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gdSir2.1 and gdSir2.3 are involved in albendazole resistance in *Giardia duodenalis* via regulation of the oxidative stress response

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ABSTRACT

Albendazole resistance in *Giardia duodenalis* includes a complex and multifactorial challenge that potentially involves non-reported pathways such as the participation of metabolic regulators. In this context, sirtuins, known as metabolic sensors in various cellular processes, have emerged as promising candidates for novel anti-parasitic treatments. To investigate their role in albendazole (ABZ) resistance, initially we analyzed the expression of sirtuins in three *Giardia* strains resistant to 8 μM, 1.5 μM and 250 μM of ABZ that were obtained in our laboratory. Additionally, we used a CRISPRi-based knockdownstrategy to repress several sirtuins in *Giardia* and analyzed the effect of sirtuins on ABZ resistance. Our findings demonstrated a significant upregulation of sirtuins gdSir2.1, gdSir2.2 and gdSir2.3 in the three distinct albendazole-resistant lines. Knockdown of gdSir2.1 and gdSir2.3 resulted in heightened parasite susceptibility to both albendazole and hydrogen peroxide. Further, our study suggested that sirtuins contribute to the regulation of reactive oxygen species (ROS) levels, oxidative DNA damage, and the expression of oxidative stress response (OSR) genes within the parasite. Collectively, our results demonstrated that gdSir2.1 and gdSir2.3 play a significant role in mediating albendazole resistance, primarily through regulating the oxidative stress response.

1. Introduction

Giardia duodenalis is an intestinal parasite and the etiological agent of giardiasis, a worldwide disease that affects more than 280 million people each year (Einarsson et al., 2016). Treatment of giardiasis relies on the use of chemotherapeutic agents such as 5-nitroimidazoles (e.g., metronidazole) and benzimidazoles (e.g., albendazole) derivatives. Albendazole (ABZ) has been proven to be an effective, low-cost alternative to the use of metronidazole, and with fewer side effects (Solaymani-Mohammadi et al., 2010). This drug has been used at a single dose (400–600 mg) in deworming campaigns in endemic regions of Africa, Asia, and Latin America (Argüello-García et al., 2020a). The use of suboptimal doses of this drug to prevent parasite infections may be associated with the development of resistant strains in the population and a latent concern in the near future (Argüello-García et al., 2015, 2020a; Quihui-Cota and Morales-Figueroa, 2012). ABZ resistance studies in *Giardia* showed an association between drug resistance and tubulin mutations, decreased drug activation, and the control of oxidative stress (Emery-Corbin et al., 2021a; Martínez-Espinosa et al., 2015; Paz-Maldonado et al., 2013a; Pech-Santiago et al., 2022). In our group, we have obtained three ABZ resistant strains to 1.3, 8 and 250 μM of ABZ and we have reported that ABZ treatment produces oxidative stress and DNA damage (Martínez-Espinosa et al., 2015), and that ABZ-resistant lines showed an increased expression of antioxidant enzymes such as NADHox, Prx1, and Fdp (Paz-Maldonado et al., 2013b). Recently, we reported that flavohemoglobin (gFlHb), another enzyme of the antioxidative system of *Giardia*, was able to metabolize ABZ to ABZ sulfoxide and that the constitutive expression of gFlHb in resistant clones led to a higher susceptibility of these clones to ABZ treatment (Pech-Santiago et al., 2022). Since the mechanism of action of ABZ involves oxidative stress, other enzymes that participate in the regulation of this type of stress may also be involved in ABZ resistance. In this context, sirtuins (SIRTs), nicotine adenine dinucleotide(+)-dependent histone deacetylases (HDAC), are able to regulate critical signaling pathways involved in numerous biological processes and these have been reported to change their expression and their activity during inflammatory conditions when an increase in ROS occurs (Merksamer et al., 2013). Currently, seven mammalian homologs of yeast Sir2 named SIRT1 to SIRT7 have been identified in humans.These, in turn, can modulate the expression and activation of other enzymes, contributing

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to the organism's response to stress (Han et al., 2016; Kim et al., 2022; Kiran et al., 2015; Mao et al., 2011; Merksamer et al., 2013; Singh et al., 2017). To date, the role of sirtuins in drug resistance in *Giardia* remains largely unknown, however, in a recent study in *T. cruzi* a mitochondrial sirtuin was associated with the oxidative stress response, and its overexpression led to a benzimidazole and nifurtimox resistance (dos Santos Moura et al., 2021). Both drugs are known to induce ROS as it has been reported for ABZ in *Giardia* (Martínez-Espinosa et al., 2015). In this study, we evaluated if *Giardia*'s sirtuins participated in ABZ resistance in the parasite through a mechanism involving the regulation of oxidative stress.

2. Materials and methods

2.1. Trophozoites culture

Giardia duodenalis trophozoites from WBC6 strain (ATCC # 30957) were axenically cultured at 37 °C in TYI-S-33 medium with 10 % Bovine Serum (Hyclone, South Logan, UT, USA) and 1 % antibiotic/antimycotic mixture (Hyclone, South Logan, UT, USA). Trophozoites resistant to 1.35 μM (R1.35), 8 μM (R8), and 250 μM (R250) were obtained as previously described (Paz-Maldonado et al., 2013a). Cells were harvested by cooling the tubes for 1 h in an ice bath. Detached trophozoites were centrifuged at 900 g at 4 °C for 5 min and the pellets were washed three times with PBS. Knockdown strains were grown in a medium supplemented with 50 μM puromycin (InvivoGen).

Table 1
gRNAs us for sirtuin downregulation.

gRNA	Sequence (5′to 3′)
ORF6942g173annF	caaaTTTAGGCCCTCCTTTAGATA
ORF6942g173annR	aaacTATCTAAAGGAGGGCCTAAA
ORF6942g1096annF	caaaTTAGTATCCACTTGCACTGG
ORF6942g1096annR	aaacCCAGTGCAAGTGGATACTAA
ORF6942g1636annF	caaaTCATCAAACATAAGTACTCT
ORF6942g1636annR	aaacAGAGTACTTATGTTTGATGA
ORF10707g104annF	caaaGACAGGATAGTCGCAAAGAG
ORF10707g104annR	aaacCTCTTTGCGACTATCCTGTC
ORF10707g825annF	caaaTAAACGTATGAAACGATCCA
ORF10707g825annR	aaacTGGATCGTTTCATACGTTTA
ORF10707g1613annF	caaaGACGTTGTACCGCCACGGCG
ORF10707g1613annR	aaacCGCCGTGGCGGTACAACGTC
ORF10708g84annF	caaaCAGAAATTCCTGCACCAGCA
ORF10708g84annR	aaacTGCTGGTGCAGGAATTTCTG
ORF10708g1069annF	caaaCCCGTGATAGCGATGGATGT
ORF10708g1069annR	aaacACATCCATCGCTATCACGGG
ORF10708g1970annF	caaaACGCTTAATGAGACCCGTGA
ORF10708g1970annR	aaacTCACGGGTCTCATTAAGCGT
ORF11676g60annF	caaaCTTTGCTCAGGACCTTGCCC
ORF11676g60annR	aaacGGGCAAGGTCCTGAGCAAAG
ORF11676g879annF	caaaACAAGGGTGCTGCTCCAGGT
ORF11676g879annR	aaacACCTGGAGCAGCACCCTTGT
ORF11676g1582annF	caaaTCTATGTATTCTGCTCCTGG
ORF11676g1582annR	aaacCCAGGAGCAGAATACATAGA
ORF16569g146annF	caaaGGTTCTAGCTAACTGCAGTA
ORF16569g146annR	aaacTACTGCAGTTAGCTAGAACC
ORF16569g684annF	caaaTGATAGGCGAAAGGCATCCG
ORF16569g684annR	aaacCGGATGCCTTTCGCCTATCA
ORF16569g1030annF	caaaGTTAGGATGCGATCTAGCAG
ORF16569g1030annR	aaacCTGCTAGATCGCATCCTAAC

gRNA designed with the CRISPR "Design and Analyze Guides" tool from Benchling against the five *Giardia* sirtuins (GL50803_10708 (gdSir1.1), GL50803_10707 (gdSir2.2), GL50803_16569 (gdSir2.3), GL50803_11676 (gdSir2.4), GL50803_6942 (gdSir2.5). ORF6942g173, ORF10707g104, ORF10708g84 & ORF16569g146 were used for the next assays.

