An extracellular redox signal triggers calcium release and impacts the asexual development of *Toxoplasma gondii*

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Abstract

The ability of an organism to sense and respond to environmental redox fluctuations relies on a signaling network that is incompletely understood in apicomplexan parasites such as *Toxoplasma gondii*. The impact of changes in redox upon the development of this intracellular parasite is not known. Here, we provide a revised collection of 58 genes containing domains related to canonical antioxidant function, with their encoded proteins widely dispersed throughout different cellular compartments. We demonstrate that addition of exogenous H_2O_2 to human fibroblasts infected with *T. gondii* triggers a Ca^{2+} flux in the cytosol of intracellular parasites that can induce egress. In line with existing models, egress triggered by exogenous H_2O_2 is reliant upon both Calcium-Dependent Protein Kinase 3 and diacylglycerol kinases. Finally, we show that the overexpression of the active catalytic domain of glutaredoxin in the parasitophorous vacuole severely impacts parasite replication. These data highlight the rich redox network that exists in *T. gondii*, evidencing a link between extracellular redox and intracellular Ca^{2+} signaling that can culminate in parasite egress. Our findings also indicate that the redox potential of the intracellular environment contributes to normal parasite growth. Combined, our findings highlight the important role of redox as an unexplored regulator of parasite biology.

57 **Introduction**

58 *Toxoplasma gondii* is a single-cell obligate intracellular parasite from the Apicomplexa phylum that
59 can infect any warm-blooded animal. Its seroprevalence is estimated at more than one-third of the
60 human population[1, 2]. Within the host, the asexual lifecycle of *T. gondii* exists as two distinct stages:
61 rapidly proliferating tachyzoites that characterize the acute infection, and slower replicating encysted
62 bradyzoites that are associated with chronic infection[3, 4]. While infections are usually benign, in
63 immunocompromised patients and foetuses[5, 6] the lytic tachyzoite lifecycle is responsible for severe
64 clinical pathology. During a lytic cycle, tachyzoites attach onto and actively penetrate host cells,
65 forming a permissive replication niche called the parasitophorous vacuole (PV). Parasites then
66 replicate by endodyogeny[7] until they eventually egress from the host cell, leading to its lytic
67 destruction. The interconversion of virulent tachyzoites with persistent encysted bradyzoites is
68 influenced by host immune pressure and is key to understanding disease recrudescence[8]. The ability
69 of *T. gondii* to infect diverse host species relates to its remarkable capacity to resist host defences,
70 efficiently recognize and quickly respond to myriad environmental clues. Among biological signal
71 cascades activated by external clues, Ca^{2+} signaling is the most notable and best studied in *T. gondii*
72 (reviewed in [9]). Ca^{2+} is a ubiquitous signaling molecule with a vital role in tachyzoite host-cell
73 invasion[10, 11], motility[12] and egress[13] through activation of effector proteins such as the plant like
74 Ca^{2+}-dependent Protein Kinase 1 (CDPK1)[14, 15] and CDPK3[16]. Alongside Ca^{2+}, other intracellular
75 signaling molecules are known to play important roles in the tachyzoite lytic cycle. These include
76 phosphatidic acid (PA)[17], and the activation of protein kinase G by cyclic guanidine monophosphate
77 (cGMP)[18].

78 Contrasting with Ca^{2+}, the study of reactive oxygen species (ROS) as signaling molecules is
79 incipient in the Apicomplexa. Hydrogen peroxide (H_2O_2) is a neutral ROS molecule with an ability to
80 cross membranes[19], and its roles in signaling are diverse. These include its interaction with
81 glutathione (GSH), and the oxidation of cysteine residues leading to allosteric changes in a variety of
82 proteins such as phosphatases, transcription factors and ion channels[20]. Typically, the oxidation of
83 redox-sensitive cysteines is a reversible process catalysed by enzymes that use GSH or nicotinamide
84 adenine dinucleotide phosphatase (NADPH)[21-24] as redox cofactors. Most studies of H_2O_2 and *T.*

85 *gondii* tachyzoites have focused on the ability of host cells to use the damaging oxidative properties of
86 ROS as a component of innate defence, and strategies employed by tachyzoites to overcome this
87 defence[25-29].

88 A *T. gondii* orthologue of the H_2O_2-detoxifying enzyme catalase is expressed in the parasite
89 cytosol, and confers protection against host oxidative stress[26, 30]. In 2004 Ding *et al.,* [26] compiled a
90 group of 14 genes related to the *T. gondii* antioxidant system. These typically localized to the parasite
91 cytosol and mitochondria, and were assigned to one of five major redox system classifications: (1)
92 metabolic genes (e.g. superoxide-dismutase and catalase); (2) thioredoxins (Trxs, proteins that
93 promote cysteine thiol-disulfide exchange); (3) Protein Disulfide Isomerases (PDIs, proteins that
94 disrupt or form cysteine disulfide bonds to assist protein folding); (4) glutaredoxin-glutathione (Grx-
95 GSH, small proteins that use GSH as a cofactor for thiol-disulfide exchange) and (5) peroxiredoxins
96 (Prxs, enzymes that detoxify hydroperoxides like H_2O_2 and organic hydroperoxides).

97 Expanding this broad description of the parasite's antioxidant system, other studies have
98 tested the association of redox with *T. gondii* signaling and cell cycle regulation. Exogenous treatment
99 of intracellular parasites with the reducing agent dithiothreitol (DTT) triggers Ca^{2+} mobilization and
100 parasite egress[31]. This egress response was triggered by the depletion of host-cell ATP resulting from
101 the activity of an exported parasite ATPase[32]. In a separate study, the oxidation-sensitive protein
102 *Tg*DJ-1 was found to associate with CDPK1 and promote microneme secretion in *T. gondii*[33]. More
103 recently, oxidative stress (generated by sodium arsenite) has been shown to trigger tachyzoite
104 differentiation into bradyzoites following phosphorylation of *T. gondii* eIF2α (TgIF2α) by the
105 translation initiation factor kinase *Tg*IF2K-B[34]. Together, these data suggest that *T. gondii* modulates
106 biological processes in response to changes in redox homeostasis.

107 Here, we use an *in silico* approach to establish a compendium of redox-associated genes and
108 provide an updated view of these genes in *T. gondii*. We then investigate the impact of H_2O_2 upon
109 parasite biology, demonstrating that a H_2O_2 signal outside the boundaries of the infected host cell can
110 be received and interpreted deep within the cytosol of intracellular parasites. We find that exogenous
111 treatment of H_2O_2 triggers mobilization of Ca^{2+} culminating in CDPK3-dependent egress. Finally, we
112 use a genetically encoded redox reporter to dissect redox oscillations prior to egress. Unexpectedly,

113 we discover that overexpression of the active catalytic domain of a human Grx in the parasite's
114 cytosol or PV delays parasite asexual replication. Our results corroborate the existence of a rich
115 variety of antioxidant proteins located in multiples cellular compartments and highlight importance of
116 redox in the basic biology of *T. gondii*.
117
118 **Results**
119 *An updated compendium of T. gondii redox-associated genes*
120 We initially sought to update our understanding of the antioxidant response and redox-signaling
121 network in *T. gondii*. We mined gene sequence and annotation information, as well as proteomic
122 datasets present on ToxoDB[35] to update the list of 14 redox associated genes previously summarised
123 by Ding *et al.,* 2004[26]. Our bioinformatic approach screened for genes containing at least one
124 functional domain of the major redox signaling groups (Trxs, PDIs, Grx-GSHs and Prxs). We
125 identified a total of 58 redox-associated genes (Supplemental material table S1), including 26 Trxs, 16
126 GRX-GSHs, six PDIs, six Prxs, and four metabolic genes (including three distinct superoxide
127 dismutases and one catalase). With the exception of the metabolic genes[26, 30, 36, 37], the majority of
128 genes representing the other redox signaling groups remain uncharacterized. For an improved view of
129 the subcellular distribution of these gene products, we extracted their primary localisation from the
130 recently published hyperLOPIT dataset[38] (Figure 1 – table supplement 1). This indicated a broad
131 distribution of these proteins throughout the cell and suggested the existence of spatially distinct
132 mechanisms of redox regulation for different subcellular compartments.

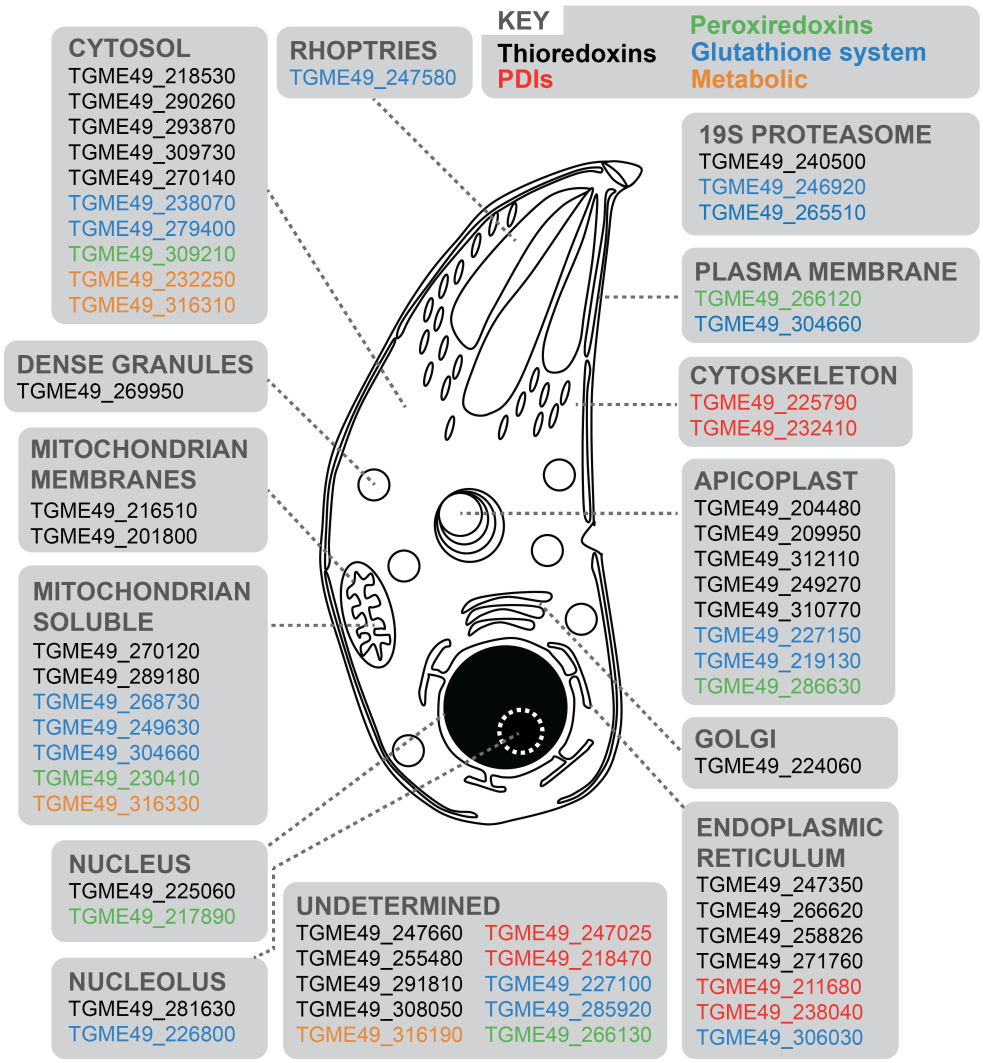

Figure 1: Schematic representation of a *T. gondii* tachyzoite displaying 58 redox-associated genes, and their primary protein location as determined by HyperLOPIT[38]. Gene ID accession numbers are provided, and genes categorized into five groups: black (Trxs); red (PDIs); green (Prxs); blue (Grx-GSHs) and orange (metabolic genes). Further details are provided in Supplement table S1.

