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Divergent Transcriptional Responses to Physiological and Xenobiotic Stress in *Giardia duodenalis*

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Understanding how parasites respond to stress can help to identify essential biological processes. *Giardia duodenalis* is a parasitic protist that infects the human gastrointestinal tract and causes 200 to 300 million cases of diarrhea annually. Metronidazole, a major antigiardial drug, is thought to cause oxidative damage within the infective trophozoite form. However, treatment efficacy is suboptimal, due partly to metronidazole-resistant infections. To elucidate conserved and stress-specific responses, we calibrated sublethal metronidazole, hydrogen peroxide, and thermal stresses to exert approximately equal pressure on trophozoite growth and compared transcriptional responses after 24 h of exposure. We identified 252 genes that were differentially transcribed in response to all three stressors, including glycolytic and DNA repair enzymes, a mitogen-activated protein (MAP) kinase, high-cysteine membrane proteins, flavin adenine dinucleotide (FAD) synthetase, and histone modification enzymes. Transcriptional responses appeared to diverge according to physiological or xenobiotic stress. Downregulation of the antioxidant system and α-giardins was observed only under metronidazole-induced stress, whereas upregulation of GARP-like transcription factors and their subordinate genes was observed in response to hydrogen peroxide and thermal stressors. Limited evidence was found in support of stress-specific response elements upstream of differentially transcribed genes; however, antisense derepression and differential regulation of RNA interference machinery suggest multiple epigenetic mechanisms of transcriptional control.

Giardia duodenalis (synonyms, *Giardia lamblia* and *Giardia intestinalis*) is a parasitic protist of the vertebrate gastrointestinal tract and the most common parasite of humans (1). The vegetative life cycle stage (trophozoite) infects around 1 billion people and causes 200 to 300 million cases of diarrheal disease (giardiasis) each year (1). Infection with *G. duodenalis* is also associated with the development of chronic diseases such as irritable bowel syndrome, chronic fatigue, and diabetes (2). At present, only two major drug classes are available to treat giardiasis: nitroimidazoles, primarily metronidazole (Mtz), and benzimidazoles, such as albendazole (3, 4). Resistance to Mtz is documented in treatment-resistant clinical isolates (5–8), and resistant lines can be generated *in vitro* (reviewed in reference 9).

The mechanism of action of Mtz is relatively poorly understood. This compound is thought to diffuse into *G. duodenalis* trophozoites as an inactive prodrug, whereupon it is enzymatically reduced (activated), yielding reactive intermediates. Mtz intermediates are thought to kill trophozoites by oxidizing proteins, lipids, and DNA (10, 11); however, the relative importance of damage to different biomolecules for Mtz-induced cytotoxicity is contested (10, 12). Resistance to Mtz involves changes in enzyme expression which limit drug activation. Nitroreductase-1, thioredoxin reductase, and pyruvate-ferredoxin oxidoreductase (PFOR) and its redox partner ferredoxin are implicated in activating Mtz and are variously downregulated in resistant lines (reviewed in reference 9). Upregulation of a putative Mtz detoxification enzyme, nitroreductase-2, is also reported in resistant lines (13), suggesting that resistance involves both avoiding drug activation and enhancing enzymatic detoxification. Understanding the initial molecular response to Mtz can yield insight into the mode of action of this drug and illuminate the cellular states that may prime the development of higher levels of resistance. Previous studies (14, 15) in which *G. duodenalis* trophozoites were subjected to sublethal concentrations of Mtz reported heightened histone 2A phosphorylation (a marker of DNA damage) and downregulation of nitroreductase 1. However, there is a paucity of system-level investigations into the response of *G. duodenalis* to Mtz-induced stress.

The nature of the Mtz response in *G. duodenalis* is of interest in the context of cellular responses to other stressors. In a natural infection, trophozoites adhere to the intestinal villi, where they are exposed to spatiotemporal fluctuations in dissolved oxygen (16) and other oxidants, including hydrogen peroxide (H_2O_2) and reactive nitrogen species, which are secreted by the host against intestinal microbes (17–20). Trophozoites may also experience anaerobic conditions before the host feeds, after secretion of antioxidant-rich bile, and close to the luminal midpoint when the cells detach to divide (16). Aside from variations in oxidant con-

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centrations, these parasites may also be exposed to temperatures as high as 40°C during fever in the host (21). Since *G. duodenalis* has evolved over millions of years in the vertebrate gut, we expect that this parasite can sense thermal stress and fluctuating intracellular redox conditions and mount molecular responses to restore homeostasis. The cellular stress response is conserved across all kingdoms of life and is characterized by four main processes: (i) cell cycle arrest, (ii) transcriptional induction or posttranslational activation of molecular chaperones, including heat shock proteins (HSPs), (iii) DNA repair, and (iv) proteasomal degradation of cellular debris (22). However, *G. duodenalis* is vastly distinct, both genetically and metabolically, from other model eukaryotes (23–25). Therefore, defining a conserved stress response in this parasite is of interest for identifying novel therapeutic targets, for understanding the evolution of drug resistance and immune evasion, and for determining the redundancy of the stress response across the eukaryotic kingdom.

The first comparison of molecular stress responses in *G. duodenalis* identified four proteins of expected molecular weights for HSPs, which were induced in response to ethanol exposure (mimicking anaerobiosis), cysteine deprivation (mimicking host malnutrition), and thermal stress (20-min exposure to 43°C) (26). Conversely, exposure to dissolved oxygen (1 h), H_2O_2 (0.1 to 1 mM; 30 min), or Mtz (58 and 580 μM; 6.5 h) elicited little HSP induction. The authors concluded that *G. duodenalis* could mount a relatively redundant response to certain stressors but lacked stress-specific responses. However, recent work has challenged this notion. For instance, trophozoites from different *G. duodenalis* genotypes (assemblages A and B) (27) were shown to downregulate HSP-coding genes (GL_98054 and GL_88765) upon exposure to H_2O_2 (28), suggesting that HSP suppression might be part of a H_2O_2-specific stress response. Furthermore, a microarray-based study revealed 11 genes that were uniquely induced in response to thermal stress and 24 that were unique to protein unfolding stress, while only a single gene was induced under both conditions (29). Promoter regions upstream of genes that were uniquely induced under thermal or unfolding stress were then shown to drive luciferase expression only in response to that stressor, suggesting that *G. duodenalis* can differentiate thermal and protein unfolding stresses (29).

A limitation of previous stress response studies in *G. duodenalis* is the failure to control for different magnitudes and durations of stress, which may confound the discovery of conserved stress responses. In the present study, we calibrated Mtz and H_2O_2 concentrations and elevated temperatures to achieve an approximate 25% reduction in trophozoite growth over 24 h. Because dissolved oxygen is evidenced to compromise the efficacy of Mtz (30, 31) and to form intracellular H_2O_2 (9, 28), we established anaerobic conditions prior to and during experimental stress exposure. As Mtz is thought to oxidize biomolecules, we hypothesized that the molecular response to this drug would be more similar to that mounted against H_2O_2, a general oxidant. Here, we present the first comprehensive comparison of transcriptional responses to different therapeutic and physiological stressors in *G. duodenalis*.

MATERIALS AND METHODS

Cell culture and reagents. *Giardia duodenalis* trophozoites (WB strain, assemblage A) were cultured in TYI-S33 medium (32) modified to contain 6 mM glucose (hereafter, TYI medium) (33). Metronidazole (Sigma-Aldrich) was dissolved to 100 mM in dimethyl sulfoxide (DMSO) and stored at 4°C. Hydrogen peroxide (H_2O_2; 30%, wt/wt, in H_2O) (HA154; Chem Supply) was diluted to 200 mM in TYI medium. Reconstituted CellTiter-Glo reagent (Promega) was stored at −20°C and thawed to room temperature ([RT] 22 to 24°C) 30 min prior to use.