2.2. CRISPRi-mediated knockdown of Giardia sirtuins

Giardia duodenalis sirtuins (GL50803_10708 (gdSir1.1), GL50803_10707 (gdSir2.2), GL50803_16569 (gdSir2.3), GL50803_11676 (gdSir2.4), GL50803_6942 (gdSir2.5) were knockdown as previously described (McInally et al., 2019). In brief, three specific gRNA against the coding region of *Giardia* sirtuins were designed with the CRISPR "Design and Analyze Guides" tool from Benchling (https://benchling.com/crispr) (Supplementary Table 1). gRNAs were annealed and cloned into Bbsl-digested dCas91pac. After sequence verification, trophozoites were electroporated on a Gene Pulser XCell (BioRad) using 50 μg of nonspecific guide plasmid as control or the knockdown plasmids. After 24 h, a final concentration of 10 μM puromycin was added to the cultures and when the cultures were confluent, the final concentration of puromycin was increased to 50 μM. The levels of knockdown in CRISPRi strains were validated through RT-qPCR, Western blot, and encystation assays. To induce encystation in trophozoites, cells were grown on TYI-S-33 medium for 24 h until an 80 % confluency was achieved, then medium was switched to encystation medium (TYI-S-33 medium pH7.8 supplemented with bile10 g/L) and incubated 24 h. Cells were then processed as described here on section 4.9 by immunofluorescence using the *Giardia*-a-Glo kit (WaterBorne), which allow us to detect cyst outer wall antigens in *Giardia duodenalis*. At least two hundred trophozoites were counted per condition in triplicates and a percentage of positive cells (fluorescent) was calculated using a ratio of positive cells/total cells counted.

2.3. Sirtuin knockdown validation

Total RNA was extracted using a commercial kit (Paris Kit, Invitrogen) and treated with DNAse (Invitrogen). RNA quality was assessed by agarose electrophoresis. cDNA was synthesized using the SuperScript III First-Strand Synthesis System (Invitrogen) according to manufacturer instructions. Quantitative PCR was performed using the SYBR Green Master Mix (Applied Biosystems) using the following primers: 10708-Sir2.1Fw 5′-GAAGCGCTTGTCCCATTCTT-3′, 10708-Sir2.1Rv 5′-ACACTGGATGGAAGTCGTGT-3'; 10707-Sir2.2Fw 5′-ACTTG-GAGCTTCTGGGAGAC-3′, 10707-Sir2.2Rv 5′-TAGCCTGCAATCTCGAC-GAT-3'. 16569-Sir2.3Fw 5′-CATGTTCGACGAGCAGTACG3-3′, 16569-Sir2.3Rv 5′-CGCTGACATGCCGTTTGATA-3'; 6942-Sir2.5Fw 5′-ACAGT-CACAGTCCAGCCTAC-3′, 6942-Sir2.5Rv 5′-CCTGCCCCAGTCCCTA-TAAG-3'. Due to the lack of commercially available antibodies against the *Giardia* sirtuins, the functionality of the silencing of the selected genes was tested using two indirect approaches: H3K18 acetylation through Western blot and detection of cyst proteins after incubating the trophozoites in encystation medium for 24 h using the *Giardia*-a-Glo kit (WaterBorne).

2.4. Western blotting

Total protein extracts of WB strain, knockdown strains, negative controls, or resistant lines were obtained using a 20 mM Tris/0.5 % Triton X-100 lysis buffer supplemented with protease inhibitors (Roche). Protein concentration was determined using the Pierce BCA Protein Kit (Thermo Scientific). Then, 50 μg of protein samples were separated in a 15 % acrylamide gel under denaturing conditions. Gels were transferred to nitrocellulose membranes for 1 h at 100 V in an ice bath. The membranes were blocked with 5 % nonfat milk for 1 h at room temperature and incubated overnight with either anti-H3K18Ac (Abcam 1191), anti-H4K12Ac (Abcam 61238), or anti-α-Tubulin (kindly donated by Dr. Keith Gull). The membranes were washed three times with PBST 0.1 % and incubated with the corresponding secondary antibody: anti-mouse HRP (Novex A16072) or anti-rabbit HRP (Cell Signaling 7074S). Then, membranes were washed three times with PBST 0.1 %. Finally, chemiluminescence detection was performed using SuperSignal West Femto Substrate (Thermo Scientific).

2.5. Viability assays

Viability assays were carried out in the presence of various concentrations of sodium butyrate (Sigma), nicotinamide (Sigma), ABZ (Sigma), or hydrogen peroxide (Meyer) in sensitive trophozoites, drug resistant lines, or knockdown strains. The trophozoite viability was determined by Trypan blue exclusion Assay or using the Luminescent ATP Detection Assay Kit (Abcam). The IC50 of each drug was calculated using a nonlinear regression in GraphPad Prism.

2.6. NAD^+/NADH quantification

NAD^+ and NADH co-substrates were quantified using the NAD/NADH Assay Kit II (colorimetric) (ab221821) according to the manufacturer's instructions. In brief, the assay was carried out using 5×10^6 trophozoites. NAD^+ was obtained through acid extraction using 0.5 perchloric acid, and NADH was obtained through alkaline extraction using 50 mM NaOH +1 mM EDTA. After adding the reaction mix, the absorbance (OD450) was measured on a microplate reader in kinetic mode for 30–90 min at 10-min intervals at room temperature, protected from the light.

2.7. Detection of reactive oxygen species

The WB strain, transfected with a non-specific sgRNA and the sirtuins knockdown strains were treated with 4 μM of ABZ or 200 μM of hydrogen peroxide for 24 h at 37 °C. ROS formation was assessed using the Image-IT LIVE Green Reactive Oxygen Species Detection Kit (Invitrogen). In brief, trophozoites were washed in PBS and suspended in 25 μM 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) at 37 °C for 30 min. Cell fluorescence was detected in a Beckman FACSCalibur Flow Cytometer.

2.8. Dot blot

DNA from the WB strain, and from the gdSir2.1 and gdSir2.3 knockout strains was extracted using Trizol-LS (Thermo Scientific). After quantification, 4 μg of DNA were dotted using a Dot Blot Apparatus (Bio-Rad) on a Nylon membrane. The membrane was blocked with a 2 % BSA-PBS solution for 1 h at room temperature. Then, the membrane was incubated with an anti-8OHdG antibody (Santa Cruz 393871) overnight at 4 °C, washed three times with PBST, and incubated with an anti-mouse IgM-HRP secondary antibody (DAKO) for 1 h at room temperature. After three extra washes with PBST, chemiluminescence was performed using SuperSignal West Femto Substrate (Thermo Scientific).

2.9. Immunofluorescence

Immunofluorescence was performed as previously described by Huber, K. et al. in 2019 (Huber et al., 2019). Trophozoites tubes were incubated in an ice bath for 15 min and collected by centrifugation (900*g*) at 4 °C for 5 min and washed two times in filtered PBS. Cells were incubated for 3 min in poly-L-lysine treated coverslips and fixed with 1 % formaldehyde in PHEM buffer for 1 h. After permeabilization with Triton 0.5 % in PHEM, coverslips were blocked for 1 h with 3 % BSA in filtered PBS pH 8. Trophozoites were incubated overnight with an anti-8OHdG antibody (Santa Cruz 66036) at 4 °C. The coverslips were washed four times with BSA 3 % and incubated for 1 h with the secondary antibody (polyvalent anti-mouse immunoglobulins FITC, Sigma) at room temperature, protected from the light. Cells were washed three times with BSA 3 % and mounted in an antifade mounting medium with DAPI (Vectashield).