147 *Extracellular hydrogen peroxide induces cytosolic Ca^{2+} flux in intracellular tachyzoites*

148 In other eukaryotic systems there are clear associations between calcium and redox signaling. For
149 example, oxidation of cysteines on the ryanodine receptor stimulates calcium release from
150 intracellular compartments[39, 40]. To investigate if a similar overlap exists between these two signaling
151 networks in *T. gondii*, we tested the ability of H_2O_2 to trigger a Ca^{2+} response in tachyzoites within a
152 host cell. We generated a Type 1 parasite (RH strain) expressing the Ca^{2+} sensor protein jRCamP1b[41]
153 fused to GFP via the T2A peptide for bicistronic protein expression (referred to as RH-GFP-T2A-
154 jRCaMP1b). The ratio of these two distinct fluorescent proteins provided an elegant tool to
155 distinguish changes in fluorescence due to cell movement from the Ca^{2+} signal (Figure 2 – figure
156 supplement 1A and B). Upon exogenous addition of 100 μM H_2O_2 we observed a distinct spike in the
157 intracellular Ca^{2+} reporter signal (Figure 2A – videos supplement 1, 2 and 3). To determine whether
158 the oxidative properties of H_2O_2 are responsible for triggering parasite Ca^{2+} release, we repeated the
159 experiment in the presence of the antioxidant α-tocopherol (Figure 2B). Pre-treatment of infected host
160 cell monolayers with 50 μM α-tocopherol abolished H_2O_2-stimulated Ca^{2+} release in intracellular
161 parasites. The addition of the Ca^{2+} ionophore A23187 caused an intense Ca^{2+} signal spike in the
162 parasite cytosol in both the presence and absence α-tocopherol (Figure 2A and 2B, respectively),
163 suggesting that the presence of the antioxidant does not interfere with Ca^{2+} stores in intracellular
164 parasites. Vehicle solvent for H_2O_2 (water) and for A231877 (DMSO) did not trigger Ca^{2+}
165 mobilisation (Figure 2 – figure supplement 1C and D, respectively).

166 We compared the magnitude of the Ca^{2+} signal increase following H_2O_2 treatment with the
167 increase triggered by A23187, a small molecule known to induce parasite egress by Ca^{2+}-dependent
168 mechanisms[42] (Figure 2C). H_2O_2 induced an overall Ca^{2+} signal increase of 35% ± 1.7. This was
169 lower than the Ca^{2+} signal increase observed when parasites were treated with A23187 alone (140 ±
170 4.3), or A23187 on cells pre-treated with 100 μM H_2O_2 (61% ± 0.9). Within restricted timeframe of
171 this experiment (10 minutes), a single egress event was observed for cells treated with 100 μM H_2O_2
172 (Figure 2C – video supplement 2). Observation of egress induced by H_2O_2 was more frequent when
173 host cells were infected with a high multiplicity of infection (MOI = 5) (Figure 2 – video supplement
174 3). We also measured the magnitude of Ca^{2+} signal increase induced by H_2O_2 in Δ*CDPK3* parasites

175 (Figure 2D). The magnitude of the Ca^{2+} signal induced by H_2O_2 in RH-GFP-T2A-
176 jRCaMP1bΔ*CDPK3* was lower (18.1% ± 4.3) compared to RH-GFP-T2A-jRCaMP1b (32.5% ± 1.1).
177 This could reflect previously observed differences in the resting Ca^{2+} levels of these lines[43]. Together,
178 these data suggest that tachyzoites within the PV can perceive and respond to oxidation events
179 initiated outside the host cell.
180
181 *Hydrogen peroxide induces parasite egress in a mechanism dependent on CDPK3*
182 Calcium flux accompanies natural egress[44], and calcium ionophores are well-characterized inducers
183 of egress[13]. To better investigate the potential of H_2O_2 to induce parasite egress, we analysed
184 populations of infected host cells by flow cytometry. The GFP signal from parasites expressing the
185 Ca^{2+} sensor and particle size were used to distinguish infected host cells from free tachyzoites (Figure
186 2 – figure supplement 2A). We tested whether treatment of infected host cells with different
187 concentrations of H_2O_2 could increase the proportion of free tachyzoites. Incubation of infected host
188 cells with H_2O_2 resulted in a dose-dependent increase in the number of free tachyzoites (Figure 2E).
189 We sought to understand how the H_2O_2 stimulated egress integrates into our current molecular
190 understanding of this process. The small molecule R59022 is an inhibitor of diacylglycerol kinase[45]
191 that inhibits egress by disrupting the formation of phosphatidic acid[17]. For all concentration tested,
192 treatment of cells with H_2O_2 resulted in a pharmacological rescue of R59022 egress inhibition.
193 However in the presence of R59022, H_2O_2 did not stimulate egress above the baseline value. This
194 could relate to the discrete points of activity for the two targets of R59022: diacylglycerol kinase 1
195 and 2[46] relative to where the H_2O_2 effect feeds into the system. The stimulation of egress by H_2O_2
196 was abolished in Δ*CDPK3* parasites (Figure 2F), demonstrating a dependency upon this kinase
197 similar to other egress agonists such as A231287. The incubation of non-infected HFFs with different
198 concentrations of H_2O_2 did not alter the proportion of events in this population (Figure 2 – figure
199 supplement 2B), confirming that the concentrations of H_2O_2 used in this protocol did not lyse the host
200 cells.
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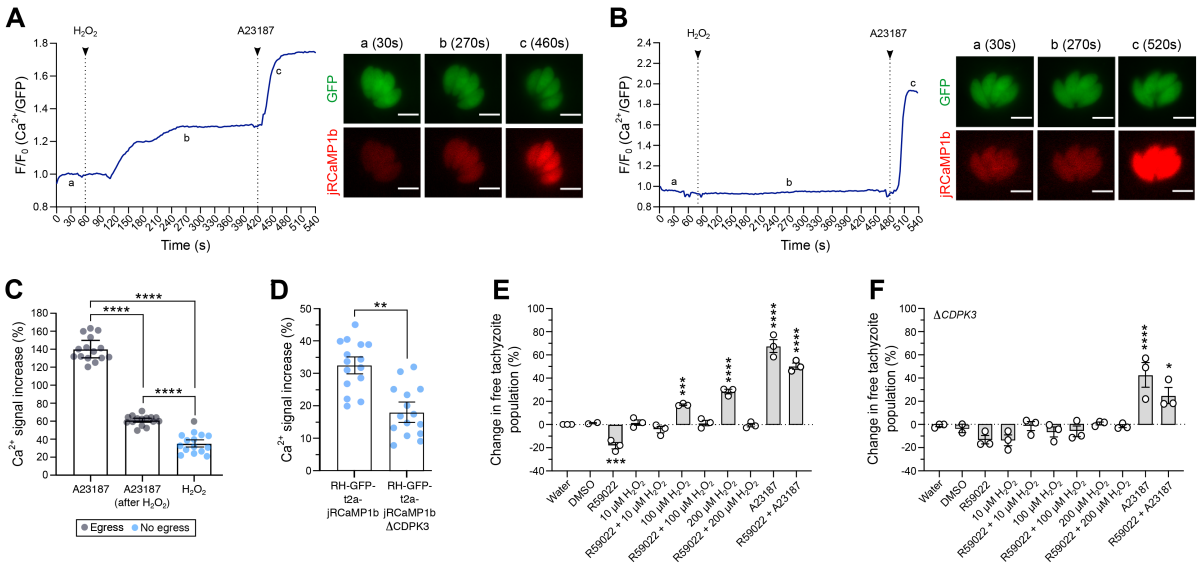

Figure 2: H_2O_2 induces Ca^{2+} release in intracellular parasites and triggers CDPK3-dependent egress. (**A**) Representative trace of 100 µM H_2O_2 induction of Ca^{2+} flux in intracellular RH-GFP-T2A-jRCaMP1b parasites. a – c: widefield microscopy images depicting changes in parasite fluorescence signal intensity for both GFP and the Ca^{2+} sensor at baseline (a at 30s), the peak of Ca^{2+} after H_2O_2 addition (b at 270s), and peak of Ca^{2+} after 1 µM A23187 addition (c at 460s). Data are representative of 27 infected vacuoles from four independent experiments. (**B**) Representative trace of 100 µM H_2O_2 induction of Ca^{2+} flux following pre-treatment of infected host cells with 50 µM α-tocopherol. a: baseline at 30s, b: trace after H_2O_2 addition at 270s and c: peak of Ca^{2+} triggered by A23187 at 520s. Data are representative of 14 infected vacuoles from three independent experiments. For (**A**) and (**B**): black arrows indicate the time of compound addition. Scale bar: 5 µM. (**C**) Intensity of parasite Ca^{2+} signal increase in RH-GFP-T2A-jRCaMP1b parasites (expressed as a percentage over baseline) following addition of: 1 µM A23187 alone, 1 µM of A23187 following 100 µM H_2O_2 pre-treatment, and 100 µM H_2O_2 alone. Gray dots indicate vacuole data points where parasite egress was observed during the measurement period, blue dots represent vacuoles where egress was not observed. (**D**) Intensity of parasite Ca^{2+} signal increase (%) over the baseline upon addition of 100 µM H_2O_2 in RH-GFP-T2A-jRCaMP1b *versus* RH-GFP-T2A-jRCaMP1bΔ*CDPK3* parasites. For (**C**) and (**D**): histograms present data mean ±SEM of three independent experiments (five vacuoles measured in each experiment), with individual vacuole data points also shown. (**E**) and (**F**): Egress assay measuring tachyzoite release after compound treatment in RH-GFP-T2A-jRCaMP1b and RH-GFP-T2A-jRCaMP1bΔ*CDPK3*, respectively. Data represent the mean ±SEM of three independent experiments (except for DMSO that has two independent experiments), with six technical replicates for each. All data were normalised to the water control. Significance was calculated using one-way Anova, Bonferroni's multiple comparisons test. P values: *< 0.05; **< 0.01, ***< 0.001 and ****< 0.0001.

