

Changes in the Expression of Human Cell Division Autoantigen-1 Influence *Toxoplasma gondii* Growth and Development

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***Toxoplasma* is a significant opportunistic pathogen in AIDS, and bradyzoite differentiation is the critical step in the pathogenesis of chronic infection. Bradyzoite development has an apparent tropism for cells and tissues of the central nervous system, suggesting the need for a specific molecular environment in the host cell, but it is unknown whether this environment is parasite directed or the result of molecular features specific to the host cell itself. We have determined that a trisubstituted pyrrole acts directly on human and murine host cells to slow tachyzoite replication and induce bradyzoite-specific gene expression in type II and III strain parasites but not type I strains. New mRNA synthesis in the host cell was required and indicates that novel host transcripts encode signals that were able to induce parasite development. We have applied multivariate microarray analyses to identify and correlate host gene expression with specific parasite phenotypes. Human cell division autoantigen-1 (CDA1) was identified in this analysis, and small interfering RNA knockdown of this gene demonstrated that CDA1 expression causes the inhibition of parasite replication that leads subsequently to the induction of bradyzoite differentiation. Overexpression of CDA1 alone was able to slow parasite growth and induce the expression of bradyzoite-specific proteins, and thus these results demonstrate that changes in host cell transcription can directly influence the molecular environment to enable bradyzoite development. Investigation of host biochemical pathways with respect to variation in strain type response will help provide an understanding of the link(s) between the molecular environment in the host cell and parasite development.**

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Introduction

Toxoplasma gondii infects a multitude of warm-blooded hosts where tachyzoite and bradyzoite life stages form in various tissues [1]. Bradyzoite development is the critical step in the pathogenesis of chronic *Toxoplasma* infection [2–4], yet the molecular details that govern this process are not clearly delineated. Bradyzoite development in human subjects has an apparent tropism for cells and tissues of the central nervous system, where clinical symptoms of toxoplasmosis are primarily manifest. Yet in a cross section of animal models, bradyzoite development differs radically, such that tissue cysts can readily be found in porcine and ovine muscle but rarely, if at all, in bovine and equine species [5–7]. The apparent molecular discrimination of host and tissue wherein parasite development will succeed is often underappreciated for *Toxoplasma* but is an accepted principle of host–parasite interactions in most apicomplexa species.

The molecular details that distinguish a host cell environment that influences *Toxoplasma* development from one that does not are unclear. The issue of parasite growth is interlinked with this question through the slowing of the parasite cell cycle that precedes the initiation of the *T. gondii* bradyzoite program [2,8,9]. The pathway of bradyzoite development initiated by either sporozoite or bradyzoite leading to the mature tissue cyst follows a progressive series of events marked by changes in the parasite cell cycle that ultimately lead to a growth-arrested parasite [2,9]. End-stage

bradyzoites enter a state of dormancy that may be equivalent to the classic G₀ phase with indeterminate life span, and as such, these parasites likely require an equally long-lived host. For these reasons, it is not surprising that tissue cysts are observed in cells of the brain [10] and mature muscle cells of susceptible hosts [6,11]. Whether this unique relationship, which appears advantageous to *T. gondii* transmission, is fortuitous or the result of evolutionary design is an important question. It is now recognized that the state of host cell life or death is altered by specific host–parasite interactions observed across several apicomplexa models [12–15], and this suggests that parasite survival and development require a specific molecular environment in the host cell. It remains to be determined whether this environment is parasite directed

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Abbreviations: BAG1, bradyzoite-specific antigen 1; CDA.1/SE20–04 and CDA1, cell division autoantigen 1; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; HFF, human foreskin fibroblast; LDH2, lactate dehydrogenase 2; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; PKG, cGMP-dependent protein kinase; PMA, phorbol esters; RNAi, RNA interference; siRNA, small interfering RNA

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Synopsis

Toxoplasma is a common opportunistic pathogen among immunocompromised populations that include subjects undergoing organ transplant, the fetus during early gestation, and persons with AIDS. The parasite escapes the host immune system by forming a dormant tissue cyst, and this chronic infection, as well as the clinical manifestation of disease, is observed primarily in cells and tissues of the brain and eye. Although it is not yet understood how the disease state is established, in this study researchers demonstrate that *Toxoplasma* can take cues from specific changes in host cell gene expression to initiate switching to the tissue cyst, and they discover that a single gene, designated human cell division autoantigen-1 (CDA1), is able to impose significant influence on the course of infection and cyst development. These studies are the first to identify a host gene that links the molecular environment in the cell to parasite development. It is interesting that the response to the host cell is not uniform among parasite strains, as acutely virulent strains appear to ignore the host and continue to proliferate until the cell is destroyed.

or the result of molecular features inherent to those cell types in which parasite development is most prevalent.