2.10. Quantitation of the expression of oxidative stress related genes (OSR) in Giardia

cDNA of WB strain and gdSir2.1 and gdSir2.3 was synthetized as previously described. We selected six genes of the antioxidant system of *Giardia* (NADH oxidase (GL50803_0033769), Flavodiiron protein (GL50803_0010358), SOR (GL50803_0061550), Prx (GL50803_0016076), Trx (GL50803_009827) and Thioredoxin(GL50803_003910) and evaluated their expression through quantitative PCR using the following primers: FwThioredox 5′-CATTTCCGCGCCCGAGTACGAG-3', RvThioredox 5′-GTGGAACGCGATGAGCTCCAGC-3'; FwTrx 5′-CACTCAGCGCCACGTCAGGATC-3′, RvTrx 5′-ACGCAGGTCTTCAGATTCGCGC-3'; FwFdp 5′-CCAGGCGTTTACTGGGTCGGGA-3′, RvFdp 5′-GAGCTCCCCTCGTCTGTGTGGT-3'; FwPrx 5′-TTCGAGGTCGACGTCGTCACCC-3′, RvPrx 5′-ATCTCCGAGGGGCAGACGAAGG-3'; FwNADHox 5′- AACAGCCGTCGCGAAGACCATC-3′, RvNADHox 5′-TTAACCGTCCCGTGGACACCGA-3'; FwSOR 5′-AACTGCCTTCACAAGCCCGACG-3′, RvSOR 5′-CAACGTGGTTCCCGCTGAGCTT-3'.

2.11. Statistical analysis

All experiments were performed with at least three biologically independent samples. Data are expressed as a mean ± SD. Comparisons were made in GraphPad Prism using a one-way ANOVA with a Tukey's post-hoc test.

3. Results

3.1. Sirtuins expression and their activity may be increased in ABZ resistant lines

To assess if sirtuins participate in ABZ resistance, we evaluated the mRNA expression levels of gdSir2.1, gdSir2.2 and gdSir2.3 in the three ABZ resistant lines (R1.35, R8 and R250) by qRT-PCR. These lines were selected by continuous subculture under increasing sub-lethal concentrations of ABZ and cloned by limiting dilution (Paz-Maldonado et al., 2013b). gdSir2.1 expression was increased in R1.35 and R250 (2.99 and 3.06-fold), while gdSir2.3 expression was increased relative to wildtype strain (WB) only in R1.35 (3.19-fold) (Fig. 1).

Nicotinamide adenine dinucleotide (NAD^+) is an important cofactor for reactions that require an electron exchange. Although it has been recently discussed, it is generally accepted that sirtuins are able to sense cellular NAD^+ levels, and its concentration modulates their activity, where a higher concentration of NAD + correlates with an increased sirtuin activation (Anderson et al., 2017; Katsyuba and Auwerx, 2017). Therefore, to indirectly evaluate if sirtuins in *Giardia* may have an increased activation, we quantified the NAD^+ and NADH concentration in R1.35, R8 and R250 lines (Fig. 2). All three resistant lines showed a higher NAD^+ concentration compared to the control (WB). These results suggest that sirtuins expression is not only increased but sirtuins may have increased activity in ABZ resistant lines. Nevertheless, this latter notion requires further insights to be unveiled, e.g. the direct evaluation of each's sirtuin activity and the expression levels of the nicotinamide phosphoribosyltransferase (NAMPT), a rate-limiting enzyme in NAD + biogenesis and sirtuin activation (Swanson et al., 2019).

3.2. Nicotinamide increases ABZ sensitivity in resistant lines

To evaluate if histone deacetylases participated in ABZ resistance, we initially assessed the IC_{50} value of the HDAC inhibitors sodium butyrate and nicotinamide on an ABZ sensitive strain (WB) (Fig. 3A). IC_{50} values for sodium butyrate were 23 and 25.7 mM, and 3.9 and 2.5 mM for nicotinamide during the 24 and 48 h, respectively. Based on these results, we selected a 24-h treatment with 10 mM of sodium butyrate and 2 mM of nicotinamide to carry further assays, since higher concentrations of these inhibitors affected the trophozoite viability (lower than 80

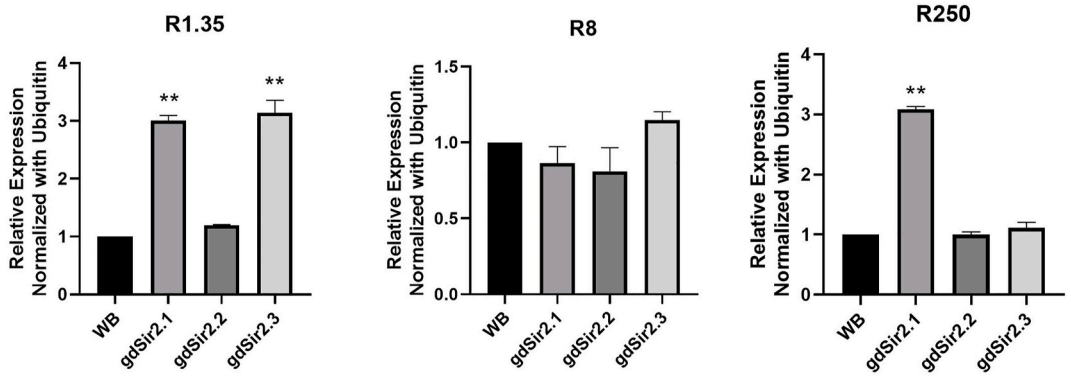


Fig. 1. Determination of mRNA expression levels of gdSir2.1, gdSir2.2 and gdSir2.3 in resistant lines and ABZ sensitive strain (WB).
gdSir2.1 expression increased in resistant lines R1.35 and R250, while gdSir2.3 expression increased only in the R1.35 line. Resistant line R8 did not show significant changes in sirtuin expression. Data are the mean ± SD of three independent qRT-PCR assays. **p < 0.01.

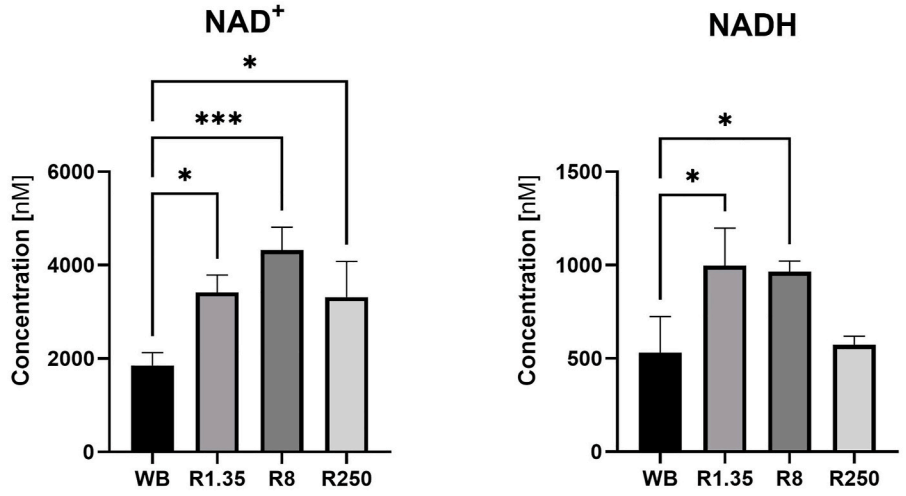


Fig. 2. Determination of NAD^+ and NADH levels in ABZ resistant lines and ABZ sensitive strain (WB).
As compared to the control culture (WB), NAD^+ concentrations are significantly increased in all ABZ resistant lines and NADH levels are increased inin R1.35 and R8 lines. Data are the mean ± SD of three independent assays. *p < 0.05, ***p < 0.005.

%).

To analyze if the selected doses of the inhibitors were effective, we first evaluated the presence of a few epigenetics marks by Western blot (Figs. S1 and S2) and selected two of them: H3K18Ac and H4K12Ac and then if the acetylation levels increased over time. In both cases, histone acetylation reached its peak at 6 h post treatment and eventually decreased to basal levels (Fig. 3B and S3).

Once we determined that treatment with the inhibitors was effective on the WB strain, we assessed on the R1.35 and R250 lines if these inhibitors would also decrease the cell viability in presence of the ABZ concentrations at which these lines are resistant. We observed a modest reduction in the viability in both resistant lines in the presence of ABZ + Nicotinamide, and this was particularly evident in the R250 line (Fig. 3C). These data suggested that type III HDACs (sirtuins) may be involved in ABZ resistance.

