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Supplementary Information for

Encephalitis is mediated by ROP18 of *Toxoplasma gondii*, a severe pathogen in AIDS patients

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Supplementary Information Text

Materials and Methods

Plasmids. Amplification of the open reading frame (ORF) encoding *T. gondii* ROP18 (Embank ID: AM075204.1) was achieved through RT-PCR from total *Toxoplasma gondii* tachyzoite RNA (17). Point mutations were introduced using the Quik Change method (Strategene, USA). The plasmid vectors pGBKT7 (Clontech, USA), pGADT7 (Clontech, USA), 3×FLAG (Sigma, USA) and pEGFP-C2 (BD-Biosciences, USA) were used to generate mammalian and yeast expression constructs carrying full-length ROP18, RTN1-C, and △20-RTN1-C (amino acids 21–208). Site-directed mutagenesis was performed using a standard molecular biology protocol. All constructs were confirmed through DNA sequencing. The wild type and mutants for RTN1-C were cloned into the bacterial expression plasmids pGEX-6P-1 (GE Healthcare, USA).

Cells and reagents. HFF(SCRC-1041), Neuro2a (CCL-131), and HEK293T cell lines (CRL-3216) were purchased from the American Type Culture Collection and cultured in DMEM (Gibco, USA) supplemented with 10% FBS (Gibco, USA) and 1% penicillin/streptomycin. Cells were maintained at 37°C in a humidified 5% CO2 atmosphere. Caspase-12 (#2202), cleaved-caspase-3 (#9664), GFP (#2956), HDAC3 (#3949) and acetyl-lysine (#9441) antibodies were purchased from Cell Signaling Technology (CST, USA). Phospho-serine/threonine antibody (ab17464), active Caspase-3 and GRP78 (ab21685) antibodies were purchased from Abcam (Abcam, U.K.). NeuN (MAB377, Millipore, USA) and RTN1-C antibodies (15048-1-AP, Proteintech, China) were also employed. CHOP (sc-4066) and GAPDH (sc-365062) antibodies were purchased from Santa Cruz (USA). *T. gondii* ROP18 antibody was kindly provided by Professor Yu Li (Anhui Medical University, China). Anacardic acid (A7236, Sigma, USA) and TSA (T1952, Sigma, USA) were also employed in the study. Secondary antibodies, including Texas Red-conjugated goat anti-rabbit IgG, FITC-conjugated goat anti-mouse IgG, and rhodamine-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, USA), were also used.

Immunofluorescent staining. The harvested brains were fixed in 4% paraformaldehyde for 48 h and embedded in paraffin. Then, 5.0-μm thick brain sections were rehydrated and rinsed in PBS. After antigen retrieval, the sections were permeabilized and blocked with PBS containing 0.5% Triton X-100 and 5% goat serum for 1 h at 37°C. The samples were stained with the indicated primary antibodies followed by fluorescent secondary antibodies. DAPI dye was used for DNA visualization. The images were captured using a fluorescence microscope (Olympus BX60, Tokyo, Japan).

Mass Spectrometry. Bands of interest from silver-stained gels were excised and then analyzed in reverse-phase nanospray liquid chromatography–tandem mass spectrometry by Proteomics Shared Resource, University of Sciences and Technology of China. The spectra from tandem mass spectrometry were automatically used for searching against the non-redundant International Protein Index human protein database (version 3.72) with the Bioworks browser (rev.3.1).

GST Pull-down assay.GST-ROP18 or GST was purified and conjugated to Glutathione-Sepharose 4B beads. Neuro2a cell lysate expressing RTN1-C or deletion mutant was incubated with GST-ROP18-conjugated or GST-conjugated beads at 4°C for 2 h. Subsequently, the beads were washed three times with pre-cooled PBS containing 1% Triton X-100, followed by three washes with PBS. The bound proteins were fixed in Laemmli loading buffer, incubated at 100°C for 10 min, and subjected to 10% SDS-PAGE, followed by immunoblot analysis.

