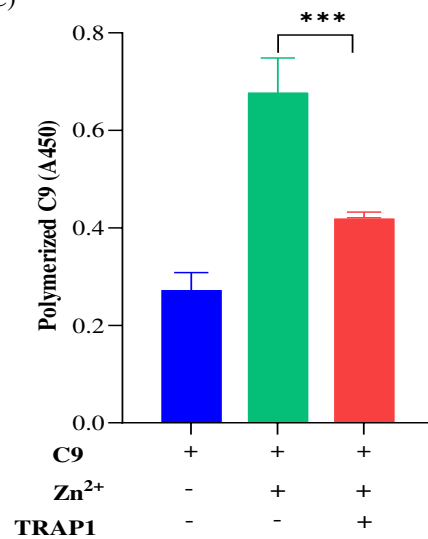
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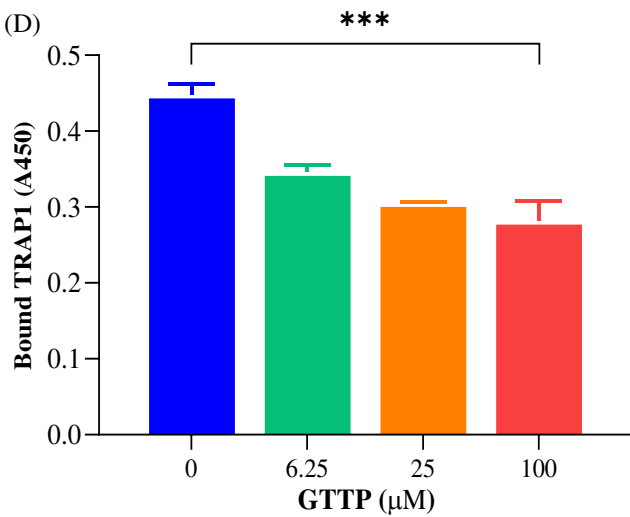
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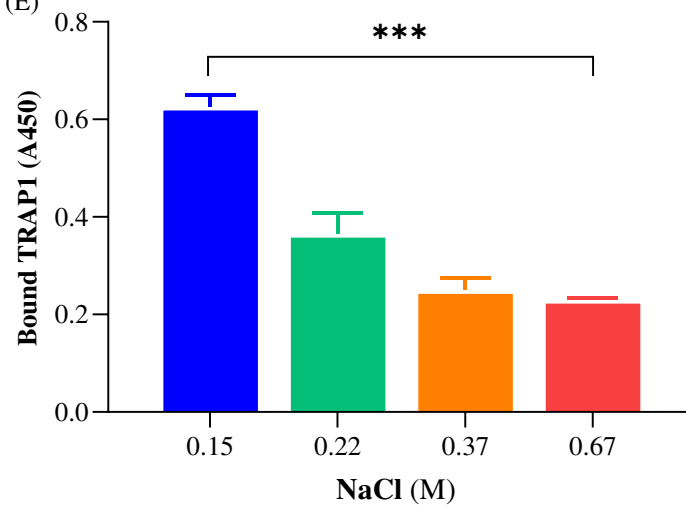
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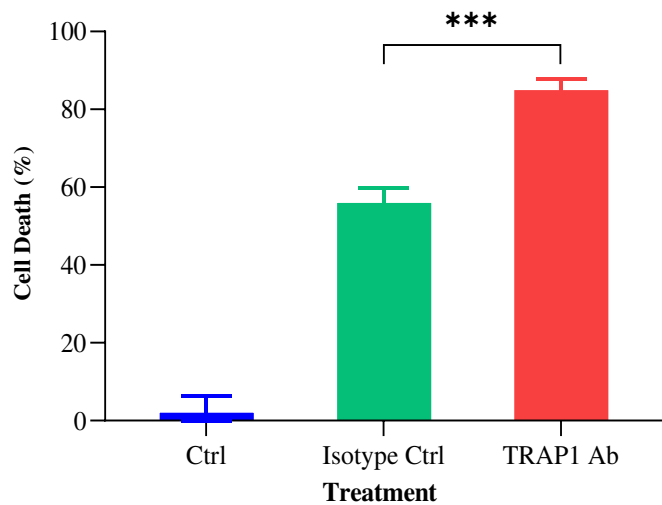


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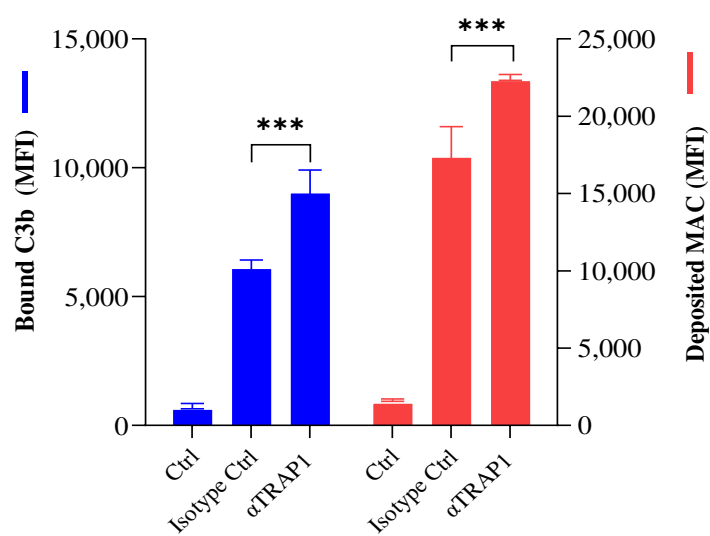


