Resveratrol induces H3 and H4K16 de-acetylation and H2A.X phosphorylation in *Toxoplasma gondii*

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Research note

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Abstract

Objective: Objective: Resveratrol (RSV) is a multi-target drug that has demonstrated activity against *Toxoplasma gondii* in macrophage and HFF cell line infection models. However, its mechanism of action has not been determined yet. With the aim of determining a possible mechanism of anti-*T. gondii* action, we analyzed the effect of RSV on histones H3 and H4 lysine 16 acetylation (H4K16). RSV-induced DNA damage of intracellular tachyzoites was assessed as well by using the DNA damage marker phosphorylated histone H2A.X (gH2AX).

Results: RSV inhibited intracellular *T. gondii* tachyzoite growth at concentrations below the toxic effect on host cells. The IC_{50} value in a 24-hours treatment was 53 mM. RSV induced a reduction in H4K16 acetylation (H4K16ac), a mark associated to transcription, DNA replication and homologous recombination repair. The same deacetylating effect was observed on histone H3. RSV also enhanced the SQE motif phosphorylation on *T. gondii* H2A.X (termed γH2A.X), a DNA damage associated PTM. Our findings suggest a possible link between RSV and DNA damage or DNA repair process maybe associated to DNA replication stress.

Introduction

Toxoplasma gondii is an important pathogen for animals and human health, particularly during pregnancy and in immunocompromised patients [1]. In humans, infection starts the asexual cycle which also occurs in other mammals and birds. The asexual phase is characterized by two stages, the rapidly replicating and highly disseminating tachyzoite, and the bradyzoite, which replicates slowly and is located in tissue cysts for the rest of the life of the animal or individual [2]. The current therapy against toxoplasmosis is effective but it is associated to adverse effects [3]. With the aim of finding new therapies in recent years, different therapeutic targets have been analyzed [4–7].

Resveratrol (RSV; 3,5,4'-trihydroxystilbene) is a natural polyphenolic phytoalexin produced in plants. RSV can alter the activity of some histone deacetylases HDACI/II, DNA methyl-transferases (DNMT) and an activator of Sir2, a sirtuin belonging to HDACIII [6]. Sir2 modulates the acetylation status of H4K16 [8, 9]. RSV acts as anti-oxidant at low doses, but it is pro-oxidant and generates DNA damage at high doses [10]. Treatment of human cell lines with RSV induced Rad9 expression, a key player in the DNA damage response (DDR), as well as H2A.X phosphorylation at SQE motif (γH2A.X), a double strand break (DSB) marker [11].

RSV administration during acute *T. gondii* infection in mice would confer protection [12] as well as moderate tissue inflammation and reduction of parasite replication [13]. Chen *et al.*, [14] observed that incubation of extracellular tachyzoites with RSV for 24 hours affected their viability. They also observed that RSV reduced tachyzoite intracellular growth and promoted autophagy in infected macrophages. Adeyemi *et al.*, [15] identified RSV as a putative drug repurposing candidate against toxoplasmosis. However, none of them analyzed the mechanism(s) by which RSV affected *T. gondii* tachyzoites.

In this work, we evaluated the effect of RSV on *T. gondii* replication and histone PTMs alteration. The effect of RSV on H3 and H4K16 acetylation level was analyzed. In addition, changes in γH2A.X levels were also analyzed in control or treated intracellular tachyzoites. Collectively, our results show that RSV inhibited *T. gondii* replication and induced H4K16 and H3 de-acetylation. Finally, γH2A.X marks were highly enhanced in tachyzoites treated with RSV suggesting and association between this treatment and DSB damage.

Materials And Methods

Parasite sources, culture and reagents

Tachyzoites of RH wild type and RH RFP (Red Fluorescent Protein) strains were cultured under standard conditions *in vitro* in immortal human foreskin fibroblasts (hTERT, ATCC® CRL-4001) monolayers. RH RFP was kindly given by Dr. Silvia N. Moreno (University of Georgia, Athens, GA, USA). Cell monolayers were infected with tachyzoites and incubated with Dulbecco's modified Eagle medium (DMEM, Invitrogen) high glucose supplemented with 10% (10X) or 1% (1X) of fetal bovine serum (FBS, Internegocios S.A., Argentina) and penicillin (10,000 units/ml) - streptomycin (10 mg/ml) solution (Gibco) at 37 °C and 5% CO_2 atmosphere. Resveratrol (Abcam,120726) solution was prepared in DMSO vehicle and stocked at 10 mg/ml.