229 *Intracellular parasites expressing GRX1-roGFP2 sensor perceive oxidation induced by H_2O_2*

230 After confirming that exogenous addition of H_2O_2 stimulated Ca^{2+} flux in intracellular *T. gondii*
231 parasites, we investigated the redox state within the parasite cytosol and PV. We reasoned that if
232 calcium release was the direct result of an oxidative signal, the redox status of cellular compartments
233 separating intracellular parasites from the extracellular environment should also be affected. To test
234 this, we generated transgenic parasite strains constitutively expressing the redox sensor protein
235 GRX1-roGFP2, in two different cellular compartments: the parasite cytosol, or targeted to the PV as a
236 consequence of an N-terminal fusion with the GRA8 signal sequence[47]. GRX1-roGFP2 is a
237 ratiometric redox reporter that detects changes in both reduced (GSH) and oxidized (GSSG)
238 glutathione[48] (Figure 3A and B). Importantly, the redox relay system underpinning GRX1-roGFP2 is
239 not affected by pH, which is known to confound data interpretation with other redox sensor
240 proteins[49]. We used GRX1-roGFP2 parasites to track dynamic changes in GSH/GSSG by
241 fluorescence microscopy, and used the normalized GSH/GSSG signal ratio to measure the intensity of
242 oxidation events (Figure 3C – figure supplement 3A). Intracellular parasites expressing the redox
243 sensor targeted to the PV (RH-GRA8-GRX1-roGFP2) or cytosol (RH-GRX1-roGFP2) detected an
244 oxidation event upon exogenous addition of 100 μM H_2O_2 (Figure 3D and E, respectively). The
245 oxidation event within the PV was of greater magnitude compared to that detected within the parasite
246 cytosol, indicating that the strength of the oxidative signal was diminished as it crossed the biological
247 membrane separating these compartments. The water control did not affect the redox signal from
248 GRX1-roGFP2 sensor (Figure 3 – figure supplement 3B).

249 Having observed that H_2O_2 could stimulate calcium flux, we investigated the reciprocal
250 nature of this relationship by testing the ability of calcium ionophores to trigger an oxidative event.
251 Using GRX1-roGFP2 parasites, we measured the resting redox state prior to ionomycin-induced
252 egress. Ionomycin-induced egress was not accompanied by detectable changes in redox within either
253 the PV or parasite cytosol (Figure 3F and G, respectively). The ionophore A23187 could not be used
254 because of saturating autofluorescence associated with this small molecule in the fluorescence
255 channel used to measure oxidation (Figure 3 – figure supplemental 3C). These data suggested that no
256 significant change in the GSH/GSSG ratio occurs before parasite egress induced by ionomycin.

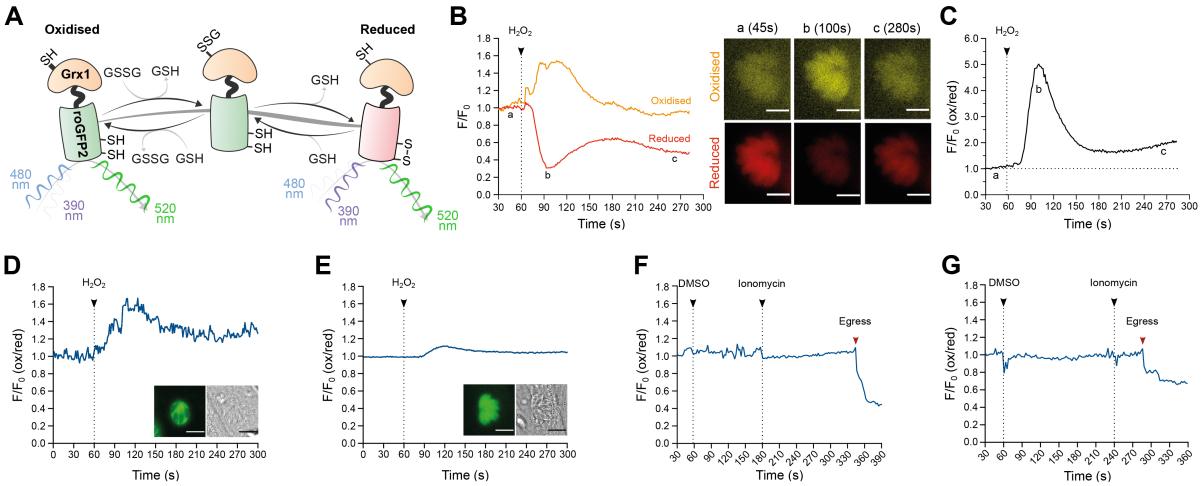

Figure 3: Oxidation induced by exogenous H_2O_2 results in a redox change within the PV and parasite cytosol. (**A**) Schematic representation of the redox sensor based on the catalytic domain of glutaredoxin (GRX1) fused with ro-GFP2 depicting how this sensor interacts with GSH/GSSG and the excitation and emission values of the reduced and oxidized states. (**B** – **C**) Proof-of-principle tracking dynamic changes of GSH/GSSG during an oxidation event in RH-GRX1-roGFP2 parasites. (**B**) Representative signal trace from the GRX1-roGFP2 sensor monitoring both fluorescence channels for reduced (red line) and oxidized (orange line) readouts over time following treatment with 10 mM H_2O_2. Microscopy images of an infected vacuole presented in pseudocolor (orange for oxidation, red for reduction) at a: baseline (45s), b: at the peak of the oxidation event (100s) and c: the return to baseline (280s). This is a representative trace from two independent experiments, with eight vacuoles. Scale bar: 5 µm. (**C**) Presents the oxidation/reduction ratio of normalized signal from graph **B** depicting the intensity changes in redox compared to baseline upon addition of 10 mM H_2O_2. (**D**) Representative trace from RH-GRA8-GRX1-roGFP2 parasites depicting 100 µM H_2O_2 induced redox change within the PV. Microscopy images of an infected vacuole highlighting the presence of the sensor within PV (left image), alongside the brightfield image of the infected host cell. (**E**) A representative trace from RH-GRX1-roGFP2 parasites depicting 100 µM H_2O_2 induction of redox change within the parasite cytosol. Microscopy images depicting an infected vacuole highlighting the presence of the sensor on cytosol (left panel image) alongside the brightfield image of the infected host cell. (**D** – **E**): Representative traces from three independent experiment, nine vacuoles for each group. Scale bar: 5 µm. (**F** and **G**): Representative trace of redox fluctuations in intracellular parasite following 1 µM ionomycin treatment for RH-GRA8-GRX1-roGFP2 (**F**), and RH-GRX1-roGFP2 parasites (**G**). (**E** – **F**): red arrows indicate the moment of parasite egress. Representative traces from three independent experiments, nine vacuoles for each group.

283 *GRX1-roGFP2 redox sensor affects parasite fitness during asexual replication*

284 We noted that one unavoidable result of using the GRX1-roGFP2 redox relay sensors would be the
285 associated overexpression of the catalytic domain of glutaredoxin (as a consequence of it being fused
286 to roGFP2). GRX1 is a small redox enzyme that confers protection against oxidative stress[49, 50], and
287 the overexpression of this enzyme would be expected to shift GSH/GSSG ratios in favour of the
288 reduced form (GSH). Correspondingly, we hypothesized that *T. gondii* strains overexpressing GRX1
289 would have their normal redox status shifted to a more reduced potential. To test whether the
290 overexpression of this redox protein affected parasite asexual growth, we generated a transgenic
291 parasite strain expressing a version of the redox sensor where we had mutated the key catalytic
292 cysteine residue of GRX to render it enzymatically inactive ($GRX1_{ser}$-roGFP2). As a result, GRX1ser-
293 roGFP2 sensor did not respond changes in GSH/GSSG following H_2O_2 treatment (Figure 4 - figure
294 supplement 4). We compared the growth of this strain with parasites expressing the catalytically
295 active version of the sensor. As before, both active and inactive versions of the GRX-fusion sensor
296 were targeted to either the parasite cytosol or PV. As a control group, we used a parasite strain
297 expressing redox-insensitive sensor GFP (RH-GFP-Luc)[51]. Parasites expressing catalytically active
298 GRX1 in the cytosol presented fewer plaques compared to RH-GFP-Luc (Figure 4A). Despite being
299 able to successfully maintain PV-targeted sensor strains in culture, all parasites lines where the sensor
300 was targeted to the PV did not form clear measurable plaques after six days of growth. Intensely
301 stained plaque-shaped boundaries visible on the HFF monolayer suggested these parasites had
302 successfully grown, but that parasite lytic growth had not outcompeted host cell monolayer recovery
303 sufficiently to produce a clear zone of lysis (Figure 4B). When either the catalytically active or
304 inactive sensors were targeted to the parasite cytosol, parasites formed smaller plaques compared to
305 the RH-GFP-Luc control (Figure 4B and 4C, respectively). These data suggested that the presence
306 roGFP2 alone might be sufficient to affect parasite growth. To directly test the influence of redox
307 environment on the lytic cycle, we grew RH-GFP-Luc parasites with 10 μM N-acetyl cysteine (NAC),
308 a small antioxidant molecule that functions by donating cysteine to increase GSH biosynthesis[52].
309 Addition of NAC decreased the size of plaques generated by RH-GFP-Luc parasites (Figure 4C),
310 suggesting that GSH/GSSG imbalance compromised parasite growth. To better understand the impact