Yeast two-hybrid analysis.RTN1-C constructed in pGADT7 (BD Bioscience) was mated with the AH109 strain transformed with pGBKT7-ROP18. In brief, the yeast cells were transformed with the bait construct using the lithium acetate method, followed by the selection of bait-containing, auxotrophic yeast cells via the appropriate nutritional marker in the selection medium. The bait-containing cells were subsequently transformed with the library constructs, and the resulting transformants were grown on medium either selecting for expression of both AD and BD vectors or an interaction between the expressed fusion proteins via nutritional reporter gene

expression. Clones that expressed all three reporter genes, HIS3, ADE2, and x-gal, were further analyzed. Interactions between bait and prey were selected via colony growth on plates lacking tryptophan, leucine, and histidine.

***In vitro* phosphorylation assay.**The GST-tagged full-length RTN1-C and its mutants were expressed in E. coli strain BL21 (DE3) and purified by using Ni-Sepharose beads (Qiagen) as previously described. For in vitro phosphorylation assay, anti-GFP immunoprecipitates from 293T cells transiently expressing ROP18-GFP were incubated with purified recombinant RTN1-C and its mutants (200 µg each) in kinase buffer (25 mM HEPES, pH 7.2, 1 mM DTT, 50 mM NaCl, 2 mM EGTA, 5 mM MgSO4) with 50 µM ATP and 0.5 µCi of [^{32}P]-ATP. The reaction mixtures (50 µl) were incubated at 30°C for 30 min and terminated by adding SDS-PAGE sample buffer. Proteins were then fractionated on SDS-PAGE. The gel was stained with Coomassie Brilliant Blue and subsequently incubated with x-ray film.

Immunoprecipitation and immunoblot analysis. The SY-5Y or Neuro2a cells infected with or without parasites were lysed in a lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM EGTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 g/ml leupeptin, and 10 g/ml pepstatin A) containing a protease inhibitor cocktail (Sigma-Aldrich). The cell lysates were separated through SDS-PAGE, transferred to nitrocellulose membranes, probed with the corresponding antibodies, and developed using an ECL kit. For immunoprecipitation, the cell lysates were precleared using anti-FLAG M2 affinity gel for 4 h with rotation at 4°C. The immunoprecipitants were washed three times with lysis buffer and three times with PBS and then eluted through boiling with Laemmli loading sample buffer. The eluates were separated using 10% SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting.

CRISPR/Cas9 Construction. To generate RTN1-C-knockout cells, three single-guide RNAs (sgRNAs) were constructed on a lentiviral backbone: sgRNA-1, forward, 5'-CGACGCTCACAACGCTGAAC-3'; sgRNA-2, forward, 5'- ATTGAAGAGCGCGCCAACGT-3'; sgRNA-3, forward, 5'- TGTGAGCGTCGTTGCCTACC-3', and a negative control (NC) sgRNA 5'-CGCTCGCGGCCCGTTCAA-3' duplex was chemically synthesized. Then, the corresponding RTN1-C-sgRNA oligos were synthesized. The resulting RTN1-C-sgRNAs for the target sitewere cloned into the vector GV371 (Genechem Co. Ltd. Shanghai, China), which contained the EGFP reporter, to produce recombinant plasmid GV371-RTN1-C-sgRNA. The other plasmid vector, Lenti-CAS9-puro, was purchased from Genepharma Co. Ltd. Virus packaging was completed in 293T cells after the co-transfection of the GV371-RTN1-C- sgRNA plasmid or the Lenti-CAS9-puro plasmid with the packaging plasmid.

HDAC colorimetric assay. Histone deacetylase activity assay was carried out using the Histone Deacetylase Colorimetric assay kit (Alexis, New York, NY, USA), according to manufacturer's specifications. Total cellular extracts of 100 mg were incubated with a colorimetric substrate at 37 1C for 1 h. The reaction was stopped with a lysine developer and the plate was incubated at 37 1C for 30min. Samples were analysed using an ELISA plate reader at 405 nm. HDAC activity was expressed as the relative optical density value per microgram of protein sample.

Detection of apoptosis. The apoptosis of Neuro2a cells were determined following the instruction of Annexin V-PE/7-AAD kit (BD, USA). Briefly, the Neuro2a cells were harvested, then washed twice with cold PBS, and incubated in 1 × binding buffer (10 mM HEPES, 0.14 M NaCl, and 0.25 mM $CaCl_2$) containing PE Annexin V and 7-AAD for 15 min. Then stained cells were analyzed using a Faces Calibur flow cytometer (BD Biosciences, USA)

tested for statistical significance using the t-test. $P<0.05$ was considered within 1 h, and the data were analyzed using FCS Express 4.0 software. Annexin V-PE+/7-AAD cells represent the early apoptotic cells, and annexinV-PE+/7-AAD+ cells represent the late apoptotic cells.