Commercial rabbit antibodies: anti-H3 (Abcam ab10799), anti-H3ac (Millipore, 06-599B), anti-H4 (Abcam ab31830) and anti-H4K16ac (Abcam, ab109463). Alexa fluor secondary antibodies: goat anti-mouse 488 (Invitrogen, A11001) and anti-rabbit 595 (Invitrogen, A11037). Alkaline phosphatase–conjugated antibodies (Santa Cruz Biotechnology).

Rabbit anti-*T. gondii* γH2A.X serum sample was obtained from Eurogentec (Belgium) on the basis of the peptide NH_2- C + GKHGV-$S_{(PO3H2)}$-QEF –COOH, designed from *T. gondii* H2A.X amino acid sequence. Rabbit anti-rH2A.X was obtained from the Animal Facility at Facultad de Ciencias Exactas y Naturales (University of Buenos Aires, Argentina) on the basis of the recombinant rH2A.X [16]. Murine anti-SAG1 was kindly provided by Dr. Marina Clemente [17].

Toxicity assay

To evaluate the cytotoxic effect of the drugs on host cells, fibroblasts (hTERT) were seeded at a 40% confluence (1.6 10^4 cells/well) in 96-well plates for 24 hours. After this period, confluence was checked by microscopy and the medium replaced with fresh media containing RSV at different concentrations. For experiments where cells were in contact with RSV for 24 hours, the concentrations ranged between 0 to 200 µM. Cytoxicity was determined by reduction of 3 (4,5 dimethyl-2-thiazoyl) -2,5-diphenyltetrazole bromide (MTT). The absorbance was measured from the bottom of the plate at 540 nm using a BioTek Synergy plate reader. Graphs indicate the relative viability of the cells with respect to the control with DMSO 0.5% in culture media (100% viability).

Replication assay

Replication was performed and assessed using RFP-expressing RH tachyzoites as previously described [18]. For intracellular exposition, hTERT cells were infected with 10,000 RH RFP parasites. The other half of the plate was used as control (host-cells + different concentrations of drugs tested). After 3 hours of infection, the medium of the whole plate was replaced by fresh media 1X containing increasing concentrations of RSV or vehicle. Parasites were then allowed to replicate for 24 hours on host cells. The RFU (Relative Fluorescent Unit) of the RFP data was collected using a Synergy H1 Hybrid Multi-Mode Microplate Reader (Biotek). Each concentration of RSV was measured in triplicates. The basal fluorescence was estimated in 234 +/- 2.3 RFU. We also calculated the IC_{50} value. IC_{50} was obtained as previously described [19] by GraphPad Prism 6. Data were normalized with 0 as the smallest value and transformed to semi-logarithmic scale (x = log(x)). After that, they were analyzed as a nonlinear regression parameter- dose-response inhibition-log(inhibitor) vs normalized response-variable slope.

Indirect immunofluorescence assay (IFA)

Intracellularly tachyzoites were incubated during 24 hours with RSV 100 µM, washed with PBS 1X and fixed with methanol for 10 min at -20 °C. Then the cells were permeabilized with 0.2% v/v Triton X-100 in PBS for 10 min and blocked with 3% w/v BSA in PBS for 30 min at room temperature (r.t.). After this, primary antibodies were added diluted in 0.5% w/v BSA in PBS for 60 min at r.t., washed three times with PBS and then incubated with the corresponding alexa fluor-conjugated secondary antibodies for 60 min at r.t. Samples were mounted on coverslips with mounting medium (Mowbiol) containing 10 µg/µl of DAPI (4,6-diamidino 2-phenyl-indole) to stain the nuclei. Primary antibodies were diluted 1:200 whereas secondary antibodies were diluted 1:2,000. Samples were analyzed by epifluorescence microscopy (Zeiss Axioscope) equipped with a 63X objective and Zeiss Axiocam 506 mono microscope camera. Images and fluorescence intensity analysis were obtained with Fiji (ImageJ) program.