311 of overexpressing the sensor on parasite growth, we counted the number of parasites per vacuole after
312 20 hours of asexual parasite replication (Figure 4D). All strains expressing the redox sensor had
313 reduced replication compared to RH-GFP-Luc (Figure 4 – figure supplement 5), with catalytic
314 inactivation of the GRX domain providing a partial rescue of the replication defect. Strains exhibiting
315 the slowest replication were those where the sensor was targeted to the PV, supporting the plaque
316 growth data in Figure 4B. Together, these data suggest parasite growth is sensitive to changes in
317 GSH/GSSG ratios.
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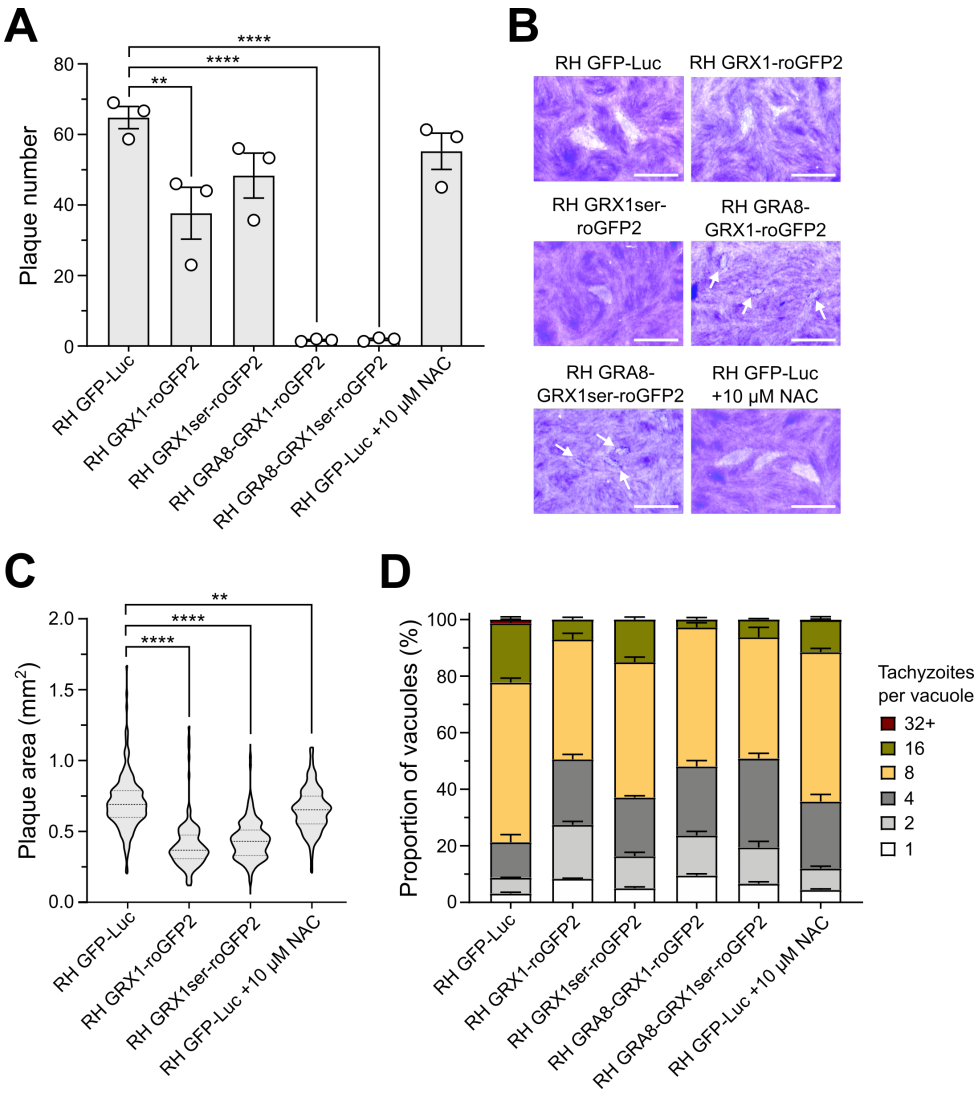

Figure 4: The GRX1-roGFP redox sensor affects *T gondii* asexual replication. (**A**) Histogram presenting plaque count data from a six-day plaque assay using RH-GFP-Luc parasites as a reference control group. (**B**) Representative images of plaques formed. Small plaques are indicated by white arrows. Scale bar: 2 mm. (**C**) Violin plot presenting the distribution of plaque areas (mm^2) for parasites expressing the redox sensor within the cytosol. (**D**) Histogram presenting the effect of the GRX1-roGFP sensors and NAC on parasite intracellular replication. (**A** – **D**) All obtained from three independent experiments, with three technical replicates. Significance was calculated using one-way Anova, Bonferroni's multiple comparisons test for (**A**) and (**B**). P values: **< 0.01, **< 0.001 and ****< 0.0001. The significance analyse for (**D**) is provide on figure supplement 5.

353 **Discussion**

354 Investigations of ROS and *T. gondii* biology initially focused on the host innate immune response.
355 Host macrophages can generate oxidative bursts, creating a toxic microenvironment to fight microbial
356 infection[53-55], with early studies interested in mechanisms used by *T. gondii* to evade oxidative
357 stress[56-58]. However, increasing evidence from multiple organisms has demonstrated that H_2O_2 has
358 functions as a signaling molecule (reviewed in [59]), suggesting the likelihood of a more complex role
359 for ROS in the pathophysiology of *T. gondii*. To sense, respond, and protect against potential
360 oxidative damage from ROS stimuli, cells employ a network of redox-associated proteins such as
361 Trxs, PDIs, Prxs, Grxs, superoxide dismutase and catalase. It was previously shown that *T. gondii*
362 possesses all these elements[26], and we provide an update on the number, diversity and cellular
363 distribution of these redox-associated proteins (Fig. 1). The function of some of these redox-
364 associated gene products in *T. gondii* parasites has been examined: disruption of catalase decreases
365 parasite virulence in mice and *in vitro* tolerance to H_2O_2 [26]; peroxiredoxin-1 interacts with histone
366 lysine methyltransferase to likely regulate gene expression by chromatin rearrangement[60]; two
367 thioredoxins have essential roles in apicoplast biogenesis[61], and another thioredoxin with cytosolic
368 localization has been associated with parasite virulence[62]. Nevertheless, functional information for
369 most of the redox-associated proteins identified in this work remains elusive.

370 The ability of low concentrations of H_2O_2 to initiate an intracellular Ca^{2+} response in
371 mammalian systems is well documented[63-65] and has helped to solidify H_2O_2 as a signaling molecule.
372 Addition of antioxidants blocks intracellular Ca^{2+} release induced by H_2O_2 in smooth muscle cells[65]
373 suggesting that the oxidative properties of H_2O_2 are required for Ca^{2+} signaling. In human endothelial
374 cells, oxidation induced by non-toxic concentrations of H_2O_2 target a Ca^{2+} channel located in acid
375 compartments[64]. Distinct from these models, this work is the first to report that an oxidation event
376 induced by H_2O_2 mobilizes intracellular Ca^{2+} in *T. gondii* tachyzoites within the infected host cell (Fig.
377 2A). This is the first time this has been shown for any apicomplexan parasite. Studying the parasite
378 within the host cell provides the best approximation of physiological conditions to observe both Ca^{2+}
379 and redox signaling. Moreover, using parasites expressing a genetically encoded fluorescent Ca^{2+}
380 sensor abrogated the need to use fluorescent Ca^{2+} indicator dyes. Use of these dyes can be damaging

381 to the cells, and typically require the use of other small molecules to avoid dye loss and
382 compartmentalization[66]. GFP co-expressed with Ca^{2+} sensor allows Ca^{2+} responses to be
383 distinguished from parasite movement which avoids the need to use inhibitors of parasite motility
384 such as cytochalasin D[67]. Finally, the concentration of 100 μM H_2O_2 has been showed to be non-
385 toxic for human fibroblast[68]. Altogether, our protocol to investigate intracellular Ca^{2+} release
386 following H_2O_2 treatment sought to avoid cellular stress that could compromise the true redox/Ca^{2+}
387 dynamic within the parasite. As previously report in muscle cells[65], the presence of antioxidant
388 inhibited the parasite Ca^{2+} response triggered by H_2O_2. Using the GRX1-roGFP2 redox sensor, we
389 confirmed that intracellular parasites directly sense an oxidation event following the exogenous
390 addition of 100 μM H_2O_2. It is not clear whether the exogenous addition of 100 μM of H_2O_2 results in
391 H_2O_2 reaching the intracellular parasites as the host cell contains an extensive network of antioxidant
392 proteins that would be expected to scavenge and neutralise H_2O_2. This could be directly addressed in
393 future experiments using *T. gondii* strains expressing a sensor to specifically detect H_2O_2[69].

394 Regardless of whether Ca^{2+} is mobilized within the parasite as a direct result of an interaction
395 with H_2O_2, or via secondary oxidation signal from the host, Ca^{2+} regulates all aspects of parasite host
396 cell invasion[9] included egress[16]. For the short time period used to track parasites by microscopy, the
397 intensity of the Ca^{2+} signal spike induced by H_2O_2 was quite modest compared to A23187, and that
398 likely explains why egress events were rare. Longer incubations with H_2O_2 induced parasite egress
399 through a mechanism that requires CDPK3 and likely phosphatidic acid. This is the first evidence that
400 suggests that oxidation can trigger *T. gondii* egress. Interestingly, at the other end of the redox
401 spectrum, the reductive molecule DDT can also mobilize Ca^{2+} and induce parasite egress[31].
402 Although the mechanism by which H_2O_2 and DDT lead to parasite egress appears to be distinct, these
403 data indicate that redox can influence the parasite's lytic cycle. We anticipate that parasite biology is
404 tuned to a specific environmental redox potential, and that the perturbation of redox homeostasis with
405 either oxidative or reductive stress elicits a phenotypic response.

406 The mechanism for how H_2O_2 mobilizes Ca^{2+} in animals is better understood but the
407 evolutionary distance between vertebrates and *T. gondii* makes direct comparisons more challenging.
408 *T. gondii* is more closely related to plants, and shares a more similar signaling toolkit[70]. It has been

409 recently reported that plants possess a cell surface H_2O_2 receptor that once activated, triggers a Ca^{2+}
410 influx into the cells through a Ca^{2+} ion channel[71]. *T. gondii* also has a H_2O_2-sensitive protein that
411 associates with the CDPK1 to promote microneme secretion[33], an event that requires Ca^{2+}
412 mobilization to allow parasite invasion[72].