Stress calibration. To compare the effect of different stressors, we incubated trophozoites for 24 h in a range of concentrations of Mtz or H_2O_2 or under various elevated temperatures to achieve an approximate 25% reduction in cell number relative to that of the controls. Trophozoites from confluent flasks were chilled on wet ice, pelleted by centrifugation (680 × *g* for 5 min at 4°C), diluted in fresh TYI medium, and added in 40-μl aliquots to wells of a 96-well clear-bottom plate (3610; Corning) to achieve 10^4 cells per well. A 200-μl aliquot of sterile water was added to the peripheral wells to limit evaporation. Plates were equilibrated at 37°C in a GasPak EZ anaerobe pouch system (260683; BD) to allow trophozoite attachment and sparging of dissolved oxygen in the medium. Serial dilutions of Mtz and H_2O_2 were prepared in TYI medium and added in 10-μl aliquots in duplicate to wells containing trophozoites. The wells of the resultant plates contained approximately 10^4 trophozoites in a total volume of 50 μl and either Mtz (2.5 to 1,000 μM), H_2O_2 (500 to 5,000 μM), 1% DMSO, or TYI medium (negative controls). Plates were incubated at 37°C in GasPak EZ anaerobe pouches with new anaerobic sachets for 24 h. To test the effect of elevated temperatures, trophozoites were added to wells in 50 μl of TYI medium and allowed to equilibrate as described above. GasPak EZ pouches were then opened, and fresh anaerobic sachets were added to replicate the procedure for Mtz and H_2O_2 exposures. On different days, plates were incubated at temperatures from 39 to 44°C, and control plates were simultaneously incubated at 37°C. To quantify cell numbers, 50 μl of CellTitre-Glo reagent was added to wells containing trophozoites, and plates were incubated for 15 min at RT with shaking. Luminescence (corresponding to the number of live cells/well) was measured using a luminometer (BioTek) and converted to a percentage of the negative-control values. For Mtz and H_2O_2 optimization, data from at least two experiments were normalized to values of the negative (1% DMSO and TYI medium) and positive (1 mM Mtz or 5 mM H_2O_2) controls, and Hill plots were fitted using the log(inhibitor) versus normalized response—variable slope module, in Prism (GraphPad). Hill equations were solved for *x* when the *y* value was 75 (i.e., 25% decrease in ATP relative to the control).

Sample generation and mRNA sequencing. For each stress condition, samples were generated in six replicates in two separate experiments (a total of 12 replicates per condition). Flat-sided tubes (Nunclon Delta) were seeded with 1.78×10^4 trophozoites in 10 ml of fresh TYI medium and incubated for 60 h, at which time trophozoites formed a confluent monolayer on the tube wall. The spent medium and suspended trophozoites were discarded, 10 ml of fresh TYI medium was added, and tubes were incubated for 2.5 h at 37°C, standing open in a GasPak EZ large incubation container (260672; BD) with three large anaerobic sachets (260678; BD) to allow sparging of dissolved oxygen. The container was briefly opened, and Mtz (20 mM stock in TYI medium) or H_2O_2 (200 mM stock in TYI medium) was added. Fresh anaerobic sachets were inserted, and tubes were incubated standing open for 24 h at 37°C. For thermal stress exposure, tubes were prepared as described above and incubated at 39°C. After incubation, tubes were capped and inverted, and a 20-μl aliquot of trophozoite suspension was deposited on a glass slide (Menzl Glazer) with a coverslip for video-microscopy (see the supplemental methods). Tubes were incubated on ice-water to allow detachment of trophozoites, which were then pelleted (680 × *g*, 5 min, 4°C), dissolved in 1 ml of TriPure reagent (Roche), and stored at −80°C. Biological replicates were thawed and randomly divided into three pairs. Aliquots of 250 μl from both members of two pairs were combined, yielding three tubes with 1 ml of dissolved trophozoite material, representing inter- and intra-experimental variation (see Fig. S1 in the supplemental material). RNA was extracted according to the manufacturer's protocol within 4 weeks of sample preparation.

The dried RNA pellet was resuspended in reverse-osmosis deionized

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water and treated with Turbo DNase (Ambion) according to the manufacturer's instructions. The quality of DNase-treated RNA was ascertained using a BioAnalyzer (Agilent). Polyadenylated RNA was purified from 10 μg of total RNA using Sera-Mag oligo(dT) beads, fragmented to a length of 100 to 500 bases, and reverse transcribed using random hexamers. Strands were labeled using the dUTP second-strand synthesis method, end repaired and adaptor ligated, and then treated with uracil-specific excision reagent (USER; NEB) before PCR amplification. Products were purified over a MinElute column (Qiagen), and single-ended strand-specific sequencing was performed using an Illumina HiSeq 2500 system (YourGene Biosciences, Taiwan).

Data processing and analysis. Raw reads were trimmed using Trimmomatic (34) (sliding window, 4 nt; minimum average PHRED quality, 20; leading and trailing, 3 nt; minimum read length, 40 nt) and mapped as single-ended reads to the accepted *G. duodenalis* coding domains (assemblage A genome, release 25) (23; http://giardiadb.org/giardiadb/) using RSEM (35), with the "forward-prob 0" flag which discards any reads that map with <100% confidence to the reverse complement of a predicted transcript (RNA-seq reads generated using the dUTP method represent the first cDNA strand, i.e., the reverse complement of the extracted RNA). Reads that did not map under these conditions were likely to include antisense transcripts. To quantify antisense transcription, unmapped reads were mapped with the "forward-prob 1" flag.

RSeQC (36) was used to calculate read mapping statistics (bam_stat module) and to confirm the orientation of mapped reads (infer_experiment module). Feature detection was calculated as a function of read mapping depth, using the counts module in Qualimap (version 1.0) (37) with the "k 10" flag, denoting a minimum mapped read threshold of 10. Saturation plots were displayed in Excel (Microsoft). Expected counts generated by RSEM were submitted to edgeR (38), and genes represented by fewer than 1 count per million counts (CPM) in fewer than three replicates were discarded. Sample libraries were normalized (trimmed mean of *M* values [TMM] method), and transcriptional abundance values were rescaled before fitting (general linear model) and identification of differentially transcribed genes (DTGs) under each condition relative to the control (false discovery rate [FDR] of 0.01). Comparisons between DTG groups required values for the number of transcripts per million transcripts (TPM), calculated by dividing the TMM-normalized CPM value for each gene by the effective gene length. Pearson correlations with previously published data (39) were facilitated by adjusting expected count data for all replicates by library size and TMM normalization to generate comparable CPM values. The number of reads per kilobase per million reads (RPKM) was used for correlations with RNA-seq data from other laboratories (28).

Gene ontology (GO) terms for the predicted *G. duodenalis* proteome were retrieved from GiardiaDB.org (release 25), and sensitive structure-based homology searches were performed for DTGs encoding hypothetical proteins, using the I-TASSER suite (40, 41) as described previously (39). Briefly, I-TASSER integrates amino acid sequence homology searching, with secondary and *de novo* structure prediction and molecular dynamics simulation, to generate a putative three-dimensional structure for a primary amino acid query. The predicted structure is compared against solved crystal structures in the Protein Data Bank (PDB) to allow functional inference (40). Protein structures were visualized using UCSF Chimera software (42). For promoter motif analysis, genes with at least 20 nucleotides of nonoverlapping (i.e., noncoding on opposite strand) sequence upstream and downstream of the start codon were curated using the genomic colocation tool at GiardiaDB.org. To accurately assess correlations between sense and antisense transcription, analysis was limited to protein-coding genes that did not overlap other genes.