Western blot

Immunoblot was done as previously described with minor modifications [16]. Intracellular parasites were incubated for 24 h with 70 µM RSV. Proteins from purified parasites (10^7 parasites/lane). Non-specific binding sites were blocked with 5% non-fat-dried milk in TBS containing 0.1% v/v Tween-20 (TBS-T) for 6 h and the membranes were then incubated overnight with primary antibodies: mouse anti-H4, rabbit anti-H4K16ac, mouse anti-H3, rabbit anti-H3ac, rabbit anti-γH2A.X, rabbit anti-H2A.X and mouse anti-SAG1. Intensities of bands were quantified from scanned images using ImageJ software and the value obtained from each band was normalized by the loading control SAG1 and then each histone PTM was normalized by their respective histone (H2A.X or H4).

Statistics

Data were expressed as mean ± SD from three to four different experiments. The variations of the data were analyzed with the GraphPad Prism (6.1) software, using Unpaired Student´s *T* test (* $p \leq 0.05$ and **** $p \leq 0.0001$) (GraphPad Prism 6.1).

Results And Discussion

In order to test our study model, uninfected or infected hTERT host cells were incubated 24 hours with different doses of RSV. Near 80% of uninfected hTERT cells remain viable at concentrations of 100 µM (Fig. 1A). In order to test the impact of RSV on *T. gondii* lytic cycle, transgenic RH RFP (Red Fluorescent Protein) tachyzoites were intracellularly grown during 24 hours in presence of RSV. As it can be observed, RSV showed a dose-dependent effect in intracellular *T. gondii* replication (Fig. 1B) with an IC_{50} value of 53 +/- 4 µM. This result agrees with those observed by Chen et al., [14], in which a 50 µM of RSV blocked *T. gondii* intracellular growth. Adeyemi *et al.*, [15] identified RSV as one of the 62 compounds with anti-*T. gondii* effect with an IC_{50} value of 1.03 µg/ml (4.4 µM) after 72 hours of incubation and in comparison with pyrimethamine. The authors also observed that HFF cells were 100% viable at 2 µg/ml (8.5 µM). The differences in IC_{50} values can be due to the exposition time with the drug. Taken bibliographic and our results together, RSV is a natural product with potential for an alternative *Toxoplasma* infection therapy.

Since HDAC enzymes are one of the multiple targets of RSV, we analyzed the acetylation levels of H3 and H4K16. Tachyzoites treated with RSV presented a significant weaker H3ac and H4K16ac labelling in comparison with DMSO (Fig. 1C, 2 A and additional file 1). We decided to corroborate these data by Western blot. RSV treatment reduced the H3ac and H4K16ac mark intensity when compared to DMSO control (Fig. 1D and 2B). This result is in agreement with another study in which RSV treatment decreased H3 and H4 acetylation levels in *Trypanosoma cruzi* [19]. More specifically, it was recently observed that RSV reduced the H4K16ac mark [20]. In addition, in yeast, Sir2, a target of RSV, negatively controlled the activation of DNA replication origins within heterochromatin and euchromatin by de-acetylating H4K16 [21]. Our results suggest that RSV could be affecting *T. gondii* transcription and/or replication trough H3 and H4K16 deacetylation. However, further studies are required to confirm this issue.

In order to determine if the effect of RSV could be associated to DSB damage, among others, γH2A.X levels (DSB level mark) were tested by Western blot. A specific rabbit anti-*T. gondii* phosphorylated peptide in the SQE motif of *T. gondii* H2A.X C-terminal end was prepared. To confirm its specificity, a Western blot against the recombinant non-phosphorylated *T. gondii* H2A.X was performed. As it can be observed, the rabbit anti-TgγH2A.X antibody does not recognize *T. gondii* H2A.X (Fig. 3A), but reacts with an expected band of 14.5-kDa in *T. gondii* lysate (Fig. 3B). After treatment with RSV, the γH2A.X signal is increased compared to DMSO control (Fig. 3B), about 5.90 times (Fig. 3C).

Here we observed that the γH2A.X level is increased in presence of RSV, a mark compatible with DNA damage. In another model, RSV at 50 µM induced DNA damage, S-phase arrest and enhanced γH2AX levels in a panel of head and neck squamous cell carcinoma lines [22]. Double strand breaks (DSB) are repaired by homologous recombination (HRR) which is triggered in the late S phase by DSB that appear during DNA replication (e.g. due to fork collapse), or non-homologous end joining (NHEJ), throughout the cell cycle [6]. Histone H4 acetylated on its lysine in position 16 (H4K16ac) is a mark that facilitates the HRR pathway [6, 23]. Putting all the data together, we propose a possible model of RSV action, at the concentrations used, which would allow us to partially explain how it could affect *T. gondii* replication

(Fig. 3D). Briefly, RSV could induce a deficiency in H3 and H4K16 acetylation, leading to altered transcription levels, DNA replication initiation and maybe the HRR pathway. This could affect the correct DNA replication and the progress of the cell cycle, keeping γH2A.X levels high. In turn, RSV could be inducing DNA damage, either by oxidation, generating fork collapse and DSB [24, 25], increasing the difficulty of repair via HRR in the absence of H4K16ac.