The development of methods to measure whole cell gene expression has opened the potential to understand the wider implications of host–parasite interactions. Genomic approaches to evaluate animal cell gene expression during *Toxoplasma* infection indicate a diverse cross section of biochemical pathways whose importance to host–parasite interaction is not well understood [16,17]. In these analyses, it is difficult to cull the important from the merely incidental, and thus, measurements of the changes in host cell gene expression without correlation to a series of definable parasite phenotypes or characteristics are limited by the noise in the experiments. In this report, we demonstrate that a trisubstituted pyrrole [18,19] can influence *Toxoplasma* development through alterations in host cell gene expression. We have resolved the set of potential host genes that are responsible for this interaction through the application of a multivariate analyses correlating defined parasite phenotypes with changes in host cell transcription and have demonstrated the importance of a single host gene in development.

Results/Discussion

A Trisubstituted Pyrrole Inhibits Parasite Growth and Induces Bradyzoite Gene Expression

Small molecule screens have identified a trisubstituted pyrrole, designated Compound 1, that dramatically slows the replication of *T. gondii* [20] and is a potent inducer of the bradyzoite-specific antigens bradyzoite-specific antigen 1 (BAG1) and cyst wall protein (Figure 1). A short treatment with Compound 1 was as effective as continuous exposure to induce BAG1 expression. Similar results were obtained when host cells were pretreated with the compound or when cells were treated after parasite infection (1- to 6-h treatments, Figure 1A), suggesting Compound 1 may act on the host cell to induce expression of bradyzoite-specific antigens. It is significant that early tissue cysts induced by Compound 1 in type III strain parasites were morphologically indistinguishable from those induced by growth in alkaline media (Figure 1B) [21,22]. This observation is further consistent with the kinetics of

induced cyst wall and BAG1 proteins when comparing induction with Compound 1 and growth in alkaline pH (Figure S1A and S1B). Compound 1, like alkaline pH, acts to change transcription of bradyzoite genes, indicating the effects likely occur via the natural bradyzoite pathway (induction of BAG1 and lactate dehydrogenase 2 [LDH2] mRNAs is shown in Figure S1C). Similar results with respect to growth inhibition and BAG1 expression in human foreskin fibroblasts (HFF cells) were observed in both infected HeLa cells and primary murine astrocytes (unpublished data), and when using the type II strains (Figure 1D [see ME49-B7] and Figure S1B [see Pr=Prugniaud]). To verify the effects of Compound 1 on the host cell, we treated both intracellular and extracellular VEG tachyzoites with 3 μ M Compound 1 for 3 h and then moved parasites to new monolayers (Figure 1C). In neither case do parasites express BAG1 at levels beyond untreated controls (less than 5%), demonstrating that BAG1 induction requires a Compound 1-treated host cell environment. A dose effect was evident at 0.5, 1, and 2 μ M Compound 1, with doses greater than 3 μ M inducing maximal BAG1 or cyst wall protein expression (unpublished data). In addition, Compound 1 before and after treatment slows and then arrests VEG parasite replication within 12 h postinfection at concentrations as low as 1 μ M, where the average number of parasites per vacuole at 72 h of postinfection is approximately four (1 μ M or greater) compared with approximately 70 for untreated controls (40-h growth shown in Figure 1D). Compound 1 alteration to the host cell was reversible over time, and independent monolayers pretreated for 3 h with 3 μ M Compound 1 induced only 21% BAG1 when parasites invaded cells at 6 h after drug removal and 5% or less BAG1 expression was observed at extended times of recovery (12 or 24 h).

Compound 1 was less able to slow parasite replication in the type I strains RH and GT-1 (from more than 60 parasites per vacuoles to less than ten in Type III strains, Figure 1D). It was further evident that type I strains express much less cyst wall and BAG1 protein in response to treatment with Compound 1 or culture in alkaline pH (Figure S1A and S1B), and it is difficult to detect the induction of BAG1 or bradyzoite-specific LDH2 mRNAs in type I strains (Figure S1C). These results demonstrate dramatic differences when comparing strain types in cells treated with Compound 1 and alkaline pH. These observations are in full agreement with direct and indirect reports of type I strain differences in bradyzoite development [21,23–32]. Thus, the relative resistance of the RH strain to form cysts is commonly known [33], and while GT-1 is capable of completing the life cycle, the original characterization of this strain showed that cysts were not apparent in mice before death, as readily observed in mice infected with the type II M-7741 strain [23]. As such, the relative resistance to the Compound 1 induction of bradyzoite development is consistent with previously known characteristics of type I strains.