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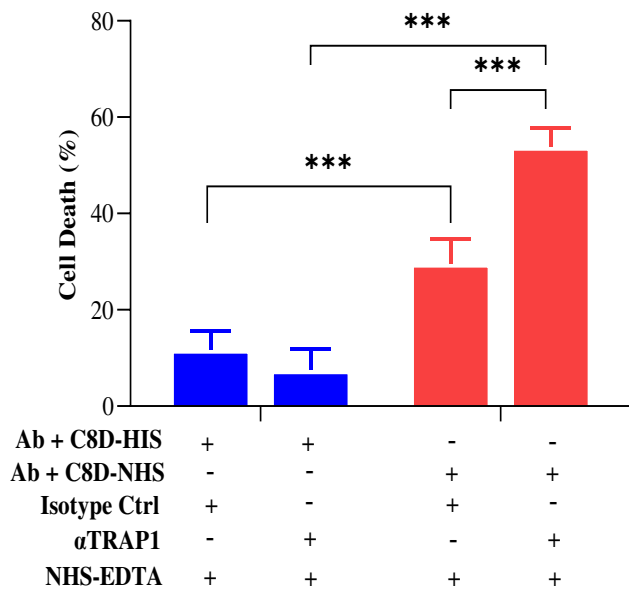
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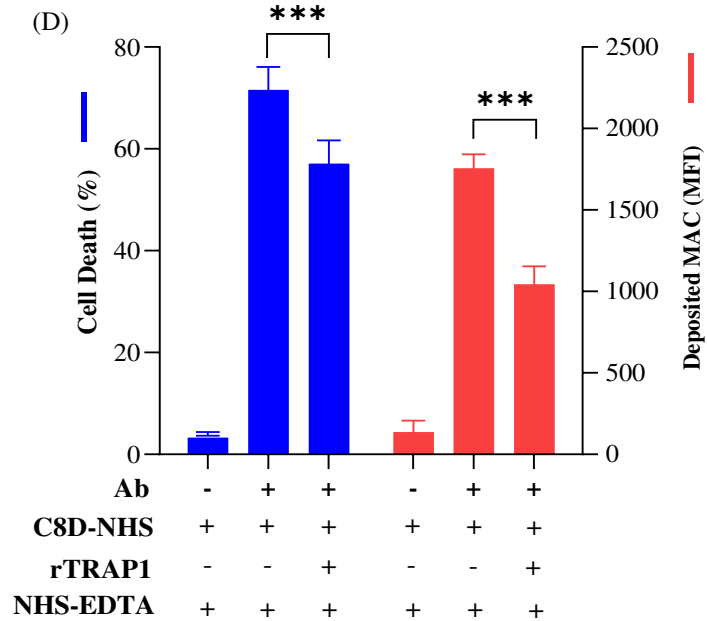
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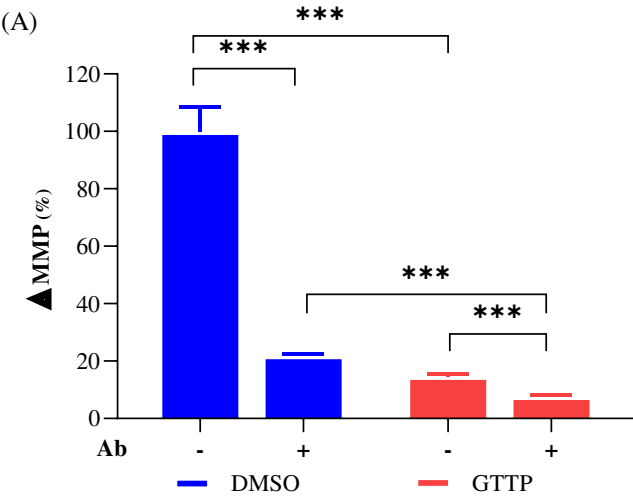
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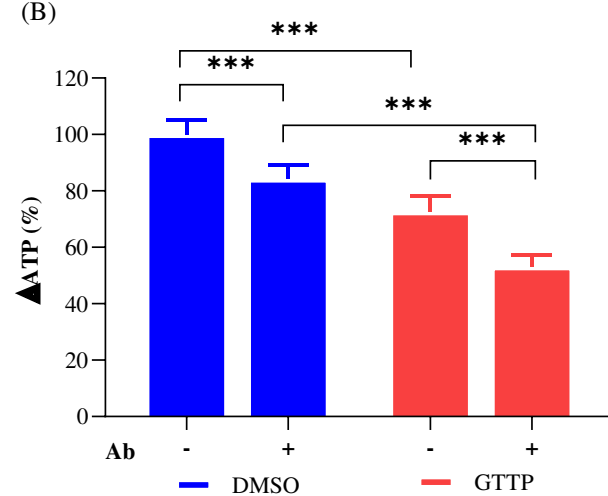
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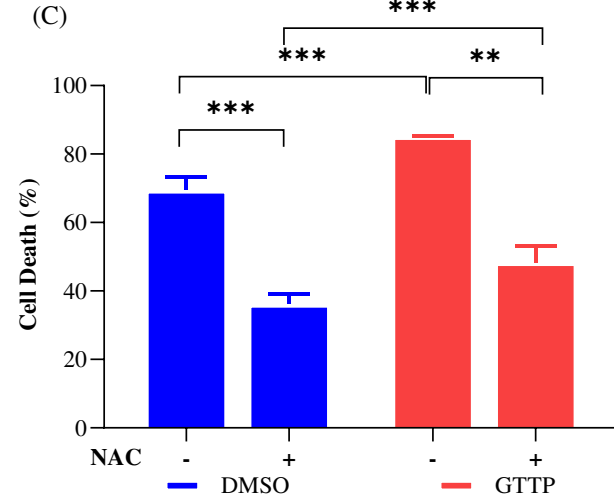