DREME software (50) was used to detect overrepresented DNA motifs in noncoding promoters in DTG groups, and Fisher tests of independence were used to identify enriched molecular function and biological process GO terms among DTGs for each stress condition, relative to a curated background of GO terms for all DTGs. For brevity in this paper, the *G. duodenalis* WB strain gene accession prefix GL50803 is abbreviated to GL.

Accession number. RNA-seq reads have been deposited in the NCBI Sequence Read Archive under BioProject number PRJNA322403.

Data availability. Relevant data are given within the paper and in its supporting files in the supplemental material except for the raw RNA-seq reads, which are available through the NCBI Sequence Read Archive.

RESULTS

Stress calibration and read mapping. Based on stress calibration experiments, 7 μM Mtz and 1.15 mM H_2O_2 were determined to exert approximately equal effects on trophozoite growth over 24 h (Fig. 1A and B). This agrees with previously published data suggesting that exposure to 5 to 10 μM Mtz induces a 25% reduction in cell growth over 24 h (15) and that 1 mM is a physiologically relevant concentration of H_2O_2 (43, 44). Incubation at either 39 or 41°C resulted in an approximate 30% reduction in cell number; however, as incubation at 42°C was highly detrimental, 39°C was selected as the more conservative condition for further experimentation (Fig. 1C). This agrees with a previous finding that *G. duodenalis* can survive prolonged exposure to temperatures of up to 40°C with little loss of viability (29). Motile and adherent cells were visible in aliquots from all replicate flasks after 24 h, indicating cellular integrity and viability prior to RNA extraction (see the video at http://gasser-research.vet.unimelb.edu.au/AAC00977-16_Supplementary_video/AAC00977-16_Supplementary_video.pptx). Based on this, we assume that the transcriptional changes associated with our stress conditions promote survival. An average of 32.8 million reads were generated for each replicate, of which at least 91% survived quality control. Approximately 90% of surviving reads mapped uniquely to predicted coding domains in the sense orientation. An average of 2.7 million unmapped reads could be mapped to the same gene models in the inverse orientation, consistent with antisense transcripts (see Table S1 in the supplemental material). The read mapping depth for all replicates was sufficient to detect transcripts from at least 5,000 coding domains (see Fig. S2 in the supplemental material). There was little difference in median CPM values for all conditions (29.2 to 34.44 CPM); however, there was a greater spread of transcriptional abundance values under thermal stress (interquartile ratio [IQR], 5.1 to 104.6 CPM) than under other conditions (IQR, ~6 to 95 CPM) (see Fig. S3).

Differential transcription statistics and stress validation. After filtering for minimum transcriptional abundance and correcting for multiple comparisons, we identified 6,273 genes that were differentially transcribed under at least one stress condition. The proportions of up- and downregulated genes were similar for each condition, with the greatest number of differentially transcribed genes (DTGs) observed under thermal stress (5,523 genes), followed by H_2O_2- and Mtz-induced stress (3,020 and 2,087 genes, respectively). The majority of Mtz-induced DTGs were unique to that condition, whereas the majority of H_2O_2 stress-induced DTGs overlapped with the thermal stress-induced DTGs (Fig. 1D and E; see also Fig. S4 in the supplemental material). When the top 100 DTGs by fold change were compared between each condition, at most approximately 10% of genes from any two groups were shared, of which many were deprecated or hypothetical (see Fig. S5). Normalized sense and antisense transcriptional abundance and fold change statistics are provided for all genes (see Tables S2

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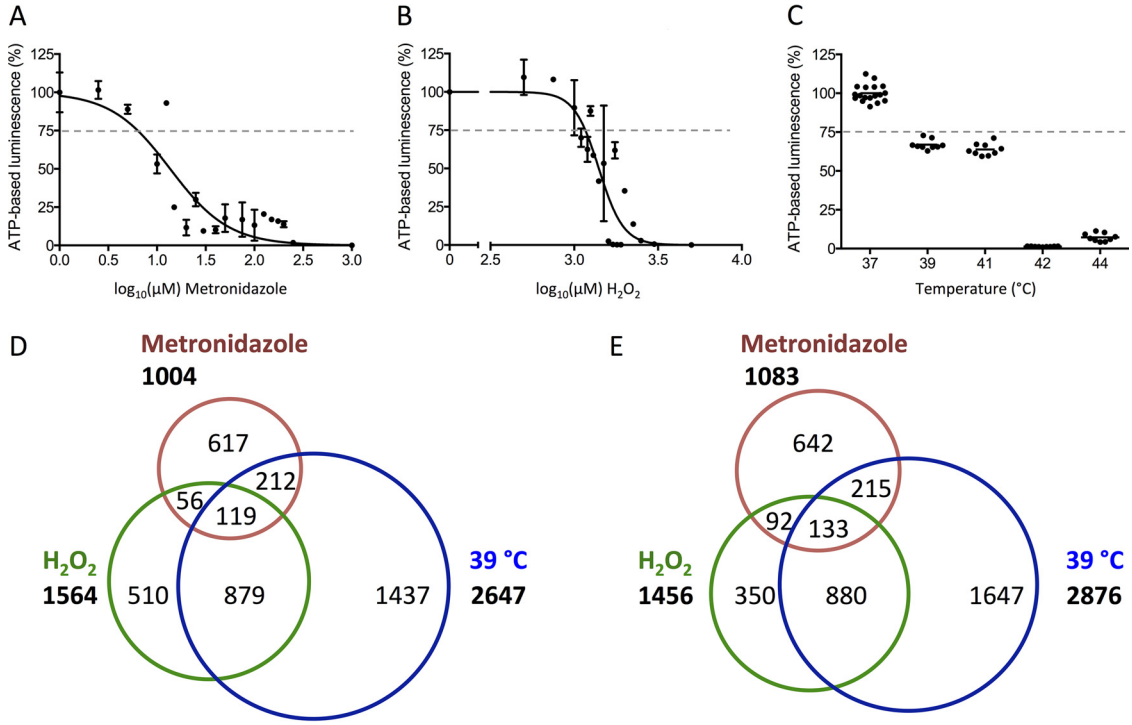

FIG 1 Stress optimization and differentially transcribed genes. Dose-response curves for *G. duodenalis* trophozoites exposed to serial dilutions of metronidazole (A), hydrogen peroxide (B), or elevated temperature (C) over 24 h. Solid lines represent Hill functions, intersected by perforated lines defining the 25% inhibitory concentration (IC_{25}) value. Venn diagrams represent significantly upregulated (D) and downregulated (E) genes at an FDR threshold of 0.01 after a 24-h exposure to each stress. Bold text indicates the total number of differentially transcribed genes under each condition.

and S3). Fold change and functional descriptions for DTGs under Mtz, H_2O_2, and thermal stress are provided in Tables S4 to S6 in the supplemental material.

In broad agreement with previous data, H_2O_2- and thermal stress-induced transcriptomes correlated equally with a previously published set of the 100 most highly transcribed genes after exposure to 150 μM H_2O_2 for 60 min ($r^2 = 0.29$) (28). Transcriptomic results for stationary-phase trophozoites grown under standard (i.e., nonanaerobic) culture conditions (39) correlated best with the transcriptome of trophozoites under H_2O_2-induced stress ($r^2 = 0.56$), whereas correlations with the anaerobic control and Mtz and thermal stress conditions were weaker ($r^2 = 0.39$ to 0.48).