Statistical analysis. We used SPSS statistical software (version 16.0, SPSS, Chicago, IL). The data are expressed as the mean ± SD. Differences between the data were statistically significant.

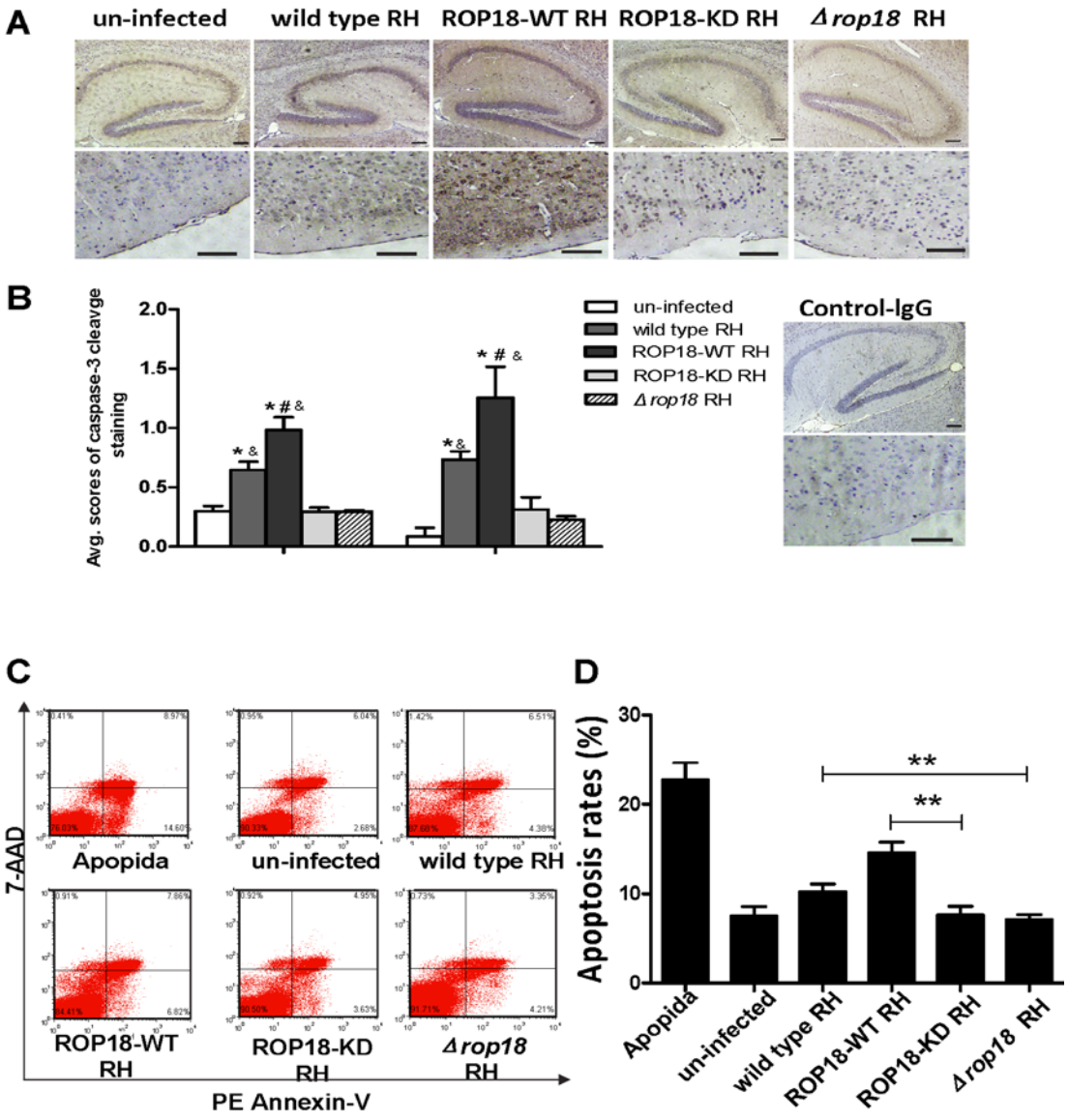

Fig. S1. ROP18 kinase activity is required for neural apoptosis.