3.3. *CRISPRi-mediated knockdown of Giardia sirtuins increases the parasite susceptibility to ABZ and H_2O_2*

To further test the possible correlation between sirtuins and ABZ resistance, we used CRISPRi to generate knockdown strains of *Giardia* sirtuins. To validate these strains, we initially quantified sirtuins expression levels in the knockdown strains compared to the WB strain. In this, we observed a downregulation of *Giardia* sirtuins between 50 and 80 % (Fig. 4A).

We then validated the knockdown of sirtuins using two approaches, based on processes in which sirtuins had been reported to be involved. These processes include encystation (CWP expression) and histone deacetylation (H3K18ac) (Carranza et al., 2016). gdSir2.1, gdSir2.2, gdSir2.3 downregulation decreased the percentage of trophozoites positive for cyst-specific proteins (Fig. 4B). Although, the downregulation of gdSir2.1, gdSir2.2 and gdSir2.5 was not statistically significant it represented an increase on H3K18 acetylation of 2.33, 1.98 and 3.15-fold, respectively while gdSir2.3 downregulation increased H3K18Ac more than seven-fold (Fig. 4C & S4).

3.4. *Sirtuins knockdowns showed a higher intracellular ROS formation during ABZ and H_2O_2 treatments and an increased susceptibility to both drugs*

We hypothesized that sirtuins could be associated with ABZ resistance through the modulation of the oxidative stress response in the parasite, since oxidative stress has been associated with the increased expression of sirtuins that in turn are able to modulate the expression and activity of antioxidant proteins, promoting cell survival (dos Santos Moura et al., 2021; Santos et al., 2016; Singh et al., 2017).

To test this hypothesis, we measured the ROS production in the parasite by flow cytometry after treatment with ABZ and H_2O_2, which

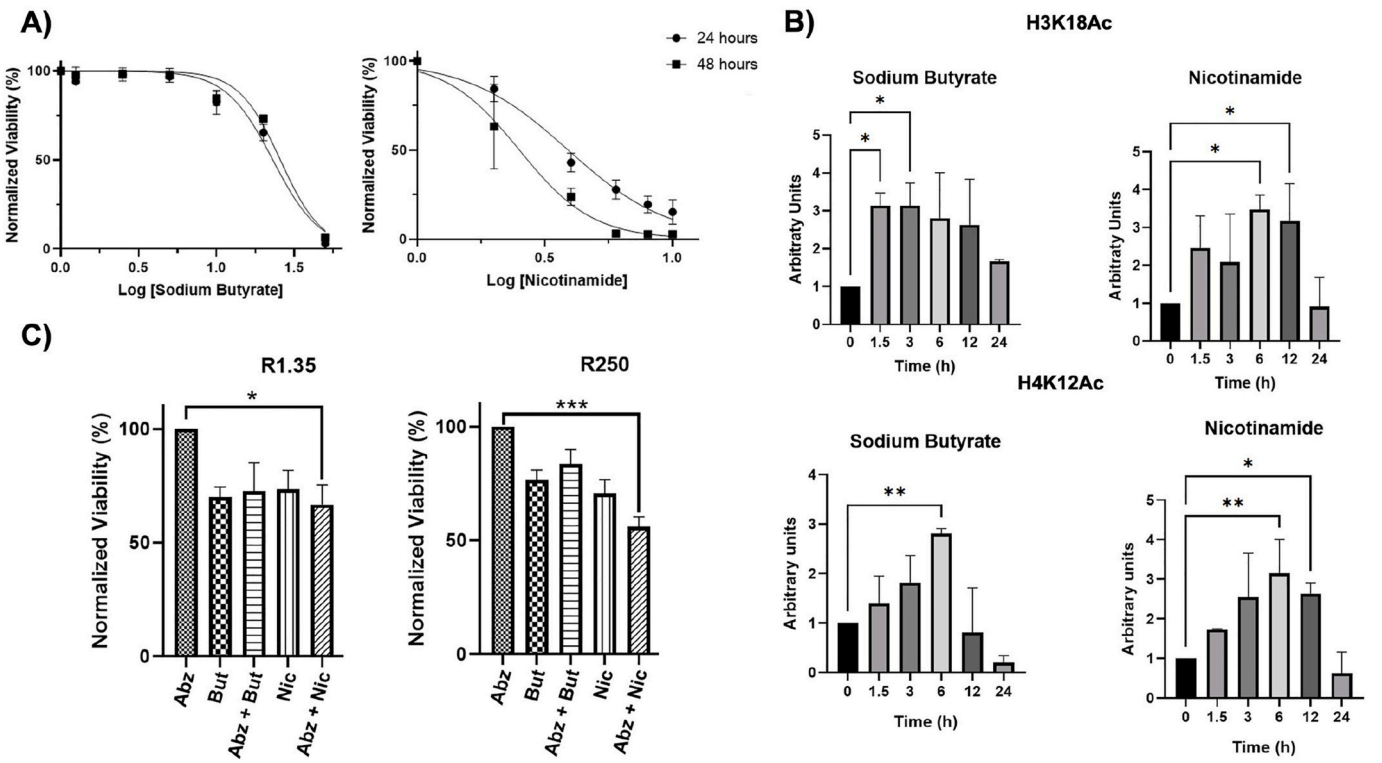


Fig. 3. Effect of histone deacetylase inhibitors in acetylation and viability of WB and ABZ resistant *G. duodenalis* strains.
(**A**) Viability curves in presence of Sodium Butyrate (But) (0, 1.25, 2.5, 5, 10, 20, 30 mM) and Nicotinamide (Nic) (0, 2, 4, 6, 8, 10 mM) for 24 (●) and 48 h (■). (**B**) Densitometry plot based on three independent Western blots assays at different times of treatment with Sodium Butyrate or Nicotinamide and its effect on the acetylation levels of H3K18 and H4K12 in WB strain. (**C**) Viability of resistant lines R1.35 and R250 in presence of ABZ and histone deacetylate inhibitors. Data are the mean ± SD of three independent assays. *p < 0.05, **p < 0.01, ***p < 0.005.

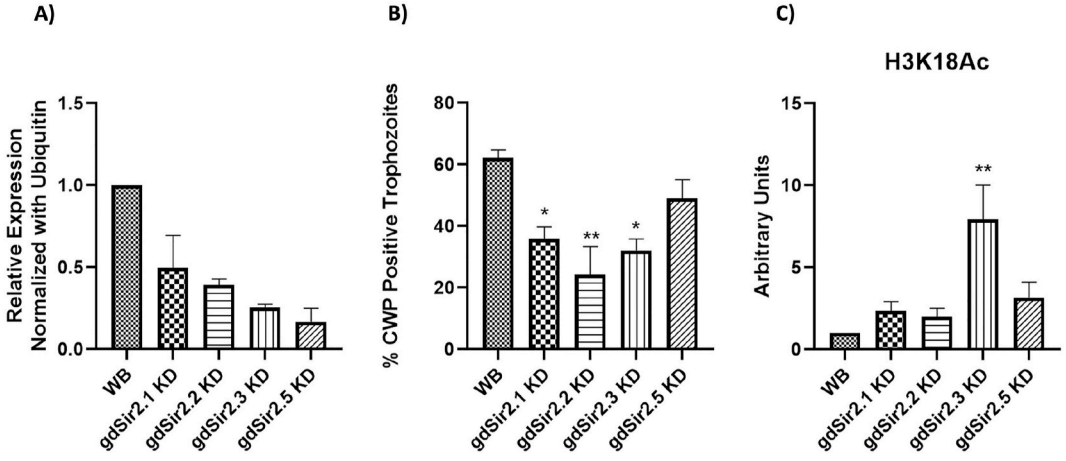
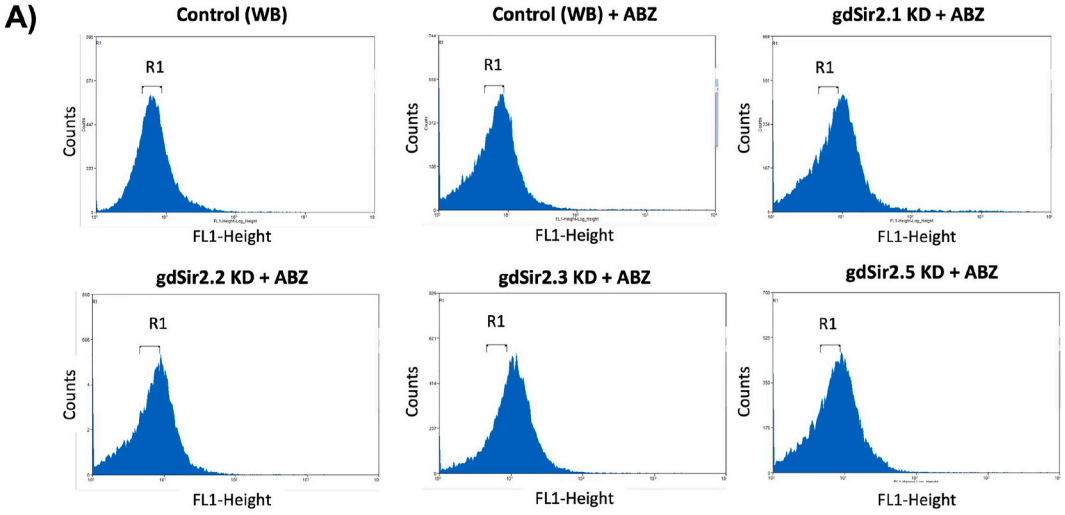