Altogether, these findings demonstrate that the effects of Compound 1 are limited to strain types most competent to complete the parasite life cycle and raise questions about the Compound 1 mechanism controlling parasite proliferation. A ligand binding assay using tritiated Compound 1 initially identified cGMP-dependent protein kinase (PKG) as a target in type I RH parasites [18], and transgenic RH parasites expressing mutant PKG were resistant to Compound 1 control as reflected by increased virulence in mice and

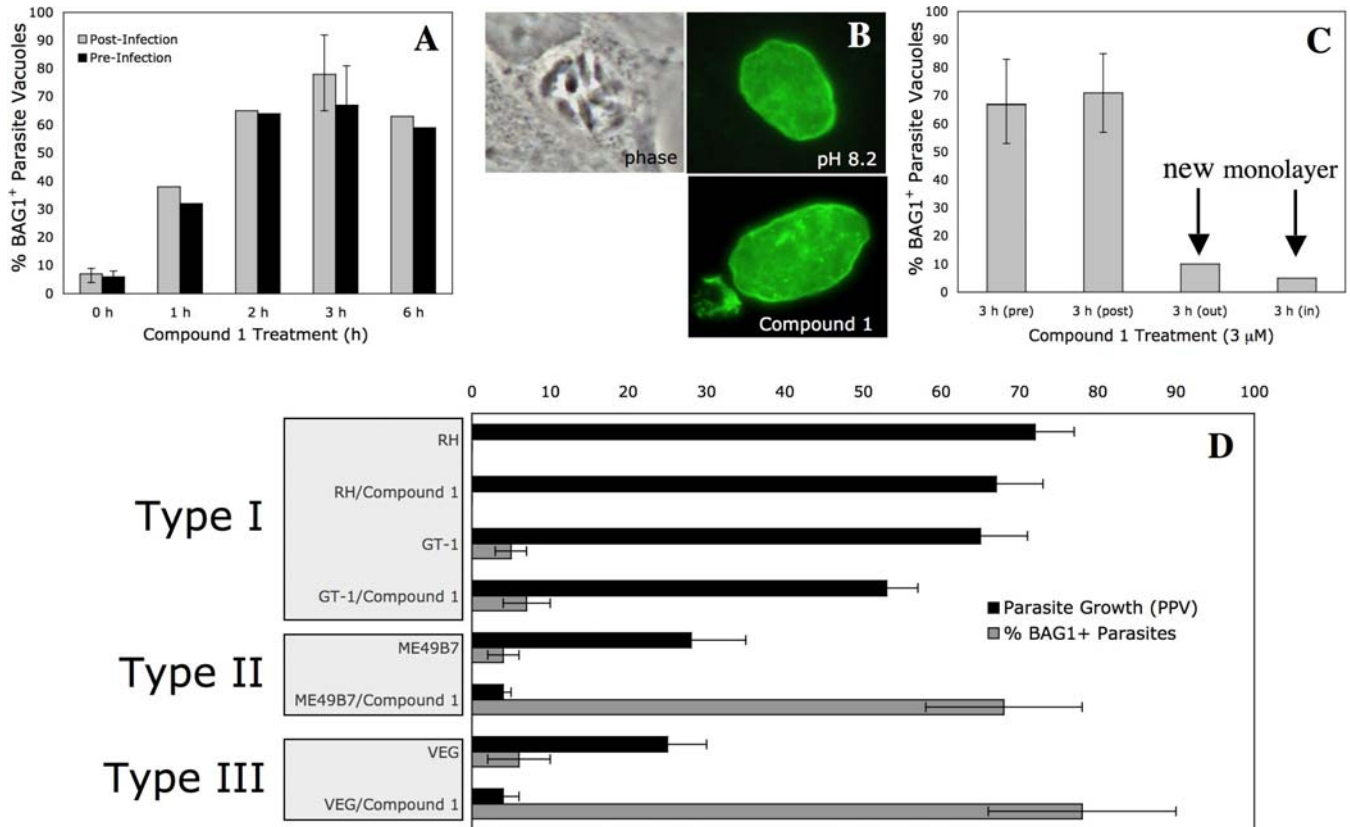


Figure 1. Compound 1–Induced Parasite Growth Inhibition and BAG1 Expression

(A) Parasites allowed to invade an HFF monolayer, and then treated 1, 2, 3, or 6 h with 3 μ M Compound 1, express maximal levels of BAG1 after 3-h treatment (light bars). Similar BAG1 expression was observed with host monolayers that were pretreated with Compound 1 (dark bars).