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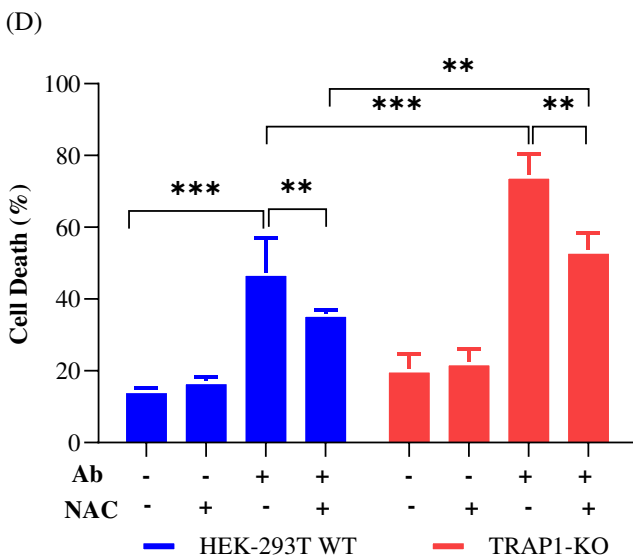
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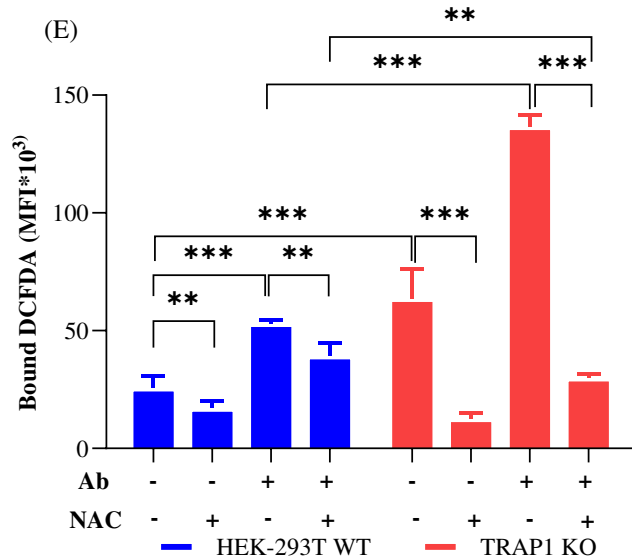
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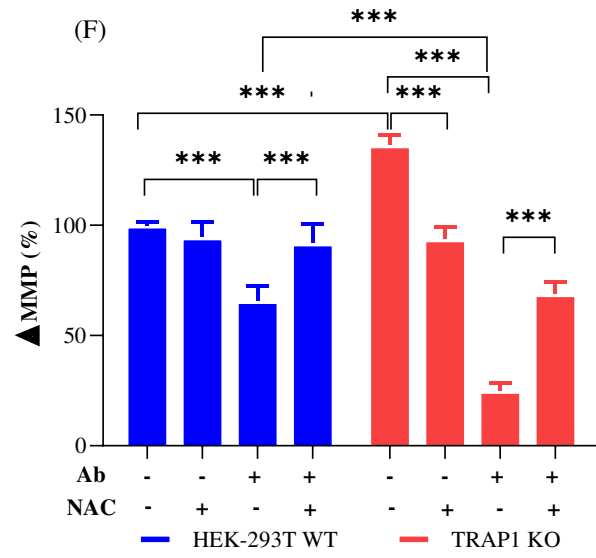
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(E)



(F)



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