Fig. 4. Validation of CRISPRi-mediated knockdown of *Giardia* sirtuins.
(**A**) mRNA expression levels of sirtuins in knockdown strains compared to the WB strain. (**B**) Percentage of trophozoites positive for encystation proteins (CWP) after a 24-h incubation with encystation medium. (**C**) H3K18 acetylation levels in knockdown strains. Data are the mean ± SD of three independent assays. *p < 0.05, **p < 0.01.

was used as positive control for oxidative stress (Martínez-Espinosa et al., 2015). Knockdown strains showed greater ROS production during ABZ treatment than control strain (WB), displacing the curve to the right, which indicates a higher signal intensity (Fig. 5A). gdSir2.3 downregulation produced a 44.27 % displacement compared to the control (WB) in ABZ treated trophozoites (Fig. 5B and C). Hydrogen peroxide showed similar results (Fig. 6A), where gdSir2.1 and gsSir2.3 downregulation produced an 8.53 % and 11.99 % displacement, which was also statistically significant (Fig. 6B and C). Taken together, these results indicated that sirtuins in *Giardia* are involved in controlling ROS levels within the cells.

Since gdSir2.1 KD and gdSir2.3 knockdown showed increased levels of ROS during ABZ and H_2O_2 treatments, we tested if these strains would also have an increased susceptibility to ABZ and H_2O_2 using a cell viability assay (Fig. 7A). The gdSir2.1 and gdSir2.3 knockdowns were 41 % and 24 % more susceptible to ABZ, and 51 % and 63 % more susceptible to hydrogen peroxide, respectively (Fig. 7B). Both enzymes seem to have a pivotal role in the control of oxidative stress since their



B)

SAMPLE	% CELS. IN R1	DISPLACEMENT (%)
Control (WB)	49.9 ± 1.025	0.00
Control (WB) + ABZ	36.7 + 1.36	24.26
gdSir2.1-KD + ABZ	32.15 ± 3.11	33.65
gdSir2.2-KD + ABZ	34.78 ± 2.23	28.22
gdSir2.3-KD + ABZ 1	26.96 ± 1.14	44.37

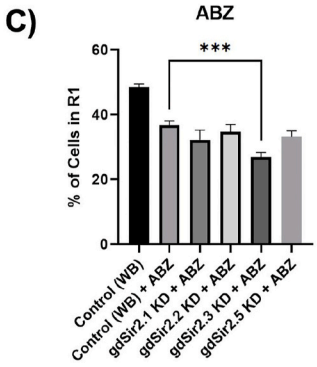


Fig. 5. Determination of reactive oxygen species (ROS) in knockdown strains during ABZ treatment.
(**A**) Graphs show ROS production monitored by flow cytometry. The shift of fluorescence in X axis indicates ROS production. (**B**) Data shows the population of cells positive for ROS (% of displacement) according to the flow cytometry data. (**C**) Graphical representation of results is included in B. Data are the mean ± SD of three independent assays. Albendazole concentration used: 1 μM ***p < 0.005.

downregulation showed a 68 % reduction in viability compared to the control at a 250 μM concentration of H_2O_2 (Fig. 7C).

3.5. Knockdown strains present increased levels of oxidative stress-induced DNA damage (8OHdG)

To corroborate if the higher ROS levels had a physiological impact in trophozoites, we evaluated DNA damage induced by oxidative stress through immunofluorescence and dot-blot using an antibody anti-8OHdG which has been previously reported (Martínez-Espinosa et al., 2015). We compared the WB strain, with the knockdown strains for gdSir2.1 and gdSir2.3, using as control WB treated with H_2O_2. Knocking down of gdSir2.1 and gdSir2.3 produces an increase in DNA damage (green fluorescence) even without treatment, similar to that observed when cells were treated with H_2O_2 (Fig. 8A). Further, we validated these results with a dot-blot. In this case, gdSir2.1 and gdSir2.3 showed a 1.8 and 2.9-fold increase of the 8OHdG mark (Fig. 8B). These are consistent with the notion that sirtuins have a protective role against oxidative stress, which can be achieved either by managing the ROS levels in the cells, or through their contribution to the DNA repair mechanisms (Feige and Auwerx, 2008; Lagunas-Rangel, 2019; Mao et al., 2011; Tian et al., 2019; Vazquez et al., 2017).

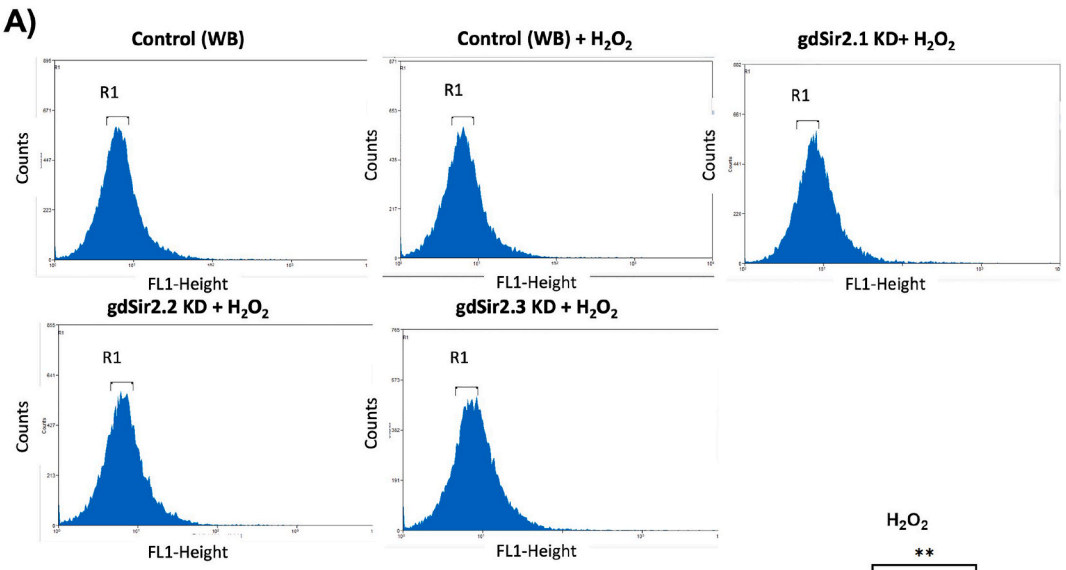
3.6. Sirtuins modulate the expression of oxidative stress response genes in Giardia

Sirtuins haven been shown to modulate the expression of oxidative stress genes in higher organisms (Chang and Guarente, 2014; Freitas et al., 2005). Therefore, the increased levels of ROS and subsequent DNA damage could be associated with alterations in the expression of enzymes of the antioxidant system of *Giardia* that are regulated by gdSir2.1 and gdSir2.3. To test this, we evaluated the mRNA expression levels of six different OSR genes (Thioredoxin, Trx, Fdp, Prx, NADHox and SOR) in knockdown strains. Downregulation of gdSir2.1 decreases the expression of Trx, Prx and SOR, while downregulation of gdSir2.3 decreases the expression of Fdp, Prx and NADHox (Fig. 9).