(*A*) Representative immunostaining of active-caspase-3 in brain sections of mice infected with ROP18-WT RH, ROP18-KD RH, *Δrop18* RH and wild type RH tachyzoites. Normal brains of the un-infected group are also included. Scale bar, 50 μm.

(*B*) Semi-quantitative scoring of active-caspase-3 staining for both hippocampus and cerebral cortex summarized in a bar graph. * $P < 0.01$ vs. un-infected group; # $P < 0.01$ vs. ROP18-KD RH strain-infected group; $^{\&}$ $P < 0.01$ vs. *Δ rop18* RH-infected group.

(*C*) Neuro2a cells were infected with ROP18-WT RH, ROP18-KD RH, *Δrop18* RH and wild type RH tachyzoites at an m.o.i. of ~3 or treated with Apopida (apoptosis inducer A) for 24 h. Then, cell apoptosis was detected by flow cytometry after Annexin V-PE/7-AAD staining. The plots are from a representative measurement.

(*D*) The quantitative data of (C) are expressed as the mean ± SD based on three different assays (n =3).*$P < 0.05$.

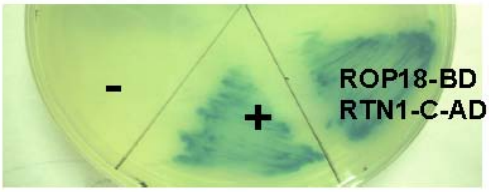

Fig. S2. Confirmation of ROP18 and RTN1-C interaction using yeast two-hybrid co-transformation assay. Yeast two-hybrid co-transformation assay was performed and assessed via growth on medium containing x-α-gal and lacking Ade, Trp, Leu, and His. Yeast cells co-transformed with BD-53 and AD-T were used as the positive control, and yeast cells co-transformed with BD-lam and AD-T were used as the negative control.

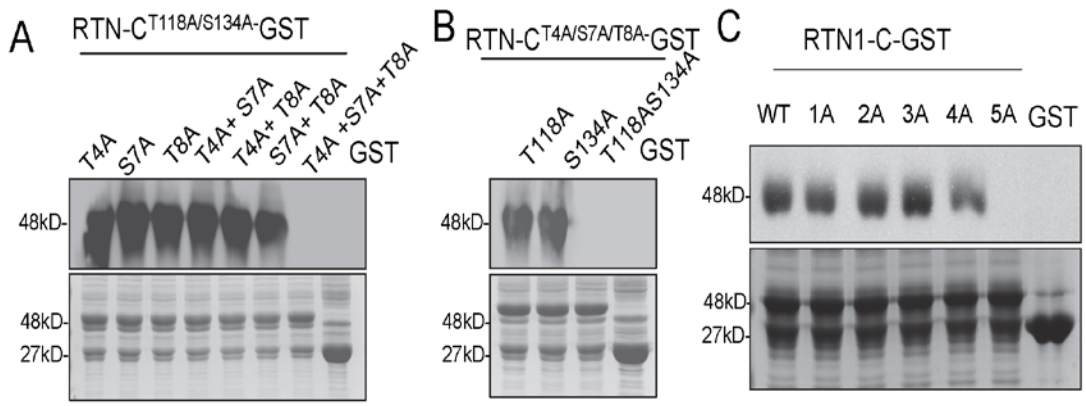

Fig. S3. RTN1-C Ser7/134 and Thr4/8/118 are substrates of ROP18.

(*A*) To confirm Thr4, Ser7 and Thr8 sites were substrates of ROP18, a GST-tagged mutated version of the full-length RTN1-C $^{T118A/S134A}$ containing T4A, S7A,T8A, T4A+S7A, T4A+T8A, S7A+T8A or T4A+S7A+T8A were subjected to in vitro kinase assays using eukaryotic express ROP18 as described.

(*B*) To confirm Thr118 and Ser134 sites were substrates of ROP18, a GST-tagged mutated version of the full-length RTN1-C $^{T4A/S7A/T8A}$ containing T118A, S134A or T118A+S134A were subjected to in vitro kinase assays using eukaryotic express ROP18 as described.