413 Our work suggests a connection between oxidation and parasite Ca^{2+} release but our data did
414 not find evidence that ionophore-induced Ca^{2+} release changes the redox state of either the parasite's
415 cytosol or PV. The use of the GRX1-roGFP2 sensor to track redox changes based on GSH/GSSG
416 allows a fast and selective assessment of redox fluctuations in real time [48], with improved dynamics
417 compared to other redox sensors like roGFP1 or roGFP2[73]. Considering that Ca^{2+} mobilization is also
418 a fast event, GRX1-roGFP2 is a suitable tool to investigate the relationship between ROS and Ca^{2+}.
419 Moreover, the fact that GRX1 does not directly interact with H_2O_2 [49] implies this sensor would not
420 interfere with any eventual interaction between H_2O_2 and its potential targets within the parasite or PV.
421 Unexpectedly, expression of GRX1-roGFP2 within *T. gondii* parasites was detrimental to asexual
422 replication. This is surprising as the GRX1-based sensor is generally well tolerated, and presents
423 negligible toxicity in neurons[49, 74]. Inactivation of the catalytic domain of GRX1 only partially
424 recovered parasite growth, suggesting that roGFP2 alone is sufficient to influence parasite replication.
425 Cells contain millimolar concentrations of GSH within their cytoplasm and organelles, and under
426 physiological conditions the cell maintains the majority of this redox buffer molecule in a reduced
427 form ([GSH]>[GSSG])[75]. Parasites overexpressing GRX1 would be expected to affect the normal
428 GSH/GSSG balance. The addition of NAC, a small molecule that can be used to generate GSH, also
429 slowed parasite growth. This supports the hypothesis that the GSH/GSSG ratio can influence parasite
430 replication. Parasites expressing GRX1ser-roGFP2 are likely have altered redox potential due ability
431 of roGFP not fused with an active GRX1 to interact directly with oxidizing molecules like H_2O_2[76].

432 Targeting of the redox sensor to the PV had the greatest effect upon parasite asexual growth.
433 During asexual replication within host cells, *T. gondii* resides within the PV. This compartment
434 separates the parasite from the host cytoplasm, providing a niche for parasite survival and
435 replication[77]. Signaling molecules from the host must cross the PV in order to reach the parasite.
436 Should an oxidative signal from the host encounter an unusually reductive environment within the PV

437 due the buffering effect of the redox sensor, the signal could be lost before reaching the parasite. Our
438 work increases our understanding of the complex redox system in *T. gondii,* and provides the first
439 evidence that parasite replication is sensitive to redox imbalance. Redox clearly has an important role
440 in *T. gondii* pathophysiology and we anticipate that the unveiling of this network will guide future
441 covalent drug discovery targeting redox-sensitive chemically reactive cysteines.
442
443 **Material and Methods**
444 **Parasite and host cell culture:** *T. gondii* tachyzoites from strain RH (Type I) lacking hypoxanthine-
445 guanine phosphoribosyl transferase gene (HXGPRT) were cultivated *in vitro* on monolayers of
446 primary human foreskin fibroblast (HFF, ATTC®) in a humidified incubator at 37°C, 5% CO_2, 3% O_2
447 atmosphere and maintained in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10%
448 of foetal bovine serum (FBS) and 2mM L-glutamine, without antibiotics. All culture were tested
449 against *Mycoplasma* infection on a monthly basis.
450
451 **Generation of plasmids and transgenic parasites:** All primers used in this study are listed in table
452 supplement 2. The GRX1-roGFP2 sensor[48, 78] was amplified from the commercially available vector
453 pEIGW-GRX1-roGFP2 (Addgene plasmid nº64990) using primers 1/2 and insert into digested (HF-
454 EcoRI & PacI) pTUB8 vector containing selectable marker for HXGPRT using Gibson Assembly®
455 Master Mix. The same strategy was used for GRA8-GRX1-roGFP2, using primers 2/3. To generate
456 vectors with the inactivated GRX1 catalytic domain ($GRX1_{cys23-26}$)[79, 80], primers 4/5 were used to
457 replace cysteine with a serine on pTUB8::GRX1-roGFP2 and primers 4/6 on pTUB8::GRA8-GRX1-
458 roGFP2, followed circularization with KLD reaction mix (NEB), resulting the vectors
459 pTUB8::$GRX1_{(ser23-26)}$-roGFP2 and pTUB8::GRA8-$GRX1_{(ser23-26)}$-roGFP2. All vectors were linearized
460 and transfected into RHΔ*ku80*Δ*HXGPRT* parasites as previously described[81]. Transfected parasites
461 were selected 24 hrs post-transfection by addition of mycophenolic acid (MPA; 25µg/mL) and
462 xanthine (XAN; 50 µg/mL) to culture medium. Strains were cloned by limiting dilution into 96 well
463 plates, and five clones selected. Genomic DNA was extracted from extracellular tachyzoites using

464 Monarch® Genomic DNA Purification Kit (New England BioLabs). Presence of GRX1-based sensors
465 was confirmed using primer pair 7/8.
466 To generate the calcium sensor construct pUPRT::GFP-T2A-jRCaMP1b, sequence encoding
467 the red fluorescent calcium sensor protein jRCaMP1b[41] was ordered from IDT as a custom synthetic
468 gene, and PCR amplified with appropriate Gibson overhangs using primers 9/10. The 5'UTR of
469 *GRA1* was PCR amplified from pTKO2c[82] using primers 11/12, and the GFP-T2A fusion sequence
470 was amplified from an unpublished in-house plasmid using primers 13/14. All three fragments were
471 subsequently cloned by Gibson assembly into PacI-digested UPRT targeting vector pUPRT-HA[83].
472 The resulting construct was linearised using NaeI and transfected into RH Δ*ku80*Δ*hxgprt* parasites to
473 generate the GFP-T2A-jRCaMP1b calcium sensor line. Transgenic parasites were subjected to 5'-
474 fluo-2'-deoxyuridine (FUDR) selection (5 µM) 24 hrs after transfection. To generate the GFP-T2A-
475 jRCaMP1b Δ*CDPK3* line, the *HXGPRT* casette (flanked by 5' and 3' DHFR UTR sequences) was
476 PCR amplified from *pGRA*-HA_HXGPRT[84] using primers 15/16 (introducing 40bp CDPK3
477 homology regions to the amplified fragment) and co-transfected into RH Δ*ku80*Δ*HXGPRT* with
478 pSag1::Cas9-U6::dbl-sgCDPK3. The pSag1::Cas9-U6::dbl-sgCDPK3 vector was generated by
479 inverse PCR amplification of the pSag1::Cas9-U6[85] vector using primer pairs 17/18 and 17/19 to
480 generate intermediate constructs pSag1::Cas9-U6::sg1CDPK3 (comprising sgRNA1) and
481 pSag1::Cas9-U6::sg2CDPK3 (comprising sgRNA2) respectively. Following circularization of both
482 intermediate constructs using KLD reaction mix, a region comprising sgRNA1 was PCR amplified
483 with primers 20/21 from pSag1::Cas9-U6::sg1CDPK3 and Gibson assembled into Kpn1/XhoI
484 linearised pSag1::Cas9-U6:: sg2CDPK3 to generate the double sgRNA plasmid pSag1::Cas9-U6::dbl-
485 sgCDPK3. Recombinant parasites were selected 24 hrs post transfection as previously described for
486 GRX1-roGFP2. Integration of the HXGPRT cassette at the CDPK3 locus was confirmed using primer
487 pairs 22/23 and 24/25 to confirm 5' and 3' integration respectively. Absence of the endogenous
488 CDPK3 locus was confirmed using primers 26/27. RH-GFP-Luc parasites (expressing GFP mutant 3
489 and firefly luciferase IAV[51]) were a gift from Dr Moritz Treeck.
490

491 **Identification of *T. gondii* genes related to redox:** Within the *Toxoplasma* database (ToxoDB[35],
492 release 50 beta 17 Dec 2020), a list of genes related to redox signaling in *T. gondii* were obtained
493 using the keywords "thioredoxin", "glutathione", "glutaredoxin", "peroxiredoxin" and "protein
494 disulfide" on gene text search option. The function domains of the canonical antioxidant groups Trxs,
495 Grx-GSH, Prxs and PDIs were confirmed for each gene using Basic Local Alignment Search Tool
496 (BLAST) at National Center for Biotechnology information (NCBI) database
497 (www.ncbi.nlm.nih.gov/). The spatial protein localization related to each redox gene were extracted
498 from the localisation of organelle proteins by isotope tagging (hyperLOPIT)[38, 86] dataset available
499 through ToxoDB.
500
501 **Plaque and replication assays:** For plaque assays, tachyzoites were harvested from infected HFF by
502 syringe passage followed by filtration (5µm). ~100 tachyzoites were added per well of a 6-well plate
503 prepared with confluent HFF monolayers, and allowed to grow undisturbed for 6 days. Plates were
504 washed with phosphate-buffer saline (PBS) and fixed with cold methanol, stained with crystal violet
505 and scanned. Plaque counts and area measurements were performed using FIJI software by drawing
506 region of interesting (ROI). For replication assays, the freshly lysed tachyzoites were added to HFF
507 monolayers grown on a µ-slide 8 well glass bottom chamber (Ibidi®) with multiplicity of infection
508 (MOI) 1. To synchronize in the infection, parasites were allowed to settle onto chilled host cells for
509 20 minutes, and then allowed to invade for 2 hours at 37˚C. Cells were washed with PBS to remove
510 extracellular tachyzoites. Cells were incubated for a further 18 hours, and subsequently fixed with 3%
511 paraformaldehyde at room temperature (RT) for 20 min, and blocked with PBS supplemented with 2%
512 FBS. Parasites per vacuole were counted by excitation with 470nm laser (4% intensity) widefield
513 Nikon Eclipse Ti-E inverted microscope equipped with an ORCA- Flash4.0 camera (Hamamatsu,
514 Japan) and NIS-Elements Viewer software (Nikon), 60x-oil objective. All parasite strains were
515 genetically encoding for GFP or roGFP2. All strains were tested tree independent times, each with
516 three technical replicates.
517