4. Discussion

Drug resistance has become a major issue worldwide, and it has limited treatment options for various parasitic infections (Rodríguez et al., 2010; Taslimi et al., 2018; Van den Kerkhof et al., 2020). Drug resistance in *Giardia duodenalis* to the most common treatment options have been previously reported (Argüello-García et al., 2020b; Emery-Corbin et al., 2021b; Emery et al., 2018). In particular, ABZ resistance has been associated with tubulin mutations, increased expression of proteins involved in cytoskeleton dynamics (α-2-giardin, ran bp-1), energetic metabolism (pgk, oct) and antioxidant response (NADHox) (Emery-Corbin et al., 2021a; Paz-Maldonado et al., 2013b). More recently, the giardial flavohemoglobin has been proposed to participate in the biotransformation of ABZ and the downregulation of this protein has been linked to the resistant phenotype in *Giardia* ABZ resistant lines (Pech-Santiago et al., 2022).

The role of sirtuins in parasites has not been extensively studied, yet some research has focused on *Plasmodium*, *Trypanosoma*, *Leishmania* and *Entamoeba* (Freitas et al., 2005; Tonkin et al., 2009; Garcia-Salcedo,



B)

SAMPLE	% CELS. IN R1	DISPLACEMENT (%)
Control (WB)	49.9 ± 1.025	0.00
Control (WB) + H_2O_2	47.44 + 1.2	2.11
gdSir2.1-KD + H_2O_2	44.32 ± 1.66	8.53
gdSir2.2-KD + H_2O_2	46.29 ± 0.69	4.48
gdSir2.3-KD + H_2O_2	42.65 ± 1.14	11.99

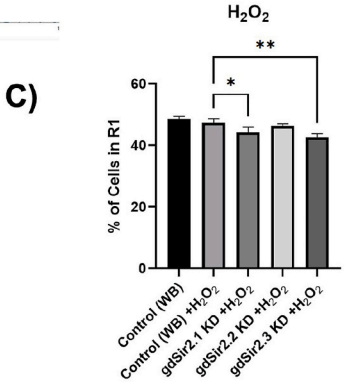
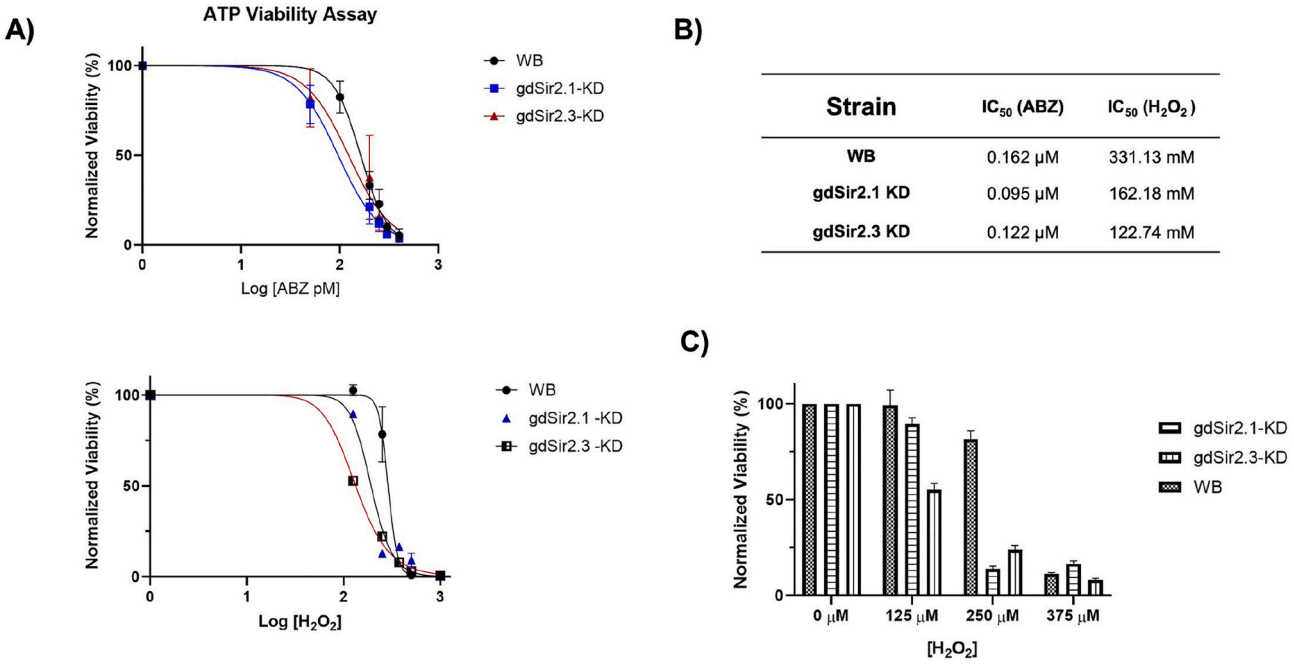


Fig. 6. Determination of reactive oxygen species (ROS) in knockdown strains during hydrogen peroxide treatment.
(**A**) Graphs show ROS production monitored by flow cytometry the shift of fluorescence in X axis indicates ROS production. (**B**) Data shows the population of cells positive for ROS (% of displacement) according to the flow cytometry data. (**C**) Graphical representation of results included in B. Data are the mean ± SD of three independent assays. Hydrogen peroxide concentration used: 200 μM *p < 0.05, **p < 0.01.

2003; dos Santos Moura et al., 2021; Tavares et al., 2008; Biswas et al., 2023). In *Giardia*, five sirtuins have been reported (gdSir2.1-gdSir2.5), however only gdSir2.1, gdSir2.3 and gdSir2.4 have been previously studied. gdSir2.4 has been associated with the regulation of *Giardia* rRNA expression, contributing to the maintenance of genomic stability (Lagunas-Rangel et al., 2021), while gdSir2.1 and gdSir2.2 have been characterized but have not been associated with a particular cellular process (Herrera T et al., 2019; Wang et al., 2016). Other studies have focused on the use of inhibitors against these enzymes to evaluate both their use as possible treatment options and in their role in antigenic variation and encystation (Carranza et al., 2016; Huber et al., 2019; Lagunas-Rangel et al., 2020). The relationship between sirtuins and drug resistance has been studied mostly in cancer, where all mammalian sirtuins are involved in tumorigenesis and cancer progression (German and Haigis, 2015; Zahedipour et al., 2020). In parasitic diseases, research has focused on the use of sirtuins as novel drug targets for treatment (Hailu et al., 2017; Zheng, 2013) ranging from protozoa as *Leishmania amazonensin* (Verçoza et al., 2017) and *Trypanosoma cruzi* (Moretti et al., 2015) to helminths as *Schistosoma mansoni* (Lancelot et al., 2015). Also, sirtuins have important roles in a plethora of biological processes of parasites; for instance, in *P. falciparum*, PfSir2A is involved in maintaining telomere length, and regulation of genes involved in virulence, antigenic variation, and pathogenesis (Freitas et al., 2005; Tonkin et al., 2009). In L. *infantum*, LiSir2rp1 has been associated with the cytoskeleton network and it is essential for the parasite survival (Tavares et al., 2008). In *T. brucei*, TbSir2rp1 expression was increased and shown able to increase cellular resistance to DNA damage under genotoxic stress (Garcia-Salcedo, 2003). In *T. cruzi*, TcSir2rp1 TcSir2rp3 have also been characterized; in this study, the increased expression of TcSir2rp1 impaired the parasite growth and differentiation, while TcSir2rp3, upon increased expression, stimulated both growth and differentiation of epimastigotes (Ritagliati et al., 2015) and more recently, the increased expression of TcSir2rp3 has been associated with an increased benznidazole (BZD) and nifurtimox (NFX) resistance (dos Santos Moura et al., 2021). The role of sirtuins in drug resistance remains largely unknown in *Giardia*, therefore in this work we have studied a novel mechanism involved in ABZ resistance that is mediated through *Giardia* sirtuins.