(*C*) Purified GST-tagged RTN1-C-1A(S7A), RTN1-C-2A(SS7/134AA), RTN1-C-3A (SS7/134AA+T4A), RTN1-C-4A (SS7/134AA+TT4/8AA), and RTN1-C-5A (SS7/134AA+TTT4/8/118AAA), were subjected to in vitro kinase assays using eukaryotic express ROP18 as described. Note that ^{32}P failed to incorporate into the RTN1-C-5A mutant proteins in the presence of ROP18.

All these samples were separated by SDS-PAGE gel and stained with Coomassie Brilliant Blue (lower) and subsequently incubated with x-ray film. The autoradiogram was also shown (upper).

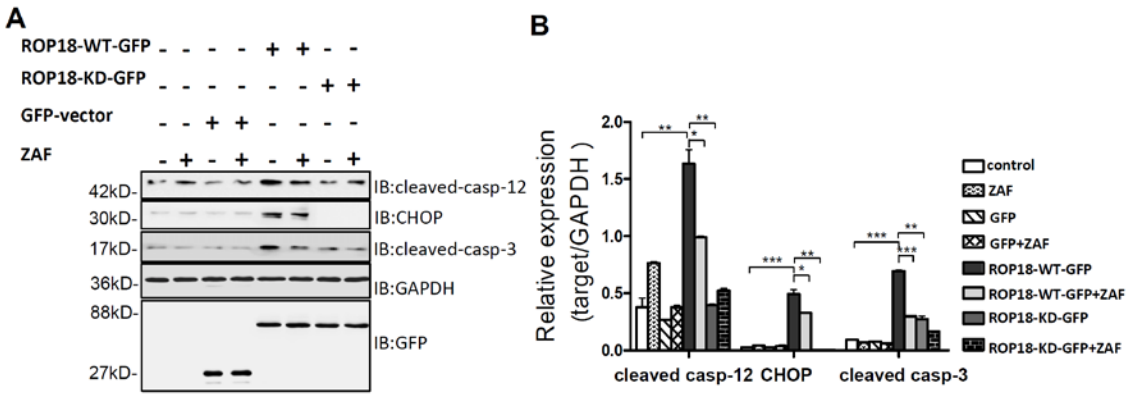

Fig. S4. Western blotting analyses of inhibitors on caspase-12, caspase-3 and CHOP expression levels in Neuro2a cells

(*A*) Western blot analysis of the expression levels of ER stress-associated apoptotic proteins induced by ROP18. After Neuro2a cells were treated with or without Z-ATAD-FMK (ZAF) for 6 h, cells were transfected with the equal volumes of ROP18-WT-GFP or ROP18-KD-GFP for 24 h. Then, cell lysates were detected through Western blot with the indicated antibodies. GAPDH was included as a loading control.

(*B*) The quantitative data of (A) panel. The quantitative data are expressed as the mean ± SD for different assays (n = 3). Neuro2a cells transfected with GFP vector were used as control. ROP18-WT-GFP + ZAF or ROP18-KD-GFP + ZAF indicate Neuro2a cells pre-treated with ZAF and then transfected with either ROP18-WT-GFP or ROP18-KD-GFP. *P < 0.05, **P < 0.01, *** P < 0.001, ROP18-WT-GFP *vs.* ROP18-KD-GFP, ROP18-WT-GFP + ZAF or control.