Limitations
Since RSV is a multitarget drug, it is difficult to establish which targets are being affected in *T. gondii*. One of the targets could be a histone acetyltransferase. However, RSV already showed a sirtuin activating activity [26]. It is possible that this is one of the targets with consequent histone deacetylation. *T. gondii* sirtuins sir2a and sir2b have not yet been characterized. Therefore, its specific functions (e.g. role in the acetylation of H4K16), genomic localization when is present in the nucleus, etc., have not yet been reported. This impairs the verification whether the observed effect of RSV could be linked to these sirtuins.

Declarations

Acknowledgments

SM Contreras (Fellow), MM Corvi (Researcher) and SO Angel (Researcher) are member of CONICET. SO Angel (Full) and MM Corvi (Adjunct) are Professors of Universidad Nacional General de San Martin (UNSAM).

Author Contributions

SMC: performed all the experiments, analyzed PTM data and wrote the paper; AG: performed Western blot assays; MMC: analyzed drug treatment data, wrote the paper and reviewed the manuscript, SOA: Conceived and designed the experiments, analyzed the data and wrote the paper

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Data Availability:

All the raw data generated are available upon reasonable request to corresponding author.

Ethics approval and consent to participate.

Not applicable.

Consent for publication

Not applicable.

Competing interests:

The authors have declared that no competing interests exist.