Further according with previous work, nitroreductase-1 (GL_22677) was transcriptionally downregulated under Mtz-induced stress (45) and also under thermal stress (see Fig. 3E). Nine of the 11 genes that were previously reported to be specific to the thermal stress response in *G. duodenalis* (29) were upregulated under our thermal stress conditions, as were 11 of 19 other genes with annotations relating to heat shock. Interestingly, the majority of these genes were also upregulated under Mtz-induced stress, whereas 12 heat shock-related genes were downregulated under H_2O_2-induced stress (see Table S7 in the supplemental material). A hypothetical protein (GL_15125), which was previously found to be upregulated under both thermal and reducing stress (29), was upregulated under both thermal and Mtz stress in our results and has predicted structural homology with a phosphatidylinositol (PI) binding protein (PDB accession number 4BJM (46) (Fig. 2).

Metronidazole-specific genes. Genes uniquely upregulated in response to Mtz-induced stress were enriched for GO terms relating to phosphorylation and phosphatidylinositol and DNA metabolism. Mannosyltransferase and two inositol phosphatases were implicated in upregulated phosphatidylinositol metabolism, as was a partial structural homologue of the kinase mTOR. Indeed, a number of kinases, including 22 Nek and Nek-like ki-

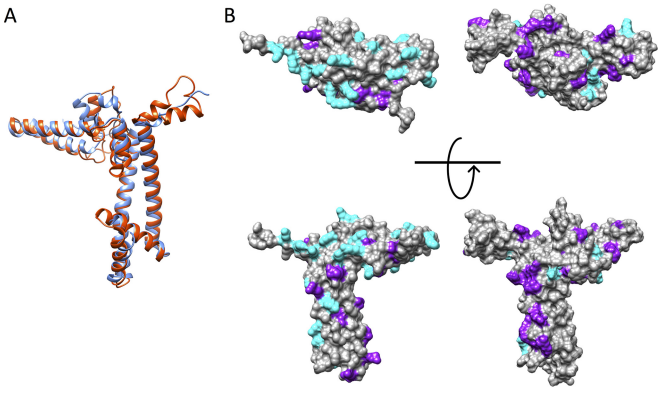

FIG 2 Comparison of the predicted structure for GL50803_15125 and the crystal structure of AvrM. (A) Predicted structure for GL50803_15125 (red), generated using I-TASSER (40), and the crystal structure of AvrM from the fungal pathogen *Melampsora lini* (blue). (B) Surface views of AvrM (left) and the predicted GL50803_15125 structure (right), with lysine (teal) and arginine (purple) residues highlighted. These residues in the AvrM N terminus (top left) are implicated in binding to phosphoinositides.

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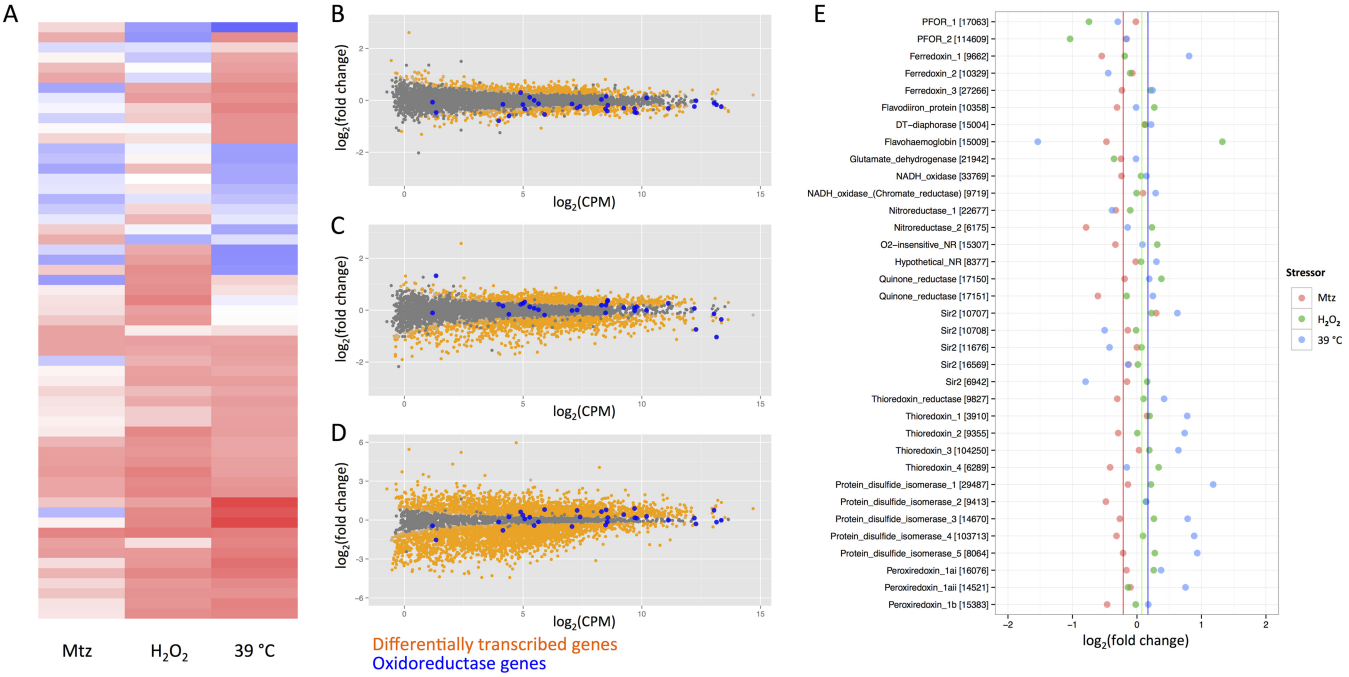

FIG 3 Antioxidant and high-cysteine membrane protein (HCMP) transcription under stress. (A) Log_2(fold change) values for HCMPs are represented in red (<0), white (0), and blue (>0), without row or column scaling. Scatter plots display differentially transcribed genes (orange) under metronidazole (B), hydrogen peroxide (C), and thermal stress (D). Antioxidant-coding genes are displayed in blue. (E) Antioxidant gene annotations including gene accession numbers (in brackets; prefix, GL50803_) and group-wise mean transcription (vertical lines) under each stress condition.

nases, were uniquely upregulated under Mtz stress. The DNA-interacting proteins Rad52, exonuclease-1, Rrm3p helicase, and PMS-1 were upregulated, as were the RNA-related proteins Dicer and pseudouridine synthase. Conversely, the vast majority of antioxidant-related genes were downregulated under Mtz-induced stress. The thioredoxin system (akin to glutathione in higher eukaryotes) and oxygen detoxification machinery were comprehensively downregulated, as were ferredoxin-1, two nitroreductases (GL_6175 and GL_15307), two putative NADPH oxidoreductases (GL_17151 and GL_17150), peroxredoxin-1b, and a glutaredoxin-related protein (Fig. 3B to E). Six Rab family members and 12 of the 21 genomically encoded α-giardins (group E annexins) (47) were also specifically downregulated, suggesting decreased vesicular trafficking and membrane fusion under Mtz stress (48, 49).

Convergent stress response genes. To identify putative general stress response genes in *G. duodenalis*, we examined genes that were up- or downregulated under all stress conditions. Enriched GO terms among 119 convergent upregulated genes related to *N*-acetyltransferase activity, ATP-dependent helicase, and cysteine-tRNA ligase activities. The genes contributing to these terms encoded glucose and histone acetyltransferases, DNA repair proteins (Rad51, TFIIH p90, and a putative ERCC-8), cysteinyl-tRNA synthetase, and a structural homologue of a catalytic aminopeptidase DIV domain. Mitogen-activated protein kinase kinase kinase (MAPKKK), flavin adenine dinucleotide (FAD) synthetase, NAD-dependent Sir2, and ubiquitin-related genes were also upregulated under all three stress conditions. Of 133 convergent downregulated genes, enriched GO terms related to molecular transport, glycolysis (specifically hexose and pyruvate metabolism), and DNA packaging. The contributing genes included ATP-binding cassette (ABC) and major facilitator superfamily (MFS) transporters, glycolytic enzymes, and several members of two gene groups that are unique to *G. duodenalis*: the high-cysteine membrane proteins (HCMPs) and 21.1-kDa ankyrin-repeat proteins (Fig. 3A).