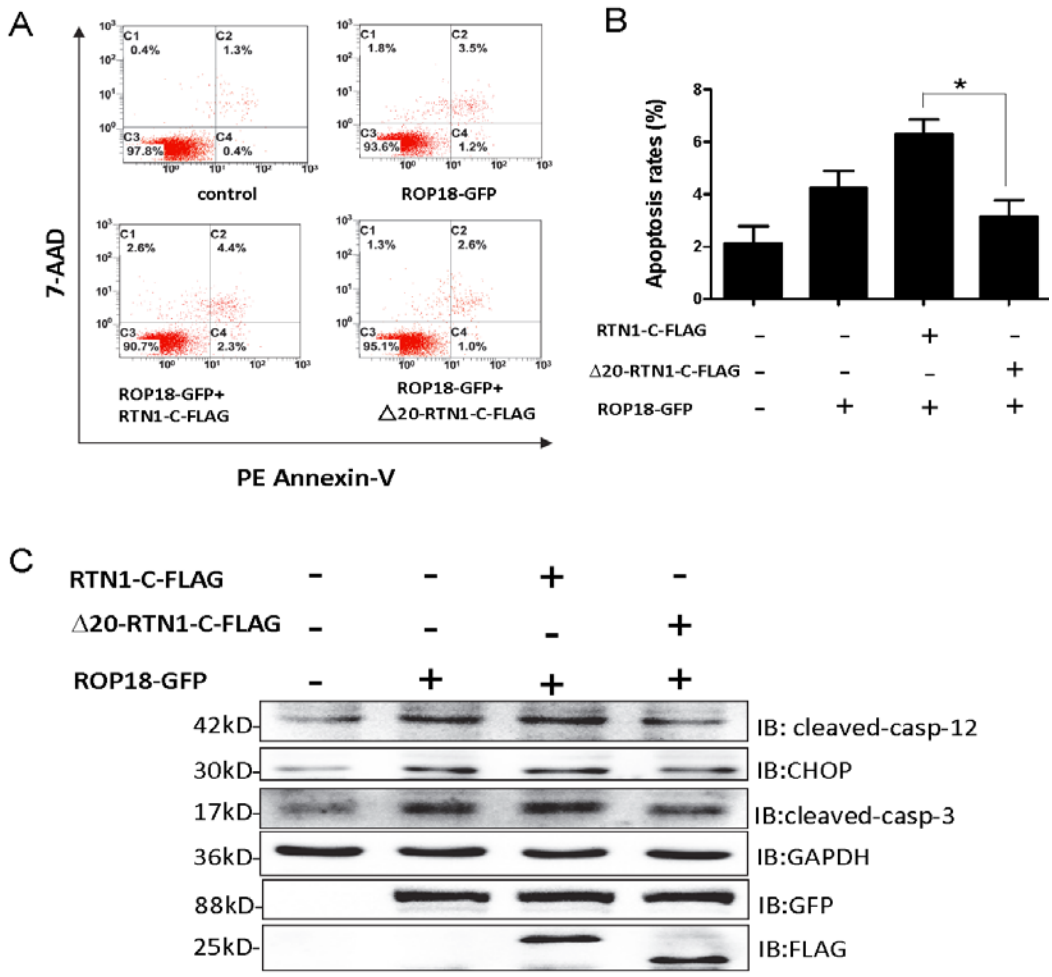

Fig. S5. The deficiency of the interaction domain of ROP18 and RTN1-C reduce the neural apoptosis.

(*A*) The Neuro2a cells were co-transfected with ROP18-GFP and RTN1-C-FLAG or △20-RTN1-C-FLAG for 24h. Then the apoptosis of the cells were detected by flow cytometry after Annexin V-PE/7-AAD staining. The plots are from a representative measurement.

(*B*)The quantitative data of (A) were expressed as mean ± SD on three different assays (n = 3). *P < 0.05.

(*C*) Representative western blot analysis of cleaved caspase-12, CHOP and cleaved caspase-3 in Neuro2a cells after co-transfection with ROP18-GFP and RTN1-C-FLAG or △20-RTN1-C-FLAG for 24h.