(B) Phase image of pH-induced vacuole is shown at 72 h postinfection. Following immunostaining with dolichos lectin, note the phenotypic similarities between cyst wall formation in alkaline media and that induced by 3 μ M Compound 1.

(C) Treatment of extracellular (out) or intracellular parasites (in) with 3 μ M Compound 1 for 3 h was unable to induce BAG1 expression following infection of untreated HFF monolayers (72 h postinfection). Compare percent BAG1 induction with VEG parasites that remained in a pre- or post-treated culture for 72 h.

(D) Type I RH and GT-1 tachyzoites inoculated into Compound 1 (3 μ M for 3 h)–pretreated HFF cells displayed little or no BAG1 expression (light bars), while in type III VEG or type II ME49B7 cultures, 68% to 75% of parasites express BAG1. Note that parasite growth is dramatically affected in VEG and ME49B7 cultures but not in RH or GT-1 parasites (black bars). The x-axis in this graph is used to plot both percent BAG1⁺ and average vacuole size. DOI: 10.1371/journal.ppat.0020105.g001

parasite mass in cell culture [20,34]. Recently, however, it has been shown that Compound 1 directly inhibits extracellular gliding motility and the invasion of RH parasites into HFF cells but not in transgenic RH parasites expressing a drug-resistant PKG allele [35]—suggesting that the alteration of parasite mobility and invasion by Compound 1 is the major action of this drug within the parasite. RH parasites carrying a wild-type PKG allele are already resistant to the inductive effects of Compound 1; thus, the published effects on type I RH parasites do not adequately explain the observed inhibition of parasite replication or induction of bradyzoite development in type II and type III strains. As such, type I strains appear to be naturally resistant to the Compound 1–induced host cell environment.

Compound 1 Treatment of HFF Cells Induces New Gene Transcription

The observed inhibition of parasite replication and the induction of bradyzoite development pathways after infection of Compound 1–conditioned cells suggest the host cell environment contains a transient signal that plays a critical role in regulating the balance between parasite proliferation

and survival within the tissue cyst. To test if that signal may result from the induction of host cell gene expression, we blocked HFF cell transcription during Compound 1 pretreatment (prior to parasite infection) with the RNA polymerase II inhibitor 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) [36]. Following a 3-h pretreatment of HFF cells with 3 μ M Compound 1 and 30 or 60 μ M DRB, parasites were allowed to invade treated cells and BAG1 expression was evaluated 72 h later (Figure 2A). Results demonstrate a 50% inhibition of Compound 1–induced BAG1 expression at 30 μ M DRB and greater than 80% inhibition using 60 μ M DRB. DRB treatment alone had no effect on parasite BAG1 expression, and thus, co-treatment with DRB (and Compound 1) largely reversed the observed BAG1 expression induced by Compound 1 alone. Importantly, the reduction of BAG1 expression caused by DRB was also accompanied by a restoration of parasite growth, such that parasites per vacuole reached approximately 54 in 60 μ M DRB/Compound 1 cultures compared with four or fewer in Compound 1 alone (Figure 2B). These results confirm the inverse relationship between cell cycle progression and bradyzoite gene expression [2,8,9,37] and also demonstrate that transcriptional