Overlapping physiological stress response genes. A greater proportion of DTGs were shared between the thermal and H_2O_2 stress responses than between these stressors and the Mtz response (see Fig. S4 in the supplemental material), suggesting divergence in transcriptional responses according to physiological or xenobiotic stress. Investigation into the function of the 879 genes upregulated under both H_2O_2 and thermal stress conditions revealed 38 of the 68 ribosomal proteins in *G. duodenalis*, together with DNA replication enzymes, cyclin-dependent kinase 1 (51), three cyclins, and the mitotic regulator Mad2 (52). Intriguingly, three of the four GARP-like transcription factors encoded in the *G. duodenalis* genome were upregulated under both physiological stressors, as were 6 of the 17 genes with predicted GARP-binding promoter elements (53). The latter genes included the antioxidant enzymes protein disulfide isomerase 1 (PDI-1), peroxiredoxin 1a, and a Dps ferretin-like protein. Six further PDIs were also upregulated. Of the 20 Nek kinases upregulated under physiological stress, a greater proportion were predicted to be catalytically active (35%) above the background genomic prevalence of active Neks (27%) (54) (see Table S2). We found 880 downregulated genes under both H_2O_2 and thermal stressors, among which were genes encoding four cysteine proteases, a cysteine desulfurase, 17 HCMPs, and 38 Nek kinases (21% predicted active). Also downregulated were genes encoding an RNA-dependent RNA polymerase, pyruvate-ferredoxin oxidoreductase 1 (PFOR-1), three of six putative phosphatidylinositol 4,5-kinases (PI4,5Ks), and six other

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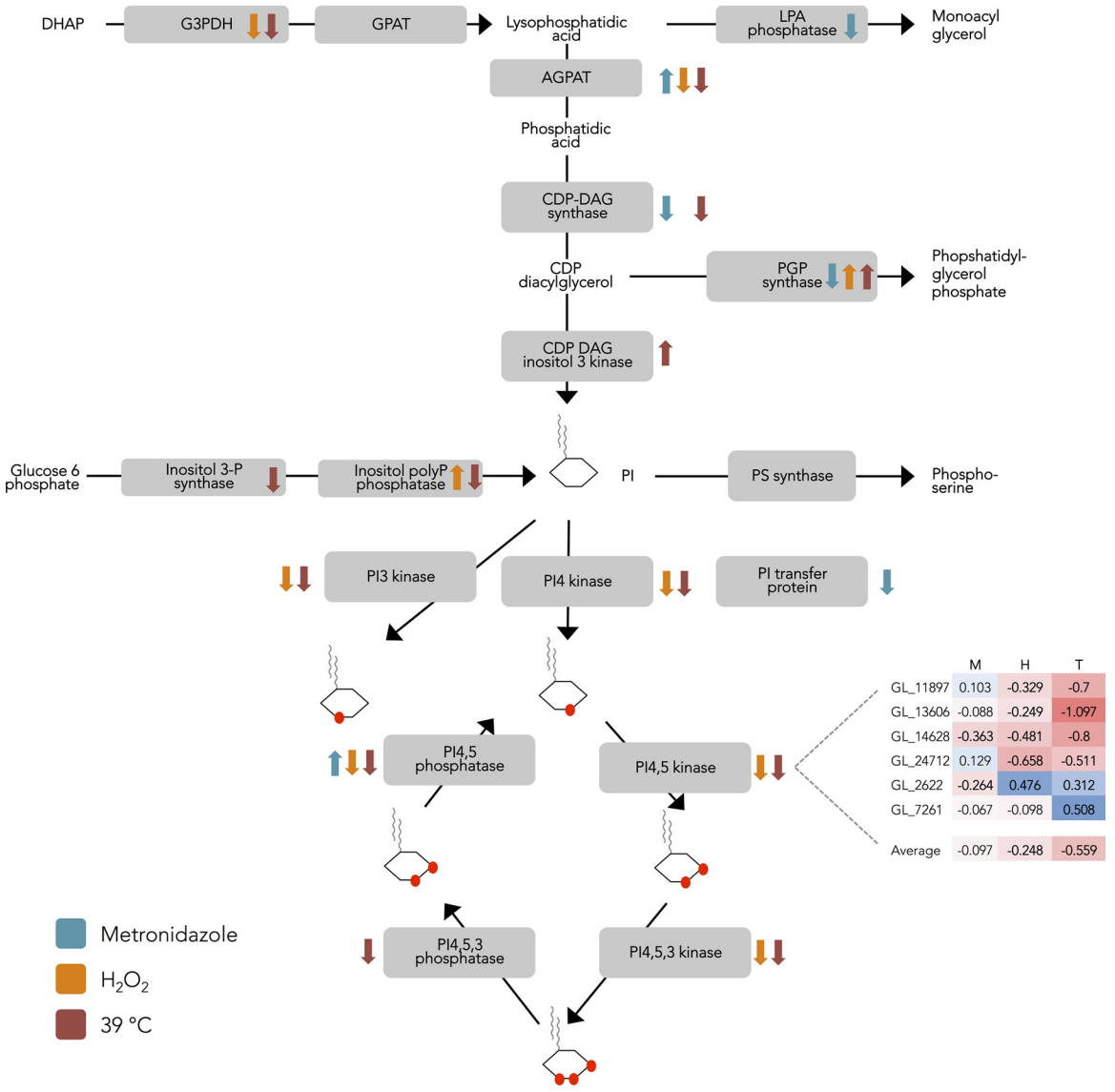

FIG 4 Differential transcription of phosphoinositide synthesis and metabolism genes under stress in *G. duodenalis*. Transcription of genes encoding enzymes involved in phosphatidylinositol (PI) metabolism appears downregulated under hydrogen peroxide and thermal stress. Trophozoites exposed to metronidazole may have a greater intracellular pool of PI4-P. Inositol phosphate is depicted as red dots. Carbon atoms in the PI ring are numbered 1 to 6 anticlockwise from the lipid moiety (wavy lines). Log_2(fold change) values for PI4,5-kinase paralogues are displayed in the table, relative to the control. Significant fold change values are displayed in bold, and average fold change for all paralogues is also indicated. Red, log_2(fold change) of <0; blue, log_2(fold change) of >0; DHAP, dihydroxyacetone phosphate; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; GPAT, glycerol-3-phosphate acyltransferase; AGPAT, acyl-glycerol-3-phosphate acyltransferase; CDP-DAG, CDP diacylglycerol; PGP, phosphatidyl glycerol phosphate; polyP, polyphosphate; PS, phosphoserine.

genes involved in phosphatidylinositol synthesis and metabolism (Fig. 4). The downregulation of Dicer, together with two annotated transcription factors and five predicted structural homologues of transcriptional repressors, suggested a cessation of transcriptional repression under physiological stressors. Finally, we previously found genes encoding homologues of two-component signaling proteins that may be differentially transcribed according to dissolved oxygen tension (39). These genes also appeared to diverge transcriptionally according to physiological or xenobiotic stress. The putative MtrR repressor (GL_2338) was downregulated under Mtz stress but uniquely upregulated under H_2O_2 stress, and the putative Rap protein (GL_1979) was upregulated under both H_2O_2 and thermal stress. Comparisons of transcriptional abundance between the control and test conditions suggested that genes induced under Mtz stress were transcribed at low levels in the control, whereas genes induced under H_2O_2 and thermal stress were relatively highly transcribed in the control (see Fig. S6 in the supplemental material).