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Figures

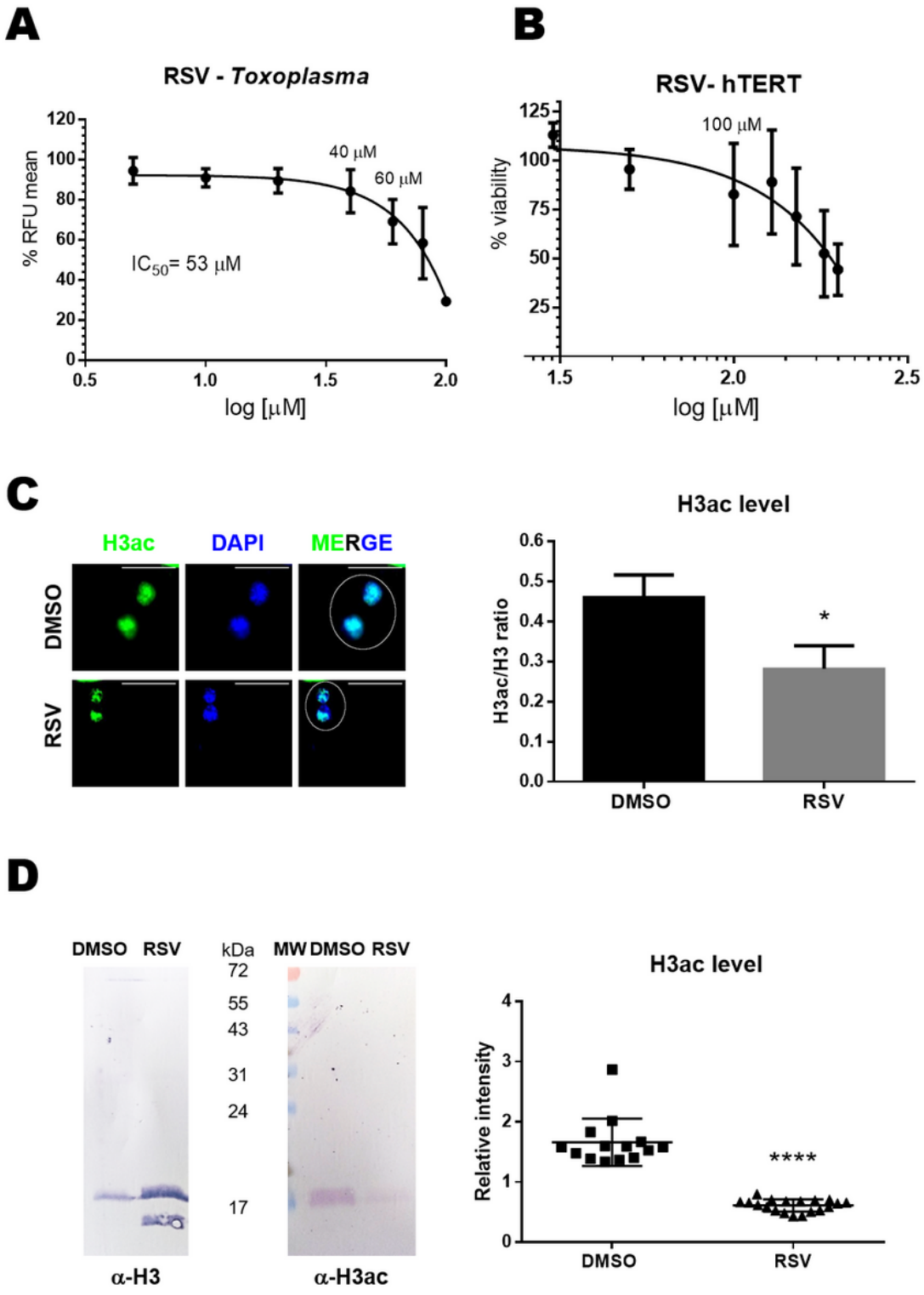

Figure 1

Effect of resveratrol (RSV) on T. gondii replication and H3 acetylation A. hTERT cell monolayers were incubated for 24 hours in the absence or presence of RSV. Cytotoxicity was estimated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide reduction (Absorbance at 540 nm) which is presented as relative to the untreated controls (defined as 100% viability). Determinations were performed on triplicate. The results are representative of three independent experiments. Untreated controls

contained 0.5% v/v DMSO. B. Effect of RSV on intracellular RH tachyzoite growth. A dose-dependent growth (0-100 M) curve relative to RFP fluorescence after 24 hours treatment is shown. Three independent experiments performed in triplicate each time and the data are presented as mean ± standard deviation (SD). C. Indirect immunofluorescence with anti-H3ac antibody (1:200). PV with two parasites were chosen to compare treatment and vehicle (white circle). The upper right white lines correspond to the scale bar: 10 µm. Nuclei were stained with DAPI. Intracellular tachyzoites were treated with RSV (100 M) or DMSO 0.5% during 24 hours. DAPI and fluorescence intensities were quantified from 10 nuclei (additional file 1) and plotted as relative intensity. The panel and graph are representative of three independent experiments with similar results. D. Western blot of T. gondii lysates with anti-H3 (1:250) or anti –H3ac (1:500). Lysates were obtained from purified intracellular tachyzoites treated with RSV (50 -70 M) or DMSO 0.5% during 24 hours. H3ac band intensities were quantified and relativized to H4 histone and plotted. Statistical analysis was performed with one-way ANOVA and Tukey's multiple comparison test (* $p \leq 0.05$ and **** $p \leq 0.0001$).