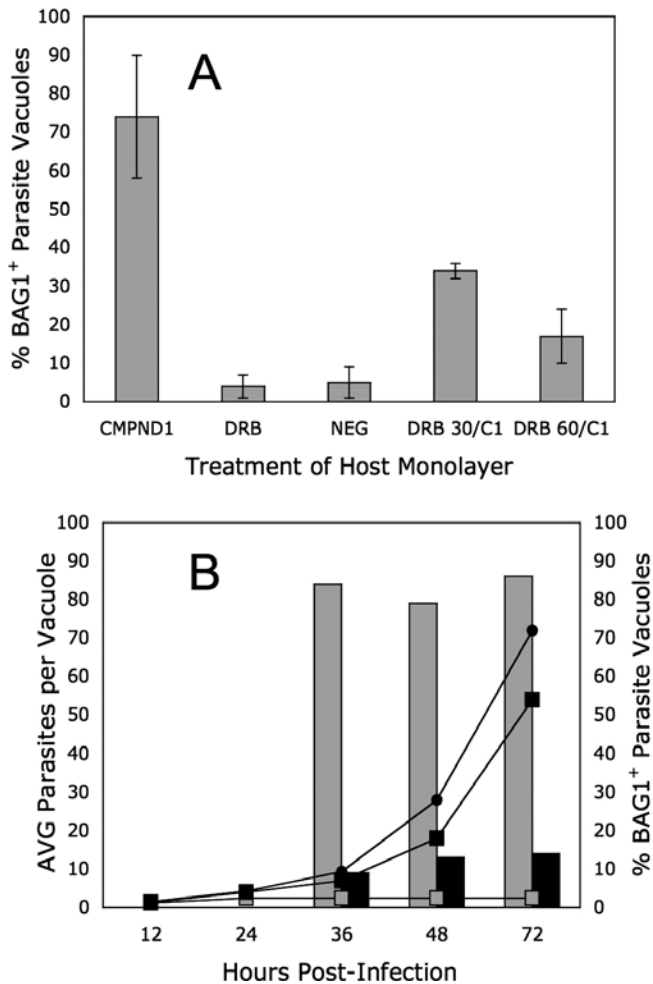


Figure 2. Compound 1–Induced Slowing of Parasite Growth and BAG1 Expression Is Mediated by New Host Cell Gene Transcription

(A) HFF cells were treated or co-treated for 3 h with 3 μ M Compound 1 and 30 or 60 μ M RNA polymerase II inhibitor DRB. Following removal of the compounds, VEG parasites were inoculated and BAG1 expression was evaluated 72 h postinfection. Antagonism of Compound 1 induction of parasite BAG1 with DRB shows a dose effect when comparing co-treatment with 30 or 60 μ M DRB and 3 μ M Compound 1 (DRB 30/C1 and DRB 60/C1, respectively). DRB alone had no effect on parasite growth or BAG1 expression, while induction by 3 μ M Compound 1 (CMPND1) is included here for reference.

(B) In untreated cultures, VEG parasites reach greater than 70 per vacuole by 72 h postinfection (black circle), but vacuoles with an average of only four parasites were observed in Compound 1–treated cells (grey box). Co-treatment with 60 μ M DRB restored parasite replication to approximately 60 parasites per vacuole (black box) by 72 h postinfection, while reducing the level of BAG1+ to 12% (dark bars) from greater than 70% in cultures treated with Compound 1 alone (light bars).

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products in the host cell environment can directly influence the induction of bradyzoite development.

Compound 1 is a trisubstituted pyrrole p38 α mitogen-activated protein kinase (MAPK) inhibitor (IC_{50} = 20 nM) [38] with similar structure to other known MAPK inhibitors [39]. In a comparison of potency to inhibit parasite growth and induce BAG1 or cyst wall protein expression in VEG strain parasites, we evaluated several commercially available reference p38 MAPK inhibitors. A trisubstituted pyrazole, designated 505126 (IC_{50} = 35 nM; EMD Biosciences, San Diego, California, United States) and a trisubstituted imida-