Enriched promoter motifs and antisense correlations. Given previous evidence that *G. duodenalis* may differentiate thermal and reducing stress and that stress-specific transcriptional responses might be mediated through different DNA elements (29), we searched for conserved motifs in noncoding promoters of DTGs (median length, 116 nt; IQR, 61 to 257 nt). Numerous significantly overrepresented motifs containing only A and T were identified (see Fig. S7 in the supplemental material), but more

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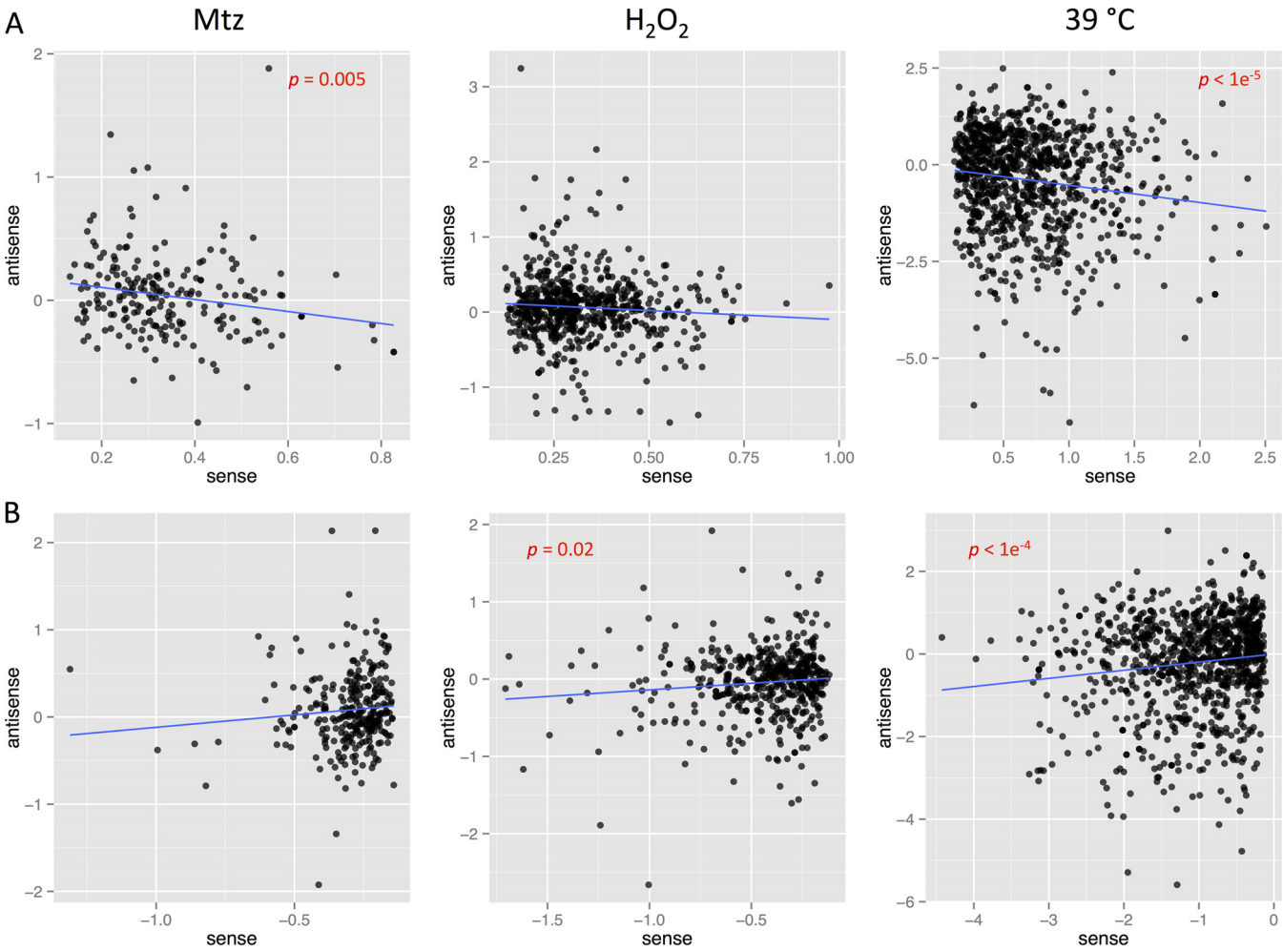

FIG 5 Change in abundance of sense and antisense transcripts from nonoverlapping differentially transcribed genes. Log_2(fold change) values for sense (*x* axis) and antisense (*y* axis) transcripts are divided according to upregulated (A) and downregulated (B) sense transcripts for each condition. Linear regression lines are shown in blue; *P* values for significant linear correlations are indicated.

complex motifs were identified upstream of genes uniquely upregulated under H_2O_2-induced stress (AGSAG), genes uniquely downregulated under Mtz stress (ADTAAA), and genes downregulated under both Mtz and thermal stresses (ACTRCC). Last, we investigated the possibility of posttranscriptional regulation by antisense transcripts under different stress conditions. Significant inverse correlation was detected between sense and antisense abundance for genes upregulated under Mtz and thermal stress, supported by relatively greater proportions of inversely differentially transcribed sense and antisense transcripts under these conditions (Mtz, 58.2%; H_2O_2, 38.4%; thermal stress, 54.0%) (see Table S8). Conversely, a positive correlation between sense and antisense transcription was observed for downregulated genes under H_2O_2 and thermal stress (Fig. 5). Significantly elevated antisense transcription from genes encoding an active Nek kinase (GL_5554), a leucine-rich repeat protein, and an ABC transporter correlated with significant suppression of sense transcripts from these genes under all stress conditions, whereas a large transcriptionally upregulated hypothetical protein (GL_34906) was associated with suppressed antisense transcription in the same manner (see Table S9). Nonoverlapping antioxidant genes tended to exhibit greater antisense transcription under Mtz stress, whereas little mean change or transcriptional suppression was observed for these genes under H_2O_2 or thermal stress, respectively (Fig. S8).

DISCUSSION

Detecting and responding to environmental stress is essential for cell survival. At the cellular level, stress can be defined as any environmental force that impairs the function of proteins, lipids, or nucleic acids and thus perturbs cellular homeostasis. Sensors that detect these perturbations generally activate signaling pathways that modulate gene activity, which, in turn, enable greater stress tolerance (22, 55). In this study, we characterized conserved and specific transcriptional responses to three different stress-inducing agents in *G. duodenalis*. Whereas previous studies did not explicitly control for differing magnitudes of stress, we optimized metronidazole (Mtz), hydrogen peroxide (H_2O_2), and thermal stressors to exert approximately equal pressure on cell growth. Previously, we reported transcriptomic evidence of oxidative stress in axenically cultured *G. duodenalis* trophozoites (39). To avoid interactions between dissolved oxygen and the stressors used in this study (30, 31), we generated anaerobic conditions

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prior to and during stress exposures. In similarity to previous studies of transcription in *G. duodenalis* and other microaerobic protozoa (28, 56, 57), the magnitude of change in transcript abundance for many genes was modest. However, concerted biological replication allowed the identification of many genes that were differentially transcribed at statistical significance (FDR, 0.01). Given the complexity of biological systems and gene-regulatory networks, we refrain from discounting the potential of any DTG to contribute to physiological changes in stressed cells and note numerous studies in model metazoan organisms that link modest transcriptional changes to significant physiological changes (58–60). Further exploration of these stress responses at the proteomic or metabolomic level may provide additional insight into their biological impact, notwithstanding the relatively reduced sensitivity of these techniques compared to that of RNA sequencing.