At first instance, sirtuins 2.1, 2.2 and 2.3 were found differentially expressed in the ABZ-resistant cell lines. This phenomenon was also observed for antioxidant enzymes in a manner independent of the ABZ concentration at which cell lines grow (Argüello-García et al., 2015, Fig. 4) albeit all ABZ resistant lines display downregulation of another antioxidant enzyme, flavohemoglobin (Pech-Santiago et al., 2022). These cell line-specific patterns of expression reflect the multifactorial nature not only of ABZ resistance but the role of several other factors underlying the antioxidant response of *G. duodenalis* coping the oxidant effect of this drug. Among these are mutations at, or in the vicinity of, the colchicine-binding site of β-tubulin itself (Emery-Corbin et al., 2021; Argüello-Garcia R, Chavez-Cano et al., unpublished), an increased thiol pool and a reduced accumulation of ABZ metabolites with concomitant FlHb dowregulation (Argüello-García et al., 2015; Pech-Santiago et al., 2022). The observed upregulation of sirtuin 2.1 in cell lines R1.35 and R250, and of sirtuin 2.3 specifically in R1.35—alongside the stable expression of sirtuin 2.2 across all lines—suggests that ABZ-induced oxidative stress activates sirtuin-specific genetic and/or epigenetic



Strain	IC_{50} (ABZ)	IC_{50} (H_2O_2)
WB	0.162 μM	331.13 mM
gdSir2.1 KD	0.095 μM	162.18 mM
gdSir2.3 KD	0.122 μM	122.74 mM

Fig. 7. CRISPRi-mediated knockdown of *Giardia* sirtuins increases parasite susceptibility to ABZ and to hydrogen peroxide.
(**A**) Viability curves of WB strain and *Giardia* knockdown strains in the presence of ABZ and hydrogen peroxide for 24 h. (**B**) IC50 values for ABZ and H_2O_2. (**C**) Graphical representation of the values of normalized viability of control strains and knockdown strains of gdSir2.1 and gdSir2.3 during a 24-h treatment with H_2O_2. Data are the mean ± SD of three independent assays.

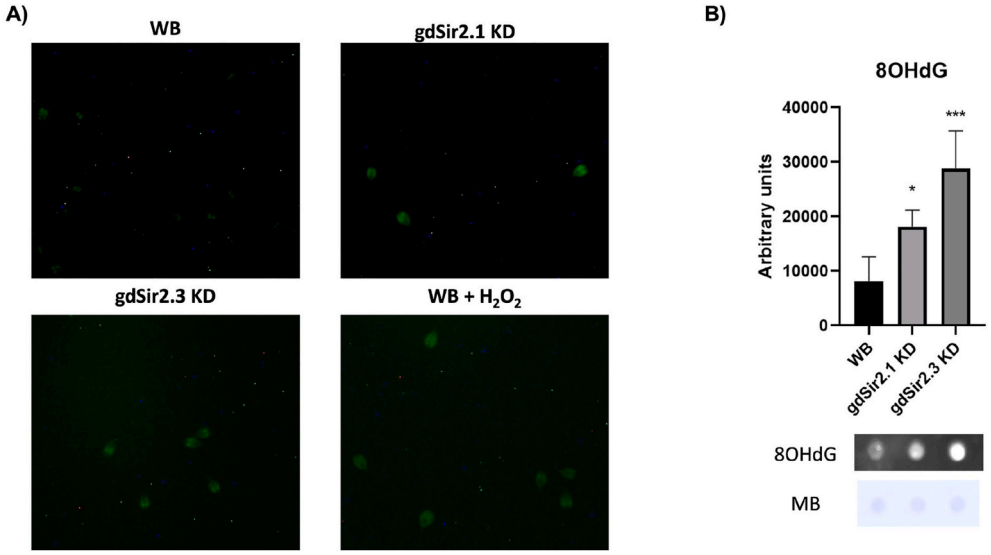


Fig. 8. Detection of oxidative damage to DNA in gdSir2.1 and gdSir2.3 knockdown strains.
(**A**) WB strain, and the gdSir2.1 and gdSir2.3 knockdown strains were fixed on slides and incubated with anti-8OHdG to detect DNA damage induced by oxidative stress. Hydrogen peroxide treatment was used as a positive control for damage (right bottom panel). (**B**) DNA from WB strain, and gdSir2.1 and gdSir2.3 knockdown strains was purified, and 4 μg of each sample was loaded by duplicate. Membrane was incubated with anti-8OHdG overnight. Data are the mean ± SD of three independent assays. *$p < 0.05$, **$p < 0.01$.

regulatory mechanisms. These mechanisms likely contribute to the differential modulation of antioxidant response pathways, including the expression and potentially the activity of ROS-processing enzymes.

In addition, we hypothesize that the increased expression of gdSir2.1 and gdSir2.3 in ABZ resistant cell lines may be related to increased sirtuin activity. While this work did not establish a direct causal relationship between NAD^+ upregulation and increased sirtuin activity, the data presented here are consistent with a model in which increased substrate availability (i.e., NAD^+) elicits a concomitant upregulation of sirtuin activity. Post-translational processes are also known to have a role in the upregulation of sirtuin activity and include: (a) enzyme modifications (PTMs) such as acetylation, sumoylation, phosphorylation among others, (b) protein-protein interactions, e.g. with cAMP, FOXO1 and p53 that may interact with the NAD + or the catalytic core domain of certain sirtuins to boost its activity, and (c) increased NAD+/NADH ratios (revised in Shahgaldi and Kahmini, 2021). Concurrent with the latter point, R1.35, R8 and R250 cell lines showed increased levels of NAD+ and NADH was increased only in R1.35 and R8 cultures (Fig. 2).

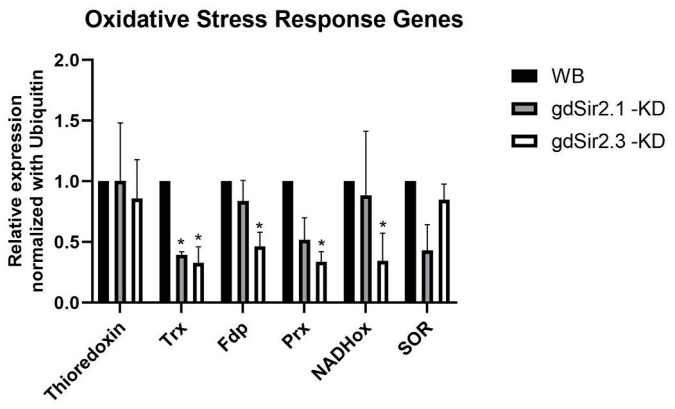


Fig. 9. Determination of mRNA expression levels of oxidative stress response (OSR) genes in gdSir2.1 and gdSir2.3 knockdown strains.
cDNA form WB strain, and gdSir2.1 and gdSir2.3 knockdown strains was used to quantify the expression levels of thioredoxin, thioredoxin reductase (Trx), flavodiiron protein (Fpd), peroxiredoxin (Prx), NADH oxidase (NADHox) and Superoxide reductase (SOR). Data are the mean ± SD of three independent assays.

In the R250 cell line, gdSir2.1 expression was upregulated, potentially reflecting a metabolic state in which elevated NAD^+ levels are required for sirtuin activation. Concurrently, reduced NADH levels may indicate enhanced metabolic fitness—characterized by lower steady-state ATP levels and an extended generation time—relative to the ABZ-sensitive WB strain (Argüello-García R, Paz-Maldonado LMT et al., unpublished). It is also noteworthy that some compounds or cellular conditions as oxidative stress, may induce transcriptional changes as the overexpression of a key, rate-limiting enzyme in sirtuin activation as NAMPT (nicotinamide phosphoribosyltransferase) that increases NAD + biogenesis thereby leading to overall or species-specific increased sirtuin activity (Swanson et al., 2019).