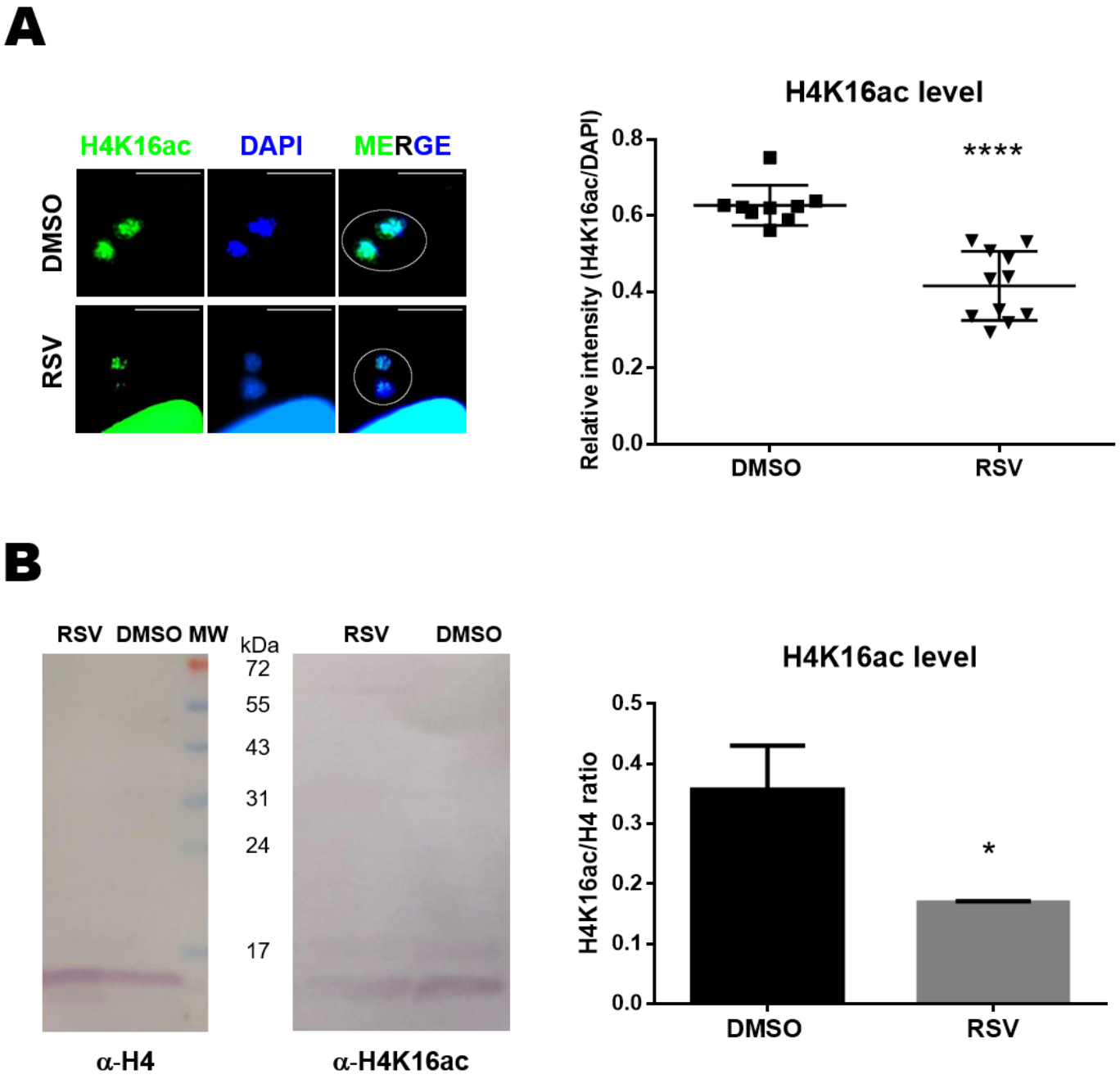

Figure 2

Effect of resveratrol (RSV) on T. gondii H4K16 acetylation A. Indirect immunofluorescence with anti-H4K16ac antibody (1:200). PV with two parasites were chosen to compare treatment and vehicle (white circle). The upper right white lines correspond to the scale bar: 10 µm. Nuclei were stained with DAPI. Intracellular tachyzoites were treated with RSV (100 M) or DMSO 0.5% during 24 hours. DAPI and fluorescence intensities were quantified from 10 nuclei (supplementary file 1) and plotted as relative intensity. The panel and graph are representative of three independent experiments with similar results. B. Western blot of T. gondii lysates with anti-H4 (1:250) or anti -H4K16ac (1:500). Lysates were obtained from purified intracellular tachyzoites treated with RSV (50 M) or DMSO 0.5% during 24 hours. H4K16ac

band intensities were quantified and relativized to H4 histone and plotted. Statistical analysis was performed with one-way ANOVA and Tukey's multiple comparison test (* $p \leq 0.05$ and **** $p \leq 0.0001$).