We found compelling evidence for a set of genes that are differentially transcribed in response to Mtz, H_2O_2, and thermal stress that may constitute general stress response genes in *G. duodenalis*. Downregulation of glycolysis could be interpreted as a mechanism to suppress metabolism and thereby concentrate resources on biomolecule maintenance and repair/degradation (22). Glucose conservation might also be important at times of stress as this molecule is a crucial source of reducing power. Upregulation of DNA repair enzymes and ubiquitin-related enzymes is consistent with the biomolecule repair and debris-clearing aspects of the classical stress response.

We observed transcriptional upregulation of a mitogen-activated protein kinase kinase kinase (MAPKKK) under all three stress conditions. In aerobic eukaryotes, disparate stress-inducing agents are thought to lead to the production of endogenous reactive oxygen species (ROS) (61). Particular MAPKs are activated by thioredoxin in response to endogenous ROS. When reduced, thioredoxin binds to and suppresses MAPK activity. Oxidation of reduced cysteine in thioredoxin by endogenous ROS leads to the dissociation of the thiol-MAPK complex and subsequent activation of stress response genes (62, 63). Given that our experiments were performed under anaerobic conditions, we expect low dissolved oxygen concentrations within trophozoites. Therefore, the upregulation of MAPKKK suggests that chemical and thermal stressors may converge on MAPK signaling pathways in *G. duodenalis* through mechanisms other than endogenous ROS generation. H_2O_2 and activated Mtz may converge on MAPK pathways by oxidizing thioredoxin; however, a mechanism of thermal stress-induced MAPK activation remains to be investigated. Three MAPKs are annotated in the *G. duodenalis* genome in addition to MAPKKK, and at least two of these kinases are transcriptionally upregulated under H_2O_2-induced and thermal stress. Taken together, these results suggest that MAP kinases may integrate and transduce signals from a variety of stress-inducing agents in *G. duodenalis*.

The upregulation of a redox-sensitive epigenetic regulator, Sir2, and flavin adenosine dinucleotide (FAD) synthetase also suggests perturbed redox conditions in trophozoites exposed to Mtz, H_2O_2, and thermal stressors. Sir2 NAD-dependent lysine deacetylases are stimulated by (oxidized) NAD and are thus sensitive to intracellular redox conditions. Many Sir2 enzymes are likely to deacetylate histones and may thereby coordinate transcriptional responses to oxidative stress (64, 65). The upregulation of a histone acetyltransferase under all stress conditions points to reversible and dynamic histone acetylation as an important mediator of the transcriptional response to different stressors in *G. duodenalis*. The *G. duodenalis* FAD synthetase is structurally similar to bifunctional FAD synthetases in prokaryotes, which both phosphorylate and adenylate flavin mononucleotide (FMN) (66). Interestingly, the nitroreductases implicated in Mtz activation and detoxification are FMN dependent, whereas oxidoreductases that are involved in oxygen and H_2O_2 detoxification are generally FAD dependent (reviewed in reference 9). Increased activity of FAD synthetase may therefore bias the antioxidant system toward ROS detoxification and away from Mtz activation. It is tempting to speculate that MAPK signaling or Sir2 activity might modulate the transcription of the FAD synthetase gene. Understanding the transcriptional regulation of this gene holds promise for interfering with global stress responses.

Of the *G. duodenalis*-specific protein families, high-cysteine membrane proteins (HCMPs) were comprehensively downregulated under all stress conditions. Although the role of HCMPs is poorly defined at present, these proteins may help to maintain the integrity of organellar membranes and the plasma membrane as reduced cysteine acts as an antioxidant. Disulfide bonding between oxidized cysteine residues is also important for stabilizing protein structure in harsh environments (67). Convergent downregulation of HCMPs under the stress conditions tested here is therefore counterintuitive. A possible explanation is that HCMPs may act as cysteine stores which are transcriptionally repressed under stress in order to divert cysteine to other proteins, for example, the thioredoxin/peroxiredoxin system. The concomitant upregulation of the cysteine tRNA synthase gene under all stress conditions and the overwhelming downregulation of genes encoding HCMPs under H_2O_2 stress (Fig. 3A) further support this hypothesis.

Transcriptional induction of heat shock proteins and chaperones was observed under Mtz and thermal stress to the near exclusion of H_2O_2 (see Table S4 in the supplemental material). This finding agrees in part with early work by Lindley and colleagues (26), which identified increased/sustained translation of several proteins with molecular weights corresponding to those of heat shock proteins in response to thermal stress, but not in response to oxygen or H_2O_2. The authors suggested that this discrepancy was due to *G. duodenalis* occupying an anaerobic niche *in vivo* and having little need for molecular responses against H_2O_2. However, this hypothesis is insufficient in light of recent work demonstrating spatiotemporal variation in oxygen, H_2O_2, and other oxidants in the gut (16). An alternative explanation, also supported by previous work (28), is that signaling pathways specific to the H_2O_2 response may circumvent or even suppress HSP transcription. The discrepancy in results for Mtz-induced HSP and chaperone induction between the present study and that of Lindley and colleagues (26) may also be attributed to different drug pressures (58 μM over 6 h versus 7 μM over 24 h used here). Clearly, further work is now required to establish the dynamics of gene induction in response to different stress pressures.

A key finding of this study, more broadly, is the lack of agreement between transcriptional responses to Mtz and H_2O_2. Mtz is widely hypothesized to kill microaerobic cells such as *G. duodenalis* by inducing oxidative stress, and H_2O_2 is a classical oxidizing agent. These chemicals were therefore expected to exert similar stresses in *G. duodenalis* and to produce similar changes in transcription. Contrary to this expectation, we observed upregulation or unchanged transcription of a number of antioxidant-coding

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genes in the presence of H_2O_2, whereas the entire antioxidant system was suppressed in the presence of Mtz. Several factors may account for this discrepancy. First, Mtz and H_2O_2 may interact with different types of biomolecules. For example, H_2O_2 is known to oxidize thiol groups in proteins such as thioredoxin (63), whereas reactive Mtz intermediates might have greater affinity for reduced cofactors (68) or nucleic acids (10). Although the importance of DNA damage in the mechanism of Mtz cytotoxicity is contested (10, 12), the increased transcription of genes encoding a pseudouridine synthase (acting to stabilize structural RNAs) (69) and several DNA repair enzymes specifically following Mtz exposure in this study suggests selective sensitivity of nucleic acids to this drug. Indeed, DNA damage may be sufficient to arrest the cell cycle, as suggested by transcriptional downregulation of the mitotic regulator Mad2 under Mtz and previous reports of H2A phosphorylation after sublethal Mtz exposure (15, 70, 71). Second, H_2O_2 requires only bivalent reduction to form H_2O, but the Mtz prodrug requires reduction with as many as six electrons until it forms an inert amine, making it more reactive (10, 72). This requirement for intracellular enzymatic reduction may, however, impose limits on the rate of Mtz activation which do not apply to H_2O_2. Yet another consequence of this difference in activation chemistry suggested in the present findings is that Mtz-induced damage can be limited by downregulating expression or activity of antioxidant enzymes, whereas H_2O_2, which is constitutively active, must be detoxified through increased/sustained antioxidant activity. Finally, while not obvious from the transcriptional results presented here, *G. duodenalis* is expected to possess mechanisms for sensing and eliciting transcriptional responses to physiologically relevant oxidants, such as dissolved oxygen, H_2O_2, and nitric oxide (16, 20, 30). Future work to identify and test the chemical specificity of these sensing mechanisms may elucidate divergent signaling pathways that could contribute to the different transcriptional responses to Mtz and H_2O_2 observed here.