zole, designated PD169316 (IC_{50} = 89 nM), were unable to induce bradyzoite development at concentrations as high as 10 μ M. A trisubstituted imidazole, designated SB203580 (IC_{50} = 34 nM), showed only partial induction (approximately 40% parasite BAG1 expression by 72 h postinfection), but an alternate trisubstituted imidazole, designated SB202190 (IC_{50} = 16 nM), was able to slow VEG strain parasite growth and induce BAG1 expression similar to that observed for Compound 1 and at concentrations as low as 30 nM (greater than 80% BAG1⁺ vacuoles and fewer than four parasites per vacuole at 72 h postinfection). Specific parasite molecules that mediate this response are presently unknown, but similar to their resistance to Compound 1, the type I strains RH and GT-1 were also resistant to pretreatment or posttreatment with these compounds and do not slow growth or express bradyzoite antigens in response to the treatment(s) used in these experiments. Co-treatment of host cells with both Compound 1 and SB202190 at these concentrations was found to abrogate the inductive effects of either and allowed VEG parasites to grow normally and without induction of BAG1 or cyst wall protein (less than 10% BAG1⁺ vacuoles and an average of 60 parasites per vacuole at 72 h postinfection). Collectively, these observations suggest that the Compound 1 mechanism to induce parasite development is distinct from that of SB202190. Given the relationship of these compounds to the MAPK pathway, we assessed the ability of lipopolysaccharide (LPS), phorbol esters (PMA)/ionomycin, anisomycin, and parasite infection to cause the activation of p38 MAPK by inducing phosphorylation in HFF cells. It was evident that of these strategies for activation, only anisomycin was able to significantly induce p38 phosphorylation in this cell type (Figure S2A). Moreover, combinations of parasite infection and LPS treatment were unable to induce p38 phosphorylation above levels observed in the untreated control (Figure S2A). We further tested the ability of SB202190, 506126, and Compound 1 to inhibit the activation of p38 and its ability to phosphorylate the known downstream targets ATF-2, MAPKAP2, and Elk-1 (Figure S2B). Results suggest that anisomycin is able to induce significant phosphorylation of p38, relative to the untreated control (Figure S2B, compare C with A). Compound 1 was able to prevent some p38 phosphorylation but had no effect on the ability of activated p38 to phosphorylate the downstream target ATF-2, based on comparison with cells treated with SB202190 and 506126. It is interesting that phosphorylation of ATF-2 appears to be enhanced by treatment with 30 nM SB202190 and 506126 when compared with cells treated with anisomycin alone and untreated controls. Finally, anisomycin-activated p38 was unable to phosphorylate either MAPKAP-2 or Elk-1 in these cells. Thus, while we cannot yet exclude a potential role for p38 (and its inhibition) in the observed effects of Compound 1 and other MAPK inhibitors in this cell type, these results suggest that other biochemical pathways play a significant role.

Gene Expression Analyses Identify Host Cell mRNAs Whose Expression Correlates with Compound 1 Induction of Bradyzoite Development

To identify the host cell mRNAs affecting the observed induction of parasite BAG1 or cyst wall protein, we used a multivariate series of DNA microarray hybridizations (defined in Table 1) on RNA samples obtained from treated HFF

cells (prior to infection) and we correlated changes in the levels of host cell mRNAs with distinct developmental outcomes that were obtained by subsequent parasite infections (the full design of these experiments and list of the final genes along with normalized levels for each condition is published in Table S1). By identifying those host mRNAs whose levels were altered in predictable ways with respect to the treatment regimens to induce parasite BAG1 expression, we were able to reduce the relevant number of potential host cell mRNAs. In Table 1, the range of host mRNAs from successive queries of the database drops from 37,000 possible genes on the array to 79 when the analyses combine Compound 1 induction with recovery for 6 or 12 h prior to infection, with the noninductive effects of DRB and 506126, and with the antagonism of Compound 1 by DRB and SB202190. The class of host cell mRNAs obtained by this analysis suggests Compound 1 has an effect on the growth state of the cell, as many of the mRNAs in the final list can be directly or indirectly associated with growth regulatory pathways of animal cells. The best-fit match (high induction and correlation) from this class of growth regulators was cell division autoantigen-1 (CDA1, also known as SE20-04). Hybridization signals for CDA1 were up 47-fold in Compound 1-pretreated host cells but were nearly unchanged in cells where the effects of Compound 1 induction were antagonized by DRB and SB202190. CDA1 is a nucleosome assembly-related protein demonstrated to be involved in cell cycle arrest and negative control of cell proliferation [40]. Overexpression of CDA1 in HeLa cells, similar to elevated levels in the Compound 1-treated human fibroblasts reported here, has been shown to arrest cell growth [40]. Interestingly, CDA1 protein can be phosphorylated in vitro by cyclin D1/CDK4, cyclin A/CDK2, and cyclin B/CDK1, and mutation of the CDK phosphorylation sites abrogates the negative growth effects of this protein [40]. In this context, CDA1 was selected for initial studies to begin exploration of host cell growth regulation and the related molecular environment potentially linked to parasite development.