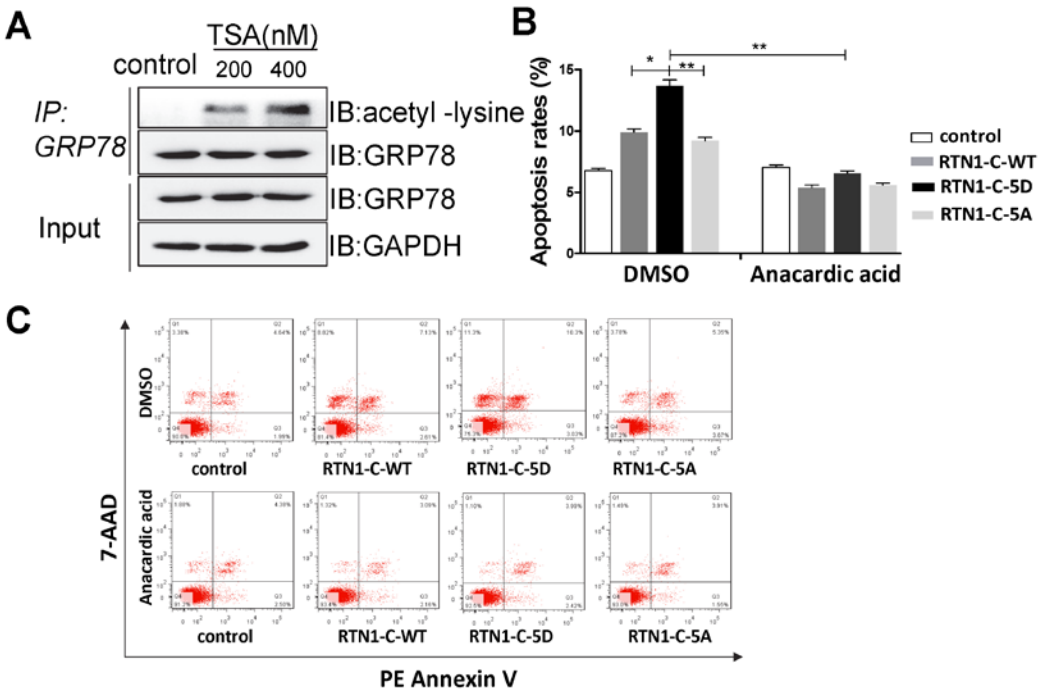

Fig. S6. Acetylation inhibition attenuated the apoptosis induced by phosphorylation of RTN1-C by ROP18.

(*A*) Immunoprecipitation assay confirmed that inhibition of HDAC activity by TSA also enhances GRP78 acetylation. SH-SY5Y cells were exposed to the HDAC inhibitor TSA at described concentrations for 12 h. Then the lysates were immunoprecipitated with anti-GRP78 and immunoblotted with both anti-acetyl lysine and anti-GRP78.

(*B*) Neuro2a cells were transfected with RTN1-C-WT-FLAG, RTN1-C-5A-FLAG or RTN1-C-5D-FLAG for 24 h with or without treatment 15 mM anacardic acid for 6 h. Then, cell apoptosis was detected by flow cytometry after Annexin V-PE/7-AAD staining.The quantitative data of the data are expressed as the mean ± SD based on three different assays (n = 3). *P < 0.05; **P < 0.01; ***P < 0.001.

(*C*) The plots of (*B*) are obtained from a representative measurement.

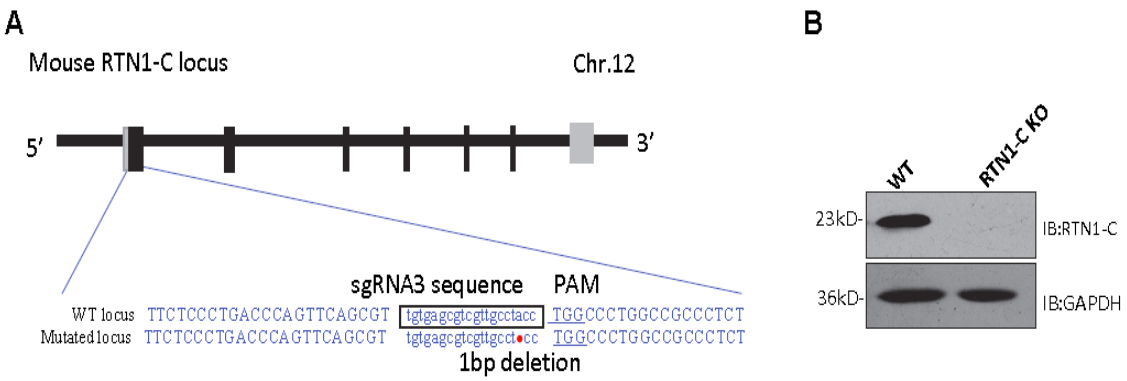

Fig. S7. Generation of RTN1-C knockout Neuro2a cells by Cas9/CRISPR-mediated genome editing.

(*A*) Schematic of the Cas9/gRNA-targeting site in the RTN1-C gene.

(*B*)Western blot analysis for RTN1-C and GAPDH protein expression in WT and RTN1-C-deficient Neuro2a cells.