It is interesting to consider how extracellular oxygen tension might influence transcriptional responses to Mtz in *G. duodenalis*. Under the anaerobic conditions used in this study, downregulation of the antioxidant system appears to be a viable transcriptional response as there is little need to combat oxidative stress. However, under higher oxygen tensions such as occur transiently *in vivo* (16), this drug evasion mechanism may not be compatible with the continued activity of antioxidant enzymes. In addition, under atmospheric oxygen tension, Mtz is rendered ineffective against *G. duodenalis* due to futile cycling between the univalently reduced nitro anion and the prodrug (reviewed in reference 9). In a metabolically related microaerophilic protozoan pathogen, *Trichomonas vaginalis*, redox-dependent constraints on gene expression are implicated in the development of different mechanisms of Mtz resistance under anaerobic and microaerobic conditions (73, 74). If similar constraints on gene expression exist under microaerobic conditions in *G. duodenalis*, it would be interesting to investigate dissolved-oxygen conditions under which the parasite might be cornered between a requirement for antioxidant activity to manage intracellular oxygen and the imperative to downregulate the same enzymes to avoid activating Mtz. Further, it is tempting to speculate that under microaerobic conditions, the cytotoxic effects of Mtz on *G. duodenalis* may be attributed to a failure to manage oxygen-derived reactive species rather than damage from Mtz-derived intermediates *per se*. Experiments exposing *G. duodenalis* to Mtz following priming with H_2O_2 or oxygen, for example, could begin to address this area (see reference 75).

A key point of difference between Mtz and physiological stressors relates to phospholipid synthesis and metabolism. Phosphorylation at different positions on membrane-linked inositol is associated with signaling for cell survival and membrane trafficking (76), and phosphoinositides are often targets of secreted effectors from bacterial and fungal pathogens (77). We observed transcriptional changes under H_2O_2 and thermal stress that suggested comprehensive downregulation of phosphatidylinositol (PI) synthesis and PI phosphorylation (PIP). Conversely, transcriptional results suggest that PI metabolism is relatively unchanged in Mtz-exposed trophozoites, wherein the PI4-P pool may increase, as suggested by upregulation of the PI4,5-phosphatase gene. PI4-P is associated with Golgi trafficking in model eukaryotes; however, the relevance of this process in the Mtz response remains to be elucidated. Differential regulation of α-giardins in Mtz and physiological stress responses is intriguing in the context of PI metabolism as particular members of this protein family, which is expanded in *G. duodenalis*, have been shown to bind both the cytoskeleton and a range of PIP species (47). Reorganization of the cytoskeleton is important for endocytosis and subsequent vesicular trafficking (78), and Rab-GTPases interact with PIPs to regulate internal organelle formation and trafficking (79). Although Mtz is thought to enter *G. duodenalis* via passive diffusion, it would be interesting to assess whether the substantial downregulation of α-giardins and Rab-GTPases observed in the presence of Mtz correlates with decreased endocytosis (80). Such suppression of communication with the extracellular environment may promote survival by further limiting the rate of Mtz internalization. Last, the importance of PI metabolism under stress in *G. duodenalis* is also supported by differential transcription of a hypothetical protein (GL_15125) with predicted structural similarity to a PI-binding effector, AvrM, from the flax rust fungus *Melampsora lini* (46). Upregulation of GL_15125 was reported under both thermal and reducing stress in a previous study (29), and we observed upregulation of the same transcript under thermal and Mtz stress. Lysine and arginine have been shown to mediate PI binding at the N terminus of AvrM (46, 81), and the predicted *G. duodenalis* protein structure exhibits similarities in the surface exposure and combined proportions of these basic residues (Fig. 2B). As the most consistently reported stress response transcript in *G. duodenalis*, the encoded hypothetical protein now clearly warrants further investigation in the context of phosphatidylinositol signaling in *G. duodenalis*.

This study provides evidence in support of a role for MAP kinase and phosphatidylinositol signaling in conserved and stress-specific responses. Multiple Nek kinases were also differentially transcribed. The Nek family is massively expanded in *G. duodenalis* although the majority of Nek proteins are predicted to be pseudokinases, many of which associate with the cytoskeleton (54). The roles of active Neks in *G. duodenalis* await experimental determination, but preferential induction of catalytically active Neks under both H_2O_2 and thermal stress suggests a role in stress-related signal transduction. Also interesting is the correlation between antisense induction and sense suppression for the predicted active Nek GL_5554, observed under all stress conditions. In this context, the experimental conditions defined here could provide a paradigm for testing the function and transcriptional regulation of active and inactive Neks in the future.

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Stress signaling cascades are likely to impinge on both genetic and epigenetic effectors. Although we found little evidence for stress-specific DNA response elements in this study, we cannot exclude the presence of motifs specific to smaller sets of coregulated genes that may be masked by the large number of functionally diverse DTGs identified under each stress condition. The GARP-like transcription factors upregulated under H_2O_2 and thermal stressors are particularly interesting. This protein family is recognized to be specific to plants and has not been identified in other protists to date (53). GARPs are predicted to bind upstream of genes encoding antioxidants (PDI-1), α-giardins, and calcium-binding enzymes, which were found to be upregulated under H_2O_2 and thermal stress in our results. Given the lack of GARP domains in the mammalian host and their putative role in regulating elements of the physiological stress response, these transcription factors are worthy candidates for further investigation as chemotherapeutic targets. GARP proteins are implicated in two-component signaling in plants (82), and we previously elucidated structural homologues of two-component signaling proteins in *G. duodenalis*, including an MtrR repressor, since localized in the trophozoite nucleus (85), that may be involved in oxidative stress signaling (39). The transcript encoding the MtrR homologue is downregulated under Mtz stress, further supporting the notion that the response to Mtz exposure, at least under anaerobic conditions, opposes the oxidative stress response.

Aside from differential transcription of epigenetic regulators associated with reversible histone acetylation, we also observed the upregulation of Dicer under Mtz-induced stress and downregulation of the same transcript under physiological stressors. Natural antisense-mediated epigenetic regulation of variant-specific surface protein (VSP) expression has been demonstrated to occur through reverse transcription of mRNA in the cytosol (83, 84). The transcript encoding the RNA-dependent RNA polymerase associated with this process was also downregulated under physiological stress. These findings, together with evidence of antisense derepression in upregulated, but not downregulated, genes (Fig. 5) strongly suggest that RNA interference (RNAi)-mediated transcriptional regulation plays an important role in stress responses. Finally, although results from transcriptomic studies (including the present study) tend to be interpreted as a reflection of a steady-state system, this is likely an oversimplification. Further investigation of transcript stability under stress and correlation of our findings with results from complementary proteomics approaches should yield further insight into gene-regulatory mechanisms in this basic eukaryote.

In conclusion, we calibrated Mtz, H_2O_2, and elevated temperatures to exert equivalent pressure on *Giardia duodenalis* growth and used the most sensitive techniques yet applied to investigate the resultant transcriptional changes. A core set of convergent stress response genes was identified, implicating DNA repair and protein turnover as the most highly conserved stress responses within *G. duodenalis*. A MAP kinase and a putative phosphatidylinositol binding protein may integrate signals from divergent stressors, with attendant redeployment of cysteine from membrane proteins to other thiol-containing proteins. We identified the comprehensive downregulation of antioxidant transcription under Mtz-induced stress, together with suppression of α-giardins and Rab GTPases, which was not detected under H_2O_2 or thermal stress. Under the latter two conditions, active Nek kinases and GARP-like transcription factors were induced, whereas genes involved in phosphoinositide signaling were suppressed, constituting substantial differences in transcriptional responses, which demonstrate the ability of *G. duodenalis* to differentiate physiological and xenobiotic stresses. Limited evidence for stress-specific DNA response elements may implicate epigenetic loci of transcriptional control, supported by antisense derepression and altered transcription of cytosolic RNA inference machinery. These findings reveal multiple exciting avenues for both enhancing the potency of Mtz and targeting conserved stress response genes for novel chemotherapeutic intervention.

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