Knockdown of Host Cell CDA1 Reverses the Compound 1 Inhibition of Parasite Replication and Induction of Parasite BAG1

To test the relevance of CDA1 to the observed Compound 1-induced growth inhibition and BAG1 expression in the parasite, we used a small interfering RNA (siRNA) SmartPool (Dharmacon, Lafayette, Colorado, United States) consisting of four distinct siRNAs against various regions of the CDA1

coding sequence, to knock down mRNA levels in Compound 1-pretreated HFF cells. VEG parasites were then introduced into this environment, and parasite vacuole size and the level of BAG1 expression were evaluated at 48 h postinfection. It was evident that the inhibition of parasite growth that occurs under Compound 1 was dramatically reversed by siRNA knockdown of CDA1 since parasite replication in these host cells was nearly equivalent to untreated controls (Figure 3A). Similarly, the knockdown of CDA1 mRNA reduced BAG1 expression to 16% of vacuoles from greater than 60% under Compound 1 alone (Figure 3A). This result was specific to CDA1 siRNA, as siRNA knockdown of Lamin A/C in HFF cells (Figure 3B) was unable to reverse Compound 1 inhibition of parasite growth or the elevation of BAG1 expression (unpublished data). Knockdown of CDA1 mRNA beginning at 24 h post-treatment (with siRNA and Compound 1) and continuing to 72 h is demonstrated by RT-PCR (Figure 3B). Transfection of HFF cells with LipofectAMINE or CDA1 siRNA alone was not found to influence parasite growth or induce BAG1 expression (unpublished data).

We tested each individual siRNA in the CDA1 Smartpool separately to corroborate the initial collective observations (Figure 3C) and determined that the siRNAs designated 1 and 4 did not reverse the growth inhibition of 3-h Compound 1 pretreatment. In contrast, siRNAs 2 and 3 alone were each able to abrogate the growth inhibitory effects of the Compound 1 treatment and allow VEG parasites to reach vacuole sizes that were 72% and 78% of that attained by the complete Smartpool (81% of untreated controls, Figure 3C). Antagonism of the Compound 1-induced growth inhibition with siRNAs 2 and 3 was dose dependent as the effectiveness of each siRNA to antagonize the Compound 1 effect was diminished at 50 and 25 nM concentrations (unpublished data). Altogether, these results demonstrate that knockdown of host cell CDA1 mRNA was able to reverse the potent effects of Compound 1 on parasite replication and bradyzoite gene expression, indicating that a single host gene (and the host pathway involved) is a critical component of the molecular environment in the host that is able to trigger parasite development. The role of host CDA1 in other methods of bradyzoite induction is unknown due to differences in cell type response and the relative slow kinetics of bradyzoite induction. Parasite development in HFF cells cultured in alkaline pH is only effective when maintained for long periods; thus, the single transfection of CDA1 siRNAs was much less effective when parasites were continually exposed to either Compound 1 or alkaline media (pH 8.2).

Table 1. Multivariate Microarray Analyses Were Used to Correlate Changes in the Levels of Host Cell mRNAs with Distinct Parasite Developmental Outcomes to Identify mRNAs Most Relevant to the Compound 1 Induction of Bradyzoite Development

Pretreatment of Host Cells	Search	Search Combination	BAG1 ⁺ Express	Number of mRNA	Δ 3-fold	Relative CDA1 mRNA
Compound 1, 3 μ M, 3 h pre, 0 h recovery	(1)	1 only	>80%	289		+47
Compound 1, 3 μ M, 3 h pre, 6 h recovery	(2)	1, 2	<50%	253		+2
Compound 1, 3 μ M, 3 h pre, 12 h recovery	(3)	1, 2, 3	<25%	213		0
DRB, control, 60 μ M, 3 h pre	(4)	1, 2, 3, 4	<10%	103		+1.2
506126, control, 30 nM, 3 h pre	(5)	1, 2, 3, 4, 5	<10%	90		0
202190 30 nM + Compound 1, 3 μ M, 3 h pre	(6)	1, 2, 3, 4, 5, 6	<10%	82		+1.5
DRB, 60 μ M + Compound 1, 3 μ M, 3 h pre	(7)	1, 2, 3, 4, 5, 6, 7	<10%	79		+1.1