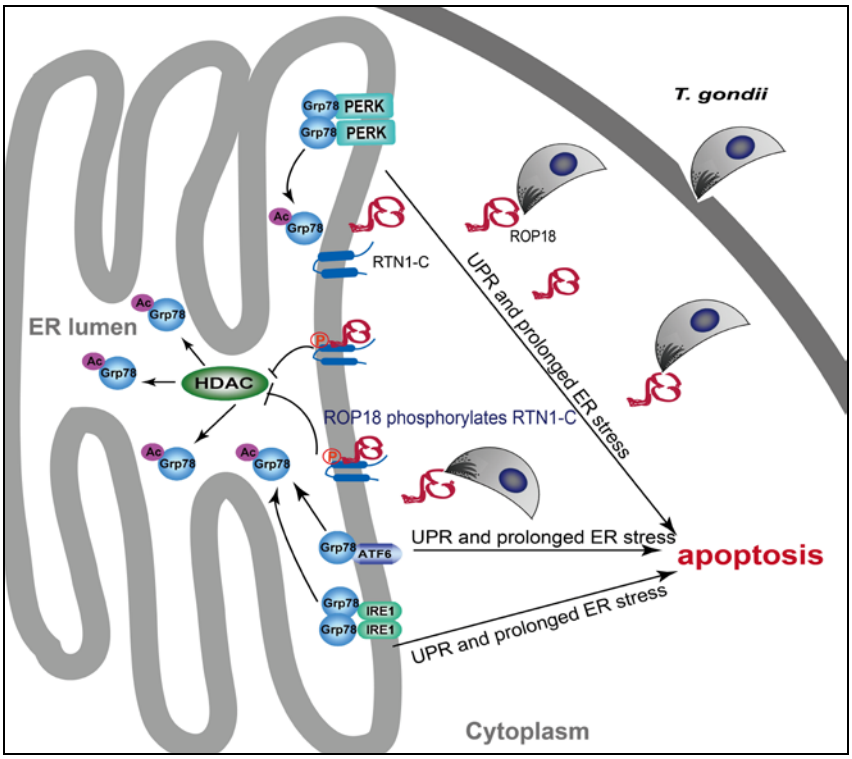

Fig. S8. Schematic drawing summarizes the phosphorylation of host RTN1-C by *T. gondii* ROP18 and its subsequent neural apoptosis.

ROP18 kinase activity phosphorylates RTN1-C to activate subsequent ER stress via the UPR (unfolded protein response). The UPR is controlled by glucose-regulated protein 78 (GRP78) and three different ER transmembrane sensor proteins: PKR-like eukaryotic initiation factor 2a kinase (PERK), inositol requiring enzyme 1 (IRE1), and activating transcription factor-6 (ATF6). Innormal and non-stressed cells, GRP78 binds to ER transmembrane sensor proteins and maintains them in an inactive form. During ER stress, an increase in unfolded substrates leads to the sequestration of GRP78, releasing the sensors to initiate UPR signals. If the ER stress is not relieved, the injured cells enter apoptosis. Histone deacetylase (HDAC) regulates the UPR via acetylation of GRP78 and when attenuated by ROP18-phosphorylated RTN1-C leads to GRP78 acetylation.

Table S1. Host proteins bound to TgROP18 identified by mass spectrometry

Band No.	NCBI accession	MM	Protein identified	Protein Probability Score	Sequence coverage & sequenced peptides	No. of matched
P1	GI:119573383	74095.7	Lamin A/C	2.56E-04	9.34% R.SGAQASSTPLSPTR.I R.SLETENAGLR.L R.NSNLVGAAHEELQQSR.I R.LADALQELR.A R.IDSLSAQLSQLQK.Q	5
P2	GI:223468676	60182.4	transcription factor p65 isoform 1	2.81E-04	6.17% R.GDYDLNAVR.L R.DLEQAISQR.I R.IQTNNNPFQVPIEEQR.G	3
P3	GI:460789	50997.4	Transformation upregulated nuclear protein	1.49E-06	10.34% R.TDYNASVSVPDSSGPER.I R.GSYGDLGGPIITTQVTIPK .D K.IILDLISESPIK.G	3
P4	GI:307311	23567.1	RTN1-C	1.41E-05	4.12% SVLQAVQK AYLELEITLSQEQ MQATADSTK	3