518 **Egress assay:** Freshly lysed tachyzoites from RH-GFP-t2a-jRCaMP1b parasites were harvested and
519 inoculated (MOI:1) onto confluent HFF cells grown on 24 well plates and grown for 18 hours. Cells
520 were washed with PBS and the growth medium replaced with phenol red free DMEM without FBS
521 and incubate for a further three hours. Medium was removed and cells were incubated for 30 minutes
522 with PBS plus drugs (A23187, R59022, H_2O_2) or vehicle controls (water, DMSO). At the end of
523 incubation, the supernatant of the wells was carefully aspirated and cells were detached by 10 min
524 incubation with 200 µL of RT Accutase™. Accutase-release cell suspensions were fixed with 200 µL
525 of 8% paraformaldehyde (20 minutes, RT). Fixed cells were transferred into falcon 5 mL tube with 35
526 µm nylon mesh cap. Free fluorescent parasite and infected HFF population were analysed and
527 quantified in a BD LRSFortessa™. A total of 5000 events were collected for each tube. Blue laser
528 (488nm) with 530/30 nm filter was used to detect the GFP signal. An uninfected HFF control was
529 used to assess the effect of each drug treatment on host cell gating. Each drug condition was tested
530 two independent times, each with six technical replicates.
531
532 **Fluorescence microscopy:** For cytosolic Ca^{2+} fluorometric measurements, RH-GFP-T2A-jRCaMP1b
533 or RH-GFP-T2A-jRCaMP1bΔ*CDPK3* parasites were added (MOI:1) to confluent HFF grown on 8
534 well glass bottom chamber and allowed to grow for 18 hours. Wells were washed with PBS and
535 media replaced by phenol red DMEM medium without FBS, and incubate for a further two hours.
536 Images of live infected HFFs were captured at 37°C, using a 60x-oil objective in the same widefield
537 microscope previously describe on replication assay. GFP (470 nm excitation/ 520 nm emission) and
538 jRCaMP1b (555 nm excitation/ 605 nm emission) with 100 milliseconds acquisition rate signal were
539 collected every second for up to a maximum 10 minutes. Drugs were applied on the wells after one
540 minute of acquisition by pipetting. Image analyses were performed using FIJI software. Raw
541 fluorescence readout (F) for each vacuole on each channel was normalised against the average of the
542 baseline signal before adding the drug (F_0) using the ratio F/F_0 bringing the resting baseline value to
543 one. To distinguish intracellular Ca^{2+} oscillation signal from vacuole movement, the
544 $F_{(jRCaMP1b)}/F_{0(jRCaMP1b)}$ values were normalised against the GFP signal $F_{(GFP)}/F_{0(GFP)}$. Ca^{2+} response
545 during a specific time is given by the formula: $(F_{(jRCaMP1b)}/F_{0(jRCaMP1b)}) / (F_{(GFP)}/F_{0(GFP)})$.

546 For redox measurements, parasites expressing ratiometric GRX1 based sensors were treated as
547 described for RH-GFP-T2A-jRCaMP1b parasites. Oxidized GRX1-roGFP2 (395 nm excitation/ 520
548 nm emission) and reduced GRX1-roGFP2 (470 nm excitation/ 520 nm emission) with 200
549 milliseconds acquisition rate signal were collected every second for up to a maximum 10 minutes.
550 Data analyses is similar to Ca^{2+}, the redox reading was obtained by formula: $(F_{(oxidised)}/F_{0(oxidised)})$ /
551 $(F_{(reduced)}/F_{0(reduced)})$.
552
553 **Chemical and Reagents:** Hydrogen peroxide 30% (w/w) solution with stabilizer; diacylglycerol
554 kinase inhibitor (R59022); A23187, Ionomycin, anhydrous methanol, and crystal violet solution were
555 obtained from Sigma-Aldrich Company Ltd. α-tocopherol phosphate disodium salt from Merk.
556 Dimethylsulfoxide (DMSO) anhydrous and paraformaldehyde 16% solution from Life Technologies.
557 Accutase cell detachment solution from Fisher Scientific Ltd.

558 **Statistical Analysis:** The data are represented as the mean ± SEM and analysed using two-tailed
559 pared Student *t* test between two groups and one-way or 2way analyses of variance (ANOVA) with
560 Bonferroni's multiple comparisons test for comparing means between ≥3. All data were analysed
561 using GraphPad Prism 9 software (California, USA). The data were considered statistically significant
562 when P values <0.05.
563
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575
576 **Competing interests**
577 The authors have declared there are no conflict or competing interest.
578
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815
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817

Gene ID (ToxoDB)	Gene annotation	Predicted localisation	Publication indicating redox function
TGME49_204480	thioredoxin domain-containing protein	apicoplast	no
TGME49_209950	thioredoxin domain-containing protein	apicoplast	no
TGME49_216510	thioredoxin, putative	mitochondria-membrane	no
TGME49_218530	PITH domain-containing protein	cytosol	no
TGME49_240500	PITH domain-containing protein	19S proteasome	no
TGME49_224060	thioredoxin, putative	golgi	no
TGME49_247350	thioredoxin domain-containing protein	ER	no
TGME49_269950	thioredoxin domain-containing protein	dense granules	no
TGME49_201800	thioredoxin domain-containing protein	mitochondria-membrane	no
TGME49_247660	thioredoxin domain-containing protein	NA	no
TGME49_255480	thioredoxin domain-containing protein	NA	no
TGME49_266620	thioredoxin domain-containing protein	ER	yes[87]
TGME49_310770	hypothetical protein / ATrx2	apicoplast	yes[88]
TGME49_265510	hypothetical protein	19S proteasome	no
TGME49_270120	thioredoxin-like protein, TLP1	mitochondria-soluble	no
TGME49_289180	thioredoxin domain-containing protein	mitochondria-soluble	no
TGME49_290260	PITH domain-containing protein	cytosol	yes[87]
TGME49_291810	thioredoxin domain-containing protein	NA	no
TGME49_293870	thioredoxin	cytosol	no
TGME49_271760	seryl-tRNA synthetase (SeRS2)	ER	no
TGME49_270140	splicing factor DIM1, putative	cytosol	no
TGME49_308050	phosducin domain-containing protein	NA	no
TGME49_309730	thioredoxin reductase	cytosol	yes [62]
TGME49_247025	PITH domain-containing protein	NA	no
TGME49_258826	thioredoxin domain-containing protein	ER	no
TGME49_312110	thioredoxin domain-containing protein	apicoplast	yes[89]
TGME49_225060	thioredoxin domain-containing protein	nucleus-chromatin	no
TGME49_211680	protein disulfide-isomerase	ER	yes[90]
TGME49_218470	protein disulfide-isomerase, putative	NA	no
TGME49_225790	thioredoxin-like-fold domain-containing protein	cytoskeleton	no
TGME49_232410	thioredoxin-like-fold domain-containing protein	cytoskeleton	no
TGME49_238040	protein disulfide-isomerase domain-containing protein	ER	no
TGME49_249270	protein disulfide isomerase-related protein (provisional), putative	apicoplast	no
TGME49_226800	GSH-synthase domain-containing protein	nucleolus	
TGME49_238070	glutaredoxin domain-containing protein	cytosol	no
TGME49_277790	glutaredoxin domain-containing protein	PM-peripheral	no
TGME49_304660	glutaredoxin domain-containing protein	mitochondria-soluble	no
TGME49_247580	glutaredoxin domain-containing protein	rhoptries	no
TGME49_227150	glutaredoxin domain-containing protein	apicoplast	no
TGME49_279400	glutaredoxin domain-containing protein	cytosol	no
TGME49_268730	glutaredoxin domain-containing protein	mitochondria-soluble	no
TGME49_246920	glutathione reductase	19S proteosome	no
TGME49_306030	glutathione s-transferase, n-terminal domain containing	ER	no
TGME49_249630	glutathione s-transferase, N-terminal domain-containing protein	mitochondria-soluble	no
TGME49_281630	lactamase-b domain-containing protein	nucleolus	no
TGME49_219130	NADPH-glutathione reductase	apicoplast	no
TGME49_227100	glutaredoxin domain-containing protein	NA	no
TGME49_285920	hypothetical protein	NA	no
TGME49_309210	thioredoxin domain-containing protein / peroxiredoxin 6, putative	cytosol	no
TGME49_230410	thioredoxin domain-containing protein / peroxiredoxin PRX3	mitochondria-soluble	yes[26, 91]
TGME49_266130	glutathione peroxidase / peroxiredoxin PRX2	NA	yes[26]
TGME49_217890	peroxiredoxin / alkyl hydroperoxide reductase	nucleus	yes[92]
TGME49_286630	peroxiredoxin / redoxin domain-containing protein	apicoplast	yes[92]
TGME49_266120	glutathione peroxidase / thioredoxin-dependent peroxidase TPX1	PM-peripheral	yes[93]
TGME49_316330	superoxide dismutase SOD2	mitochondria-soluble	yes[26]
TGME49_232250	catalase	cytosol	yes[26, 30, 94]
TGME49_316190	superoxide dismutase SOD3	NA	yes[26]
TGME49_316310	superoxide dismutase	cytosol	yes[36]

818 **Table supplement 1:** List of *Toxoplasma gondii* genes related to redox sensing systems and their
819 primary location based on hyperLOPIT datasets. Abbreviations: PITH: proteasome-interacting
820 thioredoxin. ER: endoplasmic reticulum. PM: plasma membrane. GSH: glutathione. NA: not
821 applicable (location not predicted).

822 **Supplemental video 1:** Intracellular *T. gondii* tachyzoites mobilize Ca^{2+} upon addition of H_2O_2,
823 followed by egress induced by A23187. The left panel displays the GFP channel, middle panel the
824 Ca^{2+} sensor jCaMP1b channel, and the right panel the channel merge. Time (minutes: seconds) is
825 displayed on the left top region of each channel. 100 μM of H_2O_2 was added to cells at 1:06, and 1
826 μM A23187 was added at 6:51. Video frame rate: 17 frames per second. This video is representative
827 of 27 infected vacuoles from four independent experiments. MOI: 1.
828
829 **Supplemental video 2:** Hydrogen peroxide inducing egress of *T. gondii* tachyzoites at low MOI. The
830 left panel displays the GFP channel, the middle panel the Ca^{2+} sensor jCaMP1b channel, and the right
831 panel the merge. Time (minutes: seconds) is displayed on the left top region of each channel. 100 μM
832 of H_2O_2 was added to cells at 00:45s. Video frame rate: 10 frames per second. This video presents an
833 egress event captured using low MOI (MOI=1).
834
835 **Supplemental video 3:** Hydrogen peroxide inducing egress of *T. gondii* tachyzoites at high MOI. The
836 left panel displays the GFP channel, middle panel the Ca^{2+} sensor jCaMP1b channel, and the right
837 panel the merge. Time (minutes: seconds) is displayed on the left top region of each channel. 100 μM
838 of H_2O_2 was added to cells at 00:30s. Video frame rate: 15 frames per second. This video is
839 representative of 34 infected vacuoles from three independent experiments (MOI=5).