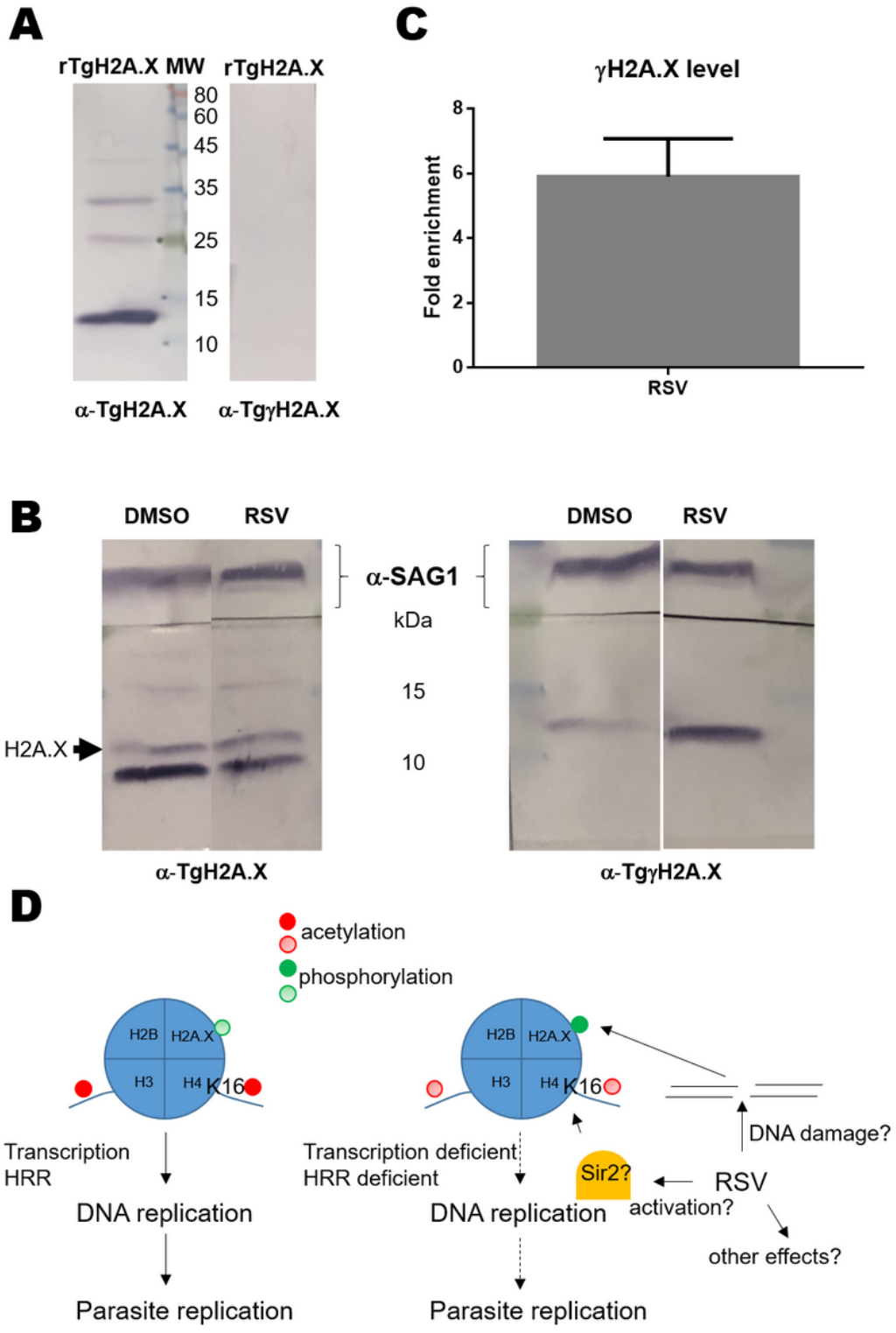

Figure 3

Effect of intracellular exposition to resveratrol (RSV) on T. gondii H2A.X phosphorylation (H2AX). A. Western blot of T. gondii recombinant H2A.X (rH2A.X; 200 ng/lane) expressed in Escherichia coli and purified by nickel resin. Rabbit anti-rTgH2A.X (-TgH2A.X, 1:5,000) and anti-T. gondii phosphorylated

peptide (□-Tg□H2A.X, 1:100). Phosphorylated peptide sequence was NH2- C+ GKHGV-S(PO3H2)-QEF - COOH. B. Western blot of T. gondii lysates with anti-SAG1 (T. gondii surface antigen 1, 1:500), anti-TgH2A.X or –anti-Tg□H2A.X. Lysates were obtained from purified intracellular tachyzoites previously treated with RSV (50 □M) or DMSO 0.5% for 24 hours. H2A.X (arrow) and □H2A.X band intensities were quantified and normalized against SAG1 band intensities. After that, relative intensity bands (□H2A.X/H2A.X) were calculated for each treatment. Normalized signal to SAG1 were calculated from relative values in comparison to DMSO control. Results are mean of three replicates ± SD. C. Quantitation of fold increase in □H2A.X levels after RSV treatment. D. Putative model of RSV action leading to a decrease in T. gondii replication. Dotted lines indicate reduced activities or processes. For acetylations and phosphorylations, color intensity represents the PTM mark level.

Supplementary Files

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