As an alternative approach, we evaluated the use of broad-spectrum inhibitors of HDACs. Our results showed that inhibition of type III HDACs (sirtuins),but not type I HDACs, decreased the viability of ABZ resistant lines. Interestingly, those lines showed an increased expression of sirtuins and higher levels of NAD^+, supporting the notion that these enzymes are involved in ABZ resistance. To further test this approach, we used a CRISPRi knockdown strategy to reduce expression of each sirtuin individually to evaluate their participation in this process. We showed that downregulation of gdSir2.1 and gdSir2.3 increased the susceptibility against ABZ, and this was even more evident during H_2O_2 treatment. To our knowledge, this is the first report that associates sirtuins with ABZ resistance in parasites.

The relationship between sirtuins and oxidative stress has been extensively studied in other systems (Balaiya et al., 2017; Merksamer et al., 2013; Wan and Garg, 2021). A general disruption of redox cellular homeostasis can induce the expression of sirtuins and promote their post-translational modification, it can also alter the interaction between sirtuins and other proteins, and change the NAD levels, which in turn, modify sirtuin activity (Santos et al., 2016). Further, in various diseases it has been reported that increased sirtuin expression has a protective role against oxidative stress (Chang and Guarente, 2014). In *T. cruzi,* TcSir2rp3 is able to interact with TcSODA *in vivo*, and this was associated with a higher activity of TcSODA, contributing to oxidative stress resistance during BZD, NFX and H_2O_2 treatment (dos Santos Moura et al., 2021). Since the production of ROS is part of the mechanism of action of ABZ (Martínez-Espinosa et al., 2015), we hypothesized that the increased expression of these enzymes in resistant lines would be similarly associated with a protective role against oxidative stress. To test this, we first evaluated if downregulation of sirtuins would promote an accumulation of ROS and ROS-induced DNA damage. The results obtained indicated that gdSir2.3 has a prevalent role regulating ROS production during ABZ treatment, since its downregulation increased the ROS intracellular levels by 43 % compared to the control. We observed similar results during H_2O_2 treatment, where downregulation of gdSir2.1 and gdSir2.3 increased ROS production between 8.53 and 11.99 %. Overall this supports that both sirtuins may to confer ROS protection to the parasite.

Since sirtuin downregulation was associated with higher levels of ROS, we evaluated if this effect could promote DNA damage. To assess this, we used an antibody against the oxidation of guanine (8OHdG). Even in basal conditions without treatment, knockdown strains maintained an increased level of DNA damage compared to the control wild-type strains. Sirtuins are strongly associated with DNA damage repair in higher organisms and parasites (Alsford et al., 2007; Garcia-Salcedo, 2003; Mao et al., 2011; Tang et al., 2019; Tian et al., 2019), and more recently, a study demonstrated that *E. histolytica* Sir2c is able to interact with EhRAD23, and increase its expression, suggesting that this enzyme may be involved to control UV stress (Biswas et al., 2023). Thus, we were interested in investigating whether sirtuins in *Giardia* are not only associated with managing ROS levels but also are involved in DNA damage repair.

Sirtuins can modulate gene expression and protein activity directly or indirectly, depending on the protein they interact with [36]. Deacetylation of P53 by SIRT1 during oxidative stress promotes its translocation to the nucleus where it acts as transcription factor for antioxidative enzymes such as SOD, catalase, and GST [37]. Here, we demonstrated that downregulation of gdSir2.1 decreases the expression of Trx, Prx and SOR, while downregulation of gdSir2.3 decreases the expression of Trx, Fdp, Prx and NADHox. These results correlate with previous reports where Prx, Fdp and NADHox had an increased expression in ABZ resistant lines (Argüello-García et al., 2009), which as stated before, have also an increased expression of gdSir2.1 and gdSir2.3. Both enzymes seem to share common target genes such as Thioredoxin reductase (Trx) and Peroxiredoxin (Prx). These observations are provocative in that thioredoxin reductase (Trx) reduces thioredoxin, and peroxiredoxin (Prx) uses the reduced form of thioredoxin to transform H_2O_2 into H_2O (Ansell et al., 2015). Since some of the antioxidant enzymes in *Giardia* use NADH as a cofactor directly (NADHox, DT-diaphorase, Rubredoxin reductase, NR1 and NR2) or indirectly (Flavodiiron protein & SOR), we propose that the overactivation of the antioxidative system during ABZ treatment may cause the increased levels of NAD^+ reported in this work. In Future investigation of the enzymes involved in NAD + biosynthesis and salvage pathways in *Giardia* would also lend insight into these mechanisms. Srtuins in *Giardia* could thus modulate the expression of antioxidative genes to regulate the oxidative stress response, hence sirtuin downregulation increases the intracellular ROS and promotes DNA damage. *Giardia* sirtuins may also be able to regulate the activity of antioxidant enzymes, as reported by dos Santos Moura in *T. cruzi* (dos Santos Moura et al., 2021).

Other plausible insight about the relationship between some sirtuins and regulation of antioxidant enzymes may rely on their subcellular localization. Regarding to the *Giardia* sirtuins, gdSir1 is cytoplasmic, gdSir2.2 and gdSir2.3 reside at nuclear periphery, gdSir2.4 localizes within nuclei and gdSir5 has nucleolar localization (Carranza et al., 2016). The fact that the cytoplasmic gdSir2.1 displays an increased expression in R1.35 and R50 cell lines is consistent with its likely interaction with antioxidant enzymes that are distributed throughout the cytoplasm in *Giardia*. The increased expression of gdSir2.3 in the R1.35 line may suggest a role in post-translational mechanisms of yet unknown nature. In contrast, the R8 line that has a sirtuin 2.1–2.3 expression profile similar to WB strain, which could suggest the sirtuin-independent regulation of oxidative stress linked to ABZ resistance. These points deserve, however, further research.

It is noteworthy that drug resistance in *Giardia* is a multifactorial process, wherein the same resistant phenotype may result from distinct mechanisms that ultimately lead to similar outcomes. For example, as reviewed by Ansell.B ***et al.***, during metronidazole resistance not all

strains show a decreased expression of NR1, and in some instances resistant lines may even show contradictory results such as an increased expression of PFOR-1 in WB C4 and 7-13-M3, but PFOR has lower expression levels in all the other lines reviewed (Ansell et al., 2015). It has also been demonstrated a high variability of subculture responses to nitroimidazole and benzimidazole exposure (Argüello-García et al., 2004). Moreover, when resistant lines are derived from the same parental strain, differences are observed in the expression levels of specific genes, without an overall consistent pattern of progressive changes in gene expression (Ansell et al., 2017; Emery et al., 2018; Paz-Maldonado et al., 2013b). It is possible that as the concentration of the drug increases, *Giardia* may engage additional mechanisms, such as genetic mutations, the activity of drug transporters, or epigenetic modifications. Together such adaptations enable the parasite to survive at concentrations as high as 250 μM of ABZ. Thus drug resistance in ***Giardia*** is likely a multifactorial adaptation rather than a single genetic or biochemical alteration, which further emphasizes the need for continued investigation into the mechanisms of drug resistance.

Taken together, we suggest that sirtuins in *Giardia*, particularly gdSir2.1 and gdSir2.3 play a role in ABZ resistance in this parasite through oxidative stress regulation mechanisms. *Giardia* sirtuin enzymes likely confer protection to the parasite against the ROS generated by ABZ and hydrogen peroxide treatment since their downregulation generates higher levels of ROS and ROS-mediated DNA damage and increases the parasite's susceptibility to these drugs. To our knowledge, this is the first report of an association between sirtuins, drug resistance, and oxidative stress in *Giardia duodenalis*.

CRediT authorship contribution statement

Adrián Chávez-Cano: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Scott C. Dawson:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **M. Guadalupe Ortega-Pierres:** Writing – review & editing, Supervision, Resources, Funding acquisition, Formal analysis, Conceptualization.

Note

Supplementary data associated with this article.

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Declarations of interest

none.

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Appendix A. Supplementary data

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