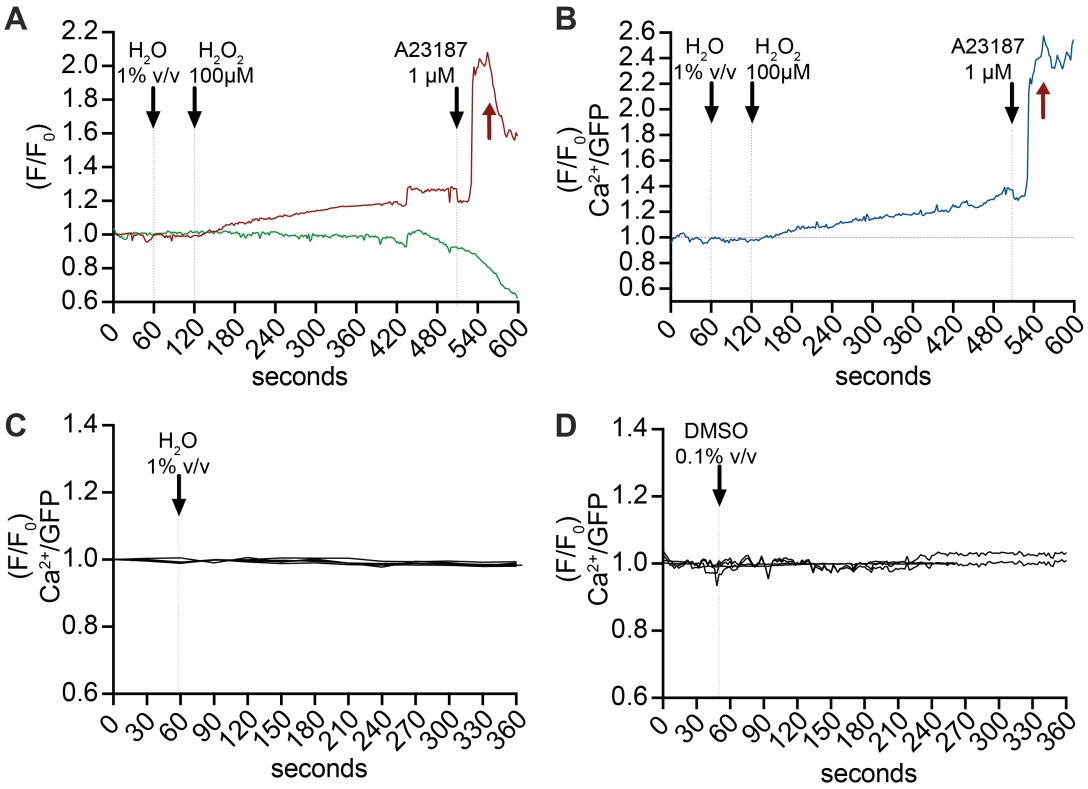


Figure supplement 1: Fluorescence tracking of Ca^{2+} signal and GFP movement on RH-GFP-t2a-jRCaMP1b parasites within host cell. (**A – B**) Representative trace of parasite vacuoles following treatment with H_2O_2 and ionophore A23187. (**A**) The graph presents the independent GFP trace (green) and Ca^{2+} signal from jRCaMP1b sensor (red). Note the parasite movement at 420s. (**B**) Graph of the Ca^{2+} signal is normalized to GFP to minimize artefact on the Ca^{2+} measurements due to parasite movement. Red arrow indicates the moment of parasite egress. (**C**) Water, the vehicle solvent for H_2O_2, does not mobilize Ca^{2+}. The graph displays the trace of three independent vacuoles from the same field of view. Data are representative of 15 infected vacuoles from four independent experiments. (**D**) DMSO, the vehicle solvent for ionophore A23187, does not mobilize Ca^{2+}. The graph displays the trace of four independent infected vacuoles from the same field of view. Data are representative of 12 rosettes from three independent experiments. (**A – D**): black arrows indicate the time of drug / solvent addition.

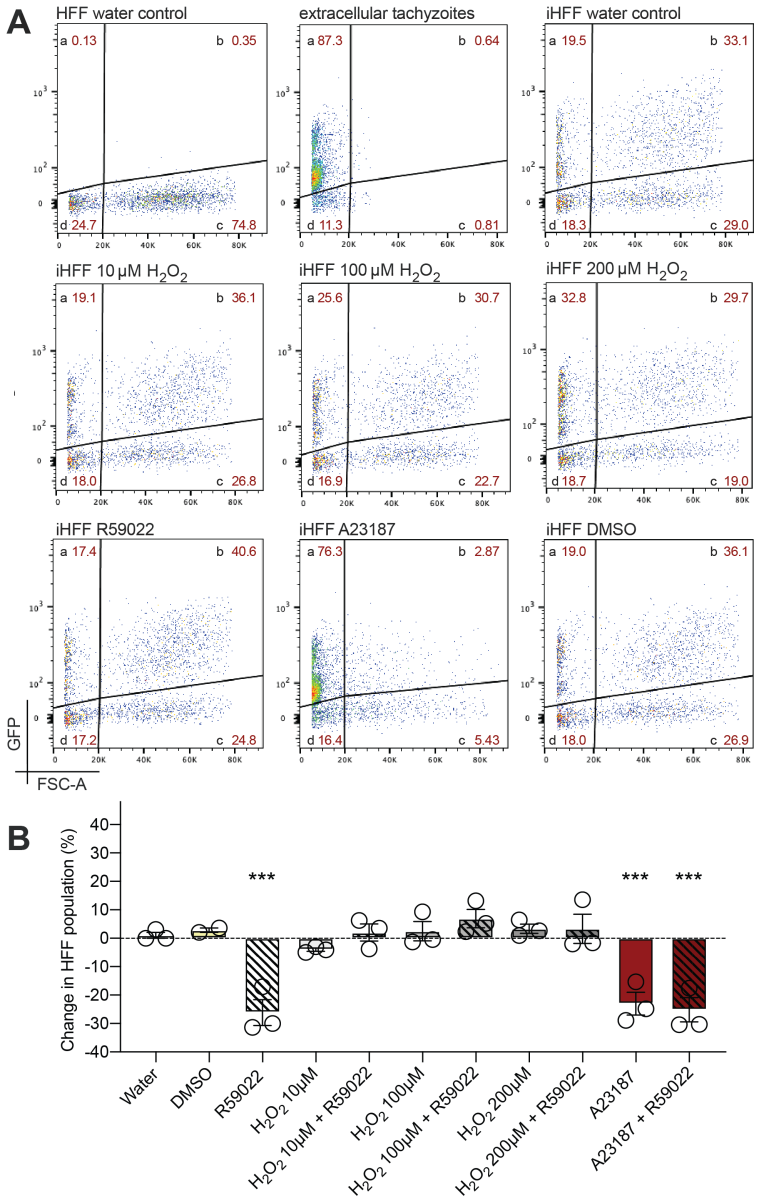
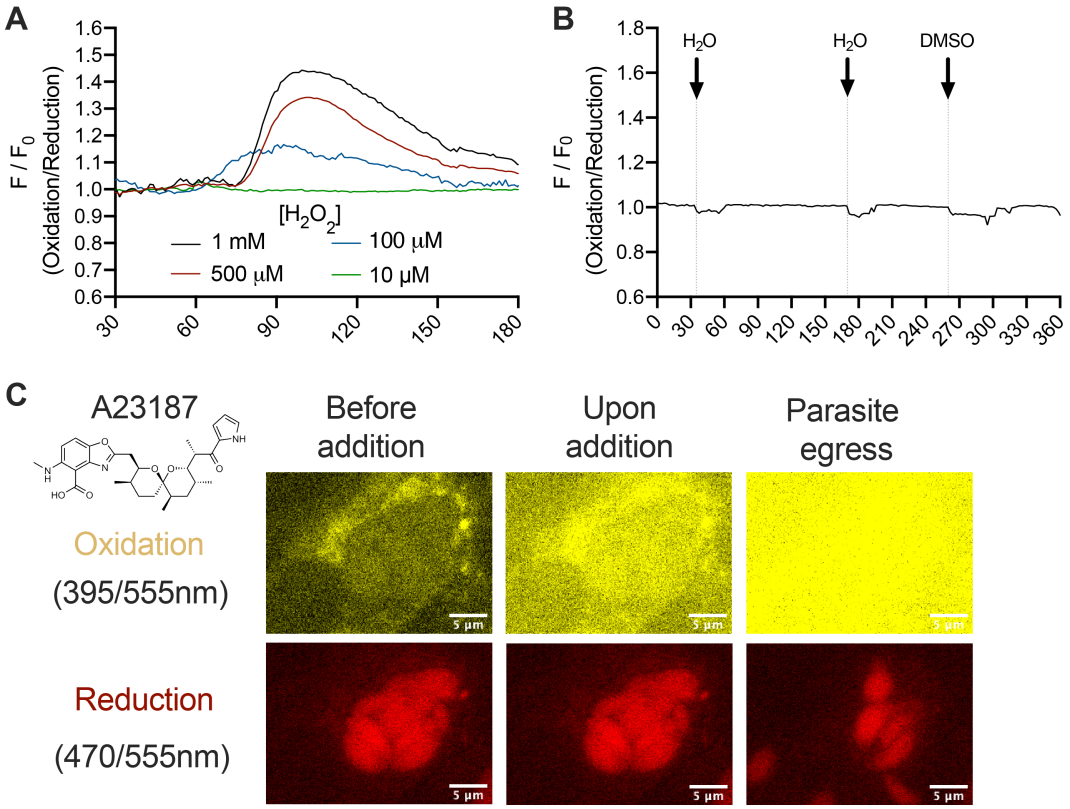


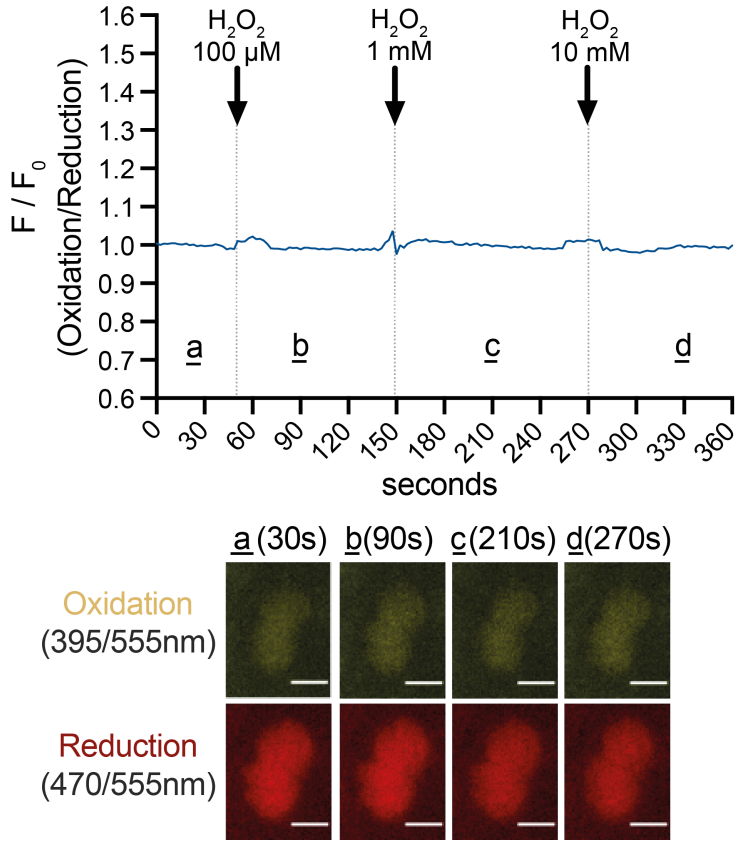
Figure supplement 2: Quantification of RH-GFP-T2A-jCamP1b parasite egress by flow cytometry. (**A**) Representative gating using GFP (Blue laser, 488nm, filter 530/30) against Forward scatter (FSC-A). HFF water control: uninfected human foreskin fibroblast (HFF) are mainly localised in quadrant c. Lysed tachyzoites: free RH-GFP-T2A-jCamP1b parasites are mainly localised in quadrant a. iHFF water control: infected host cells are detected in quadrant b. By using the GFP it is possible to distinguish free fluorescent parasite from iHFF in a population and assess the egress rates throughout different treatments. (**B**) Effect of drug incubation on non-infected HFF. The graph presents a change on event number within the non-fluorescent HFF gate. Data represent the mean ±SEM of three independent experiments, six technical replicates on each. Significance was calculated using one-way Anova, Bonferroni's multiple comparisons P value ***< 0.001.



875

876 **Figure supplement 3:** Exploring the redox sensitivity of RH-GRX1-roGFP2 parasites to H_2O_2. (**A**)
877 Tracking the GSH/GSSG change upon different concentration of H_2O_2. Among the concentration
878 tested, 10 µM was the only one that did not induce a change in redox within the parasite cytosol. Data
879 are representative of five infected vacuoles from each concentration, one independent experiment. (**B**)
880 Water (vehicle control for H_2O_2) and DMSO (vehicle control for A23187) do not trigger change in
881 GSH/GSSG. Data are representative of 12 vacuoles from three independent experiments. (**C**)
882 Autofluorescence effect of A23187 drug on oxidation channel makes this ionophore unsuitable for
883 ratiometric analyses of GRX1-roGFP2 sensor. Widefield microscope imaging depicting an egress
884 induce by A23187. The structure of A23187 is shown above the microscopy images.

885



Supplemental Figure 4: Inactivation of the catalytic domain of glutaredoxin 1 makes the GRX1-roGFP2 redox sensor insensitive to changes in GSH/GSSH. Parasite expressing GRX1ser-roGFP2 do not display fluorescent changes in either channel (reduction or oxidation) upon addition of H_2O_2. Data are representative of 12 infected vacuole, three independent experiments. Black arrows indicate the time of H_2O_2 addition.

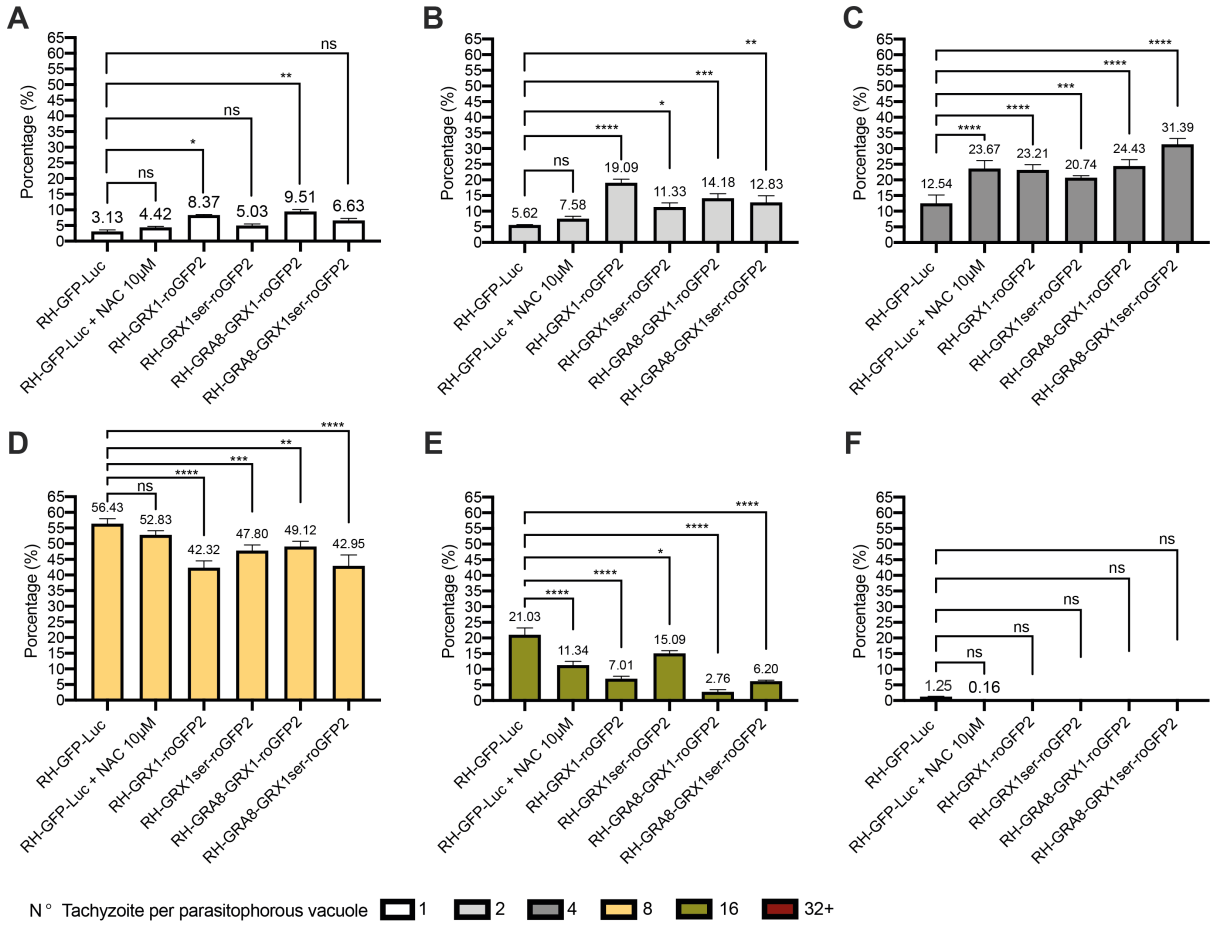


Figure supplement 5: Effect of redox sensors on *T. gondii* parasite growth. Each graph presents parasites/vacuole counts after 20 hours of intracellular growth. The mean values are displayed over each bar for three independent experiments. (**A**) Percentage (%) of vacuoles contain one parasite. (**B**) % of vacuoles with two parasites. (**C**) % of vacuoles with four parasites. (**D**) % of vacuoles with eight parasites. (**E**) % of vacuoles with 16 parasites. (**F**) % of vacuoles with more than 32 parasites. Significance was calculated using two-way Anova, Bonferroni's multiple comparisons test. P values: *< 0.05; **< 0.01, ***< 0.001 and ****< 0.0001.

Primer name	Primer number	Sequence (5'-3')
Fw-GRX1-roGFP2	1	ctcgttggcatttttcttgatggctcaagagtttgtgaac
Rv-GRX1-roGFP2	2	agtgagcacaacggtgattattacttgtacagctcgtccatg
Fw-GRA8-GRX1-roGFP2	3	ctcgttggcatttttcttgatggctttaccattgcgtgttt cggccacggtgttcgtggtcttcgctgtctttggtgtagct cgcgccatggggaaggtggttgtgttc
Fw-GRX1ser23-26	4	ccagcccaccagcccgtacagcag
Rv-GRX1ser23-26	5	atgaacacaaccaccttcccagg
Rv-GRA8-GRX1ser23-26	6	atgaacacaaccaccttccccat
Fw-pTUB8 sequence	7	cagaagacatccaccaaacg
Rv-pTUB8 sequence	8	gctgcgaaacactcctatttag
Fw-jRCaMP1b	9	actttcgtcgtagtcttaatctacttcgctgtcatcatttgtac
Rv-jRCaMP1b	10	tccaggaccaatgctgcagaacgagcttg
Fw-5'UTR of GRA1	11	tgctcaccatcttgcttgatttcttcaaag
Rv-5'UTR of GRA1	12	ccggactacgcgtagttaattcgaaggctgtagtactg
Fw-GFP-T2A-fusion	13	tctgcagcattggtcctggattttcttctacatc
Rv-GFP-T2A-fusion	14	atcaagcaagatggtgagcaagggcgag
Fw-HXGPRT cassette	15	caagaatccccactccaagcatgcaggcgcagctggagaatggcca gaggcaggcatc
Rv-HXGPRT cassette	16	caaccggctgcatgcacgacgaagacatgtgcacctaaagccgcggaa gatccgatcttg
Rv-pSAg1::CAS9-U6::sg1/2 CDPK3	17	aacttgacatccccatttac
Fw-pSag1::Cas9-U6::sg1 CDPK3	18	gacgccagcctcgaaaaggggttttagagctagaaatagc
Fw-pSag1::Cas9-U6::sg2 CDPK3	19	tcagtccgtagttgggacaggttttagagctagaaatagc
Fw-sg1CDPK3	20	gtaatacgactcactatagggcgaattgggtacccaagtaagc agaagcacgct
Rv-sg1CDPK3	21	gtaaaagcttatcgataccgtcgacctcgagaattaaccctc actaaagg
Fw-5'HXGPRT cassette at CDPK3 locus	22	ggagtcacgcctgagtttga
Rv-5'HXGPRT cassette at CDPK3 locus	23	ggcctacgtgacttgctgat
Fw-3'HXGPRT cassette at CDPK3 locus	24	cttcaatgggtttggacgcc
Rv-3'HXGPRT cassette at CDPK3 locus	25	gaacaaagggggtcggtcat
Fw-CDPK3 locus absent check	26	gcgcgttctcaggatgttcgt
Rv-CDPK3 locus absent check	27	cagtgtatctgcaacaaccaga

913

914 **Table supplement 2:** List of primers used to generate plasmid and check sequence / integration in
915 this study.

916

917

918

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