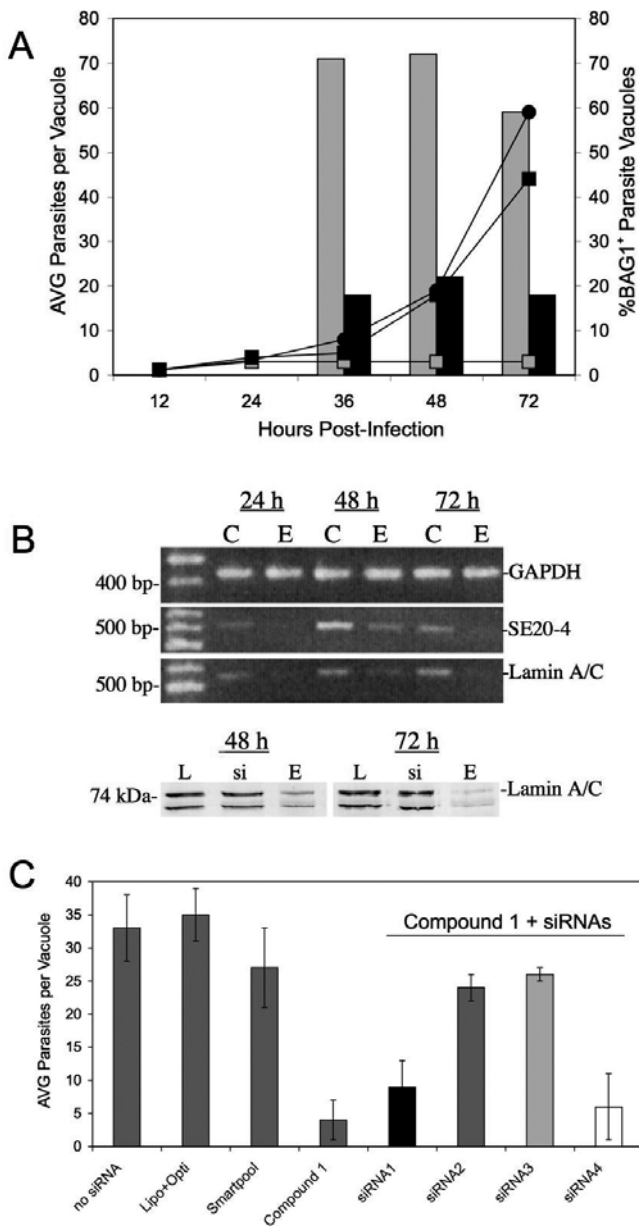


Figure 3. siRNAs against CDA1 Antagonize Compound 1 Inhibition of Parasite Growth

(A) HFF cells transfected with the siRNA SmartPool against CDA1 and co-treated with Compound 1 prior to parasite infection allow normal parasite growth (black boxes) and BAG1 expression (dark bars). Compare growth in untreated controls (black circles) with growth (grey boxes) and BAG1 expression (light bars) in Compound 1-treated cells.

(B) Concurrent with the restoration of normal parasite growth and BAG1 expression, we observed the loss of Compound 1-induced CDA1 mRNAs as measured by RT-PCR of total RNA at 24, 48 following transfection with siRNA and Compound 1 pretreatment. Lamin A/C controls demonstrate siRNA-mediated knockdown of individual genes. Transfection with siRNAs against Lamin A/C, with nonspecific siRNAs or with LipofectAMINE alone, was unable to antagonize Compound 1 inhibition of parasite growth or induction of BAG1 expression (results not shown). RT-PCR primers specific to GAPDH were used as a control for RNA quality. RT-PCR experiment: C = Compound 1 pretreatment (3 h/3 μ M) only; E = HFF cells pretreated with both Compound 1 and siRNA(s) against either CDA1 or Lamin A/C. Protein blot: L = HFF cells pretreated with LipofectAMINE, but without siRNAs; si = HFF cells pretreated with siRNAs against either CDA1 or Lamin A/C, but without transfection reagent (LipofectAMINE); E = same as above.

(C) Co-transfection of HFF cells with siRNAs 1 to 4 separately and then treatment with Compound 1 prior to parasite infection demonstrate that

siRNAs 2 and 3 were responsible for antagonizing the Compound 1-induced inhibition of parasite growth as measured by average parasites per vacuole at 48 h postinfection. Compare light bars for siRNAs 2 and 3 with untreated parasites (labeled no siRNA), and also with Compound 1-treated parasites (labeled Compound 1). siRNAs 1 and 4 do not significantly antagonize the Compound 1-induced inhibition of parasite replication.

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Interferon γ (100 U/ml) was unable to significantly inhibit parasite growth in HFF cultures treated prior to parasite infection or with continuous exposure after infection (unpublished data), a finding consistent with reported resistance of HFF cells to this cytokine [21,41].

CDA1 Inhibits Parasite Replication and Induces Expression and Assembly of Cyst Wall Protein

To determine if CDA1 alone was able to initiate early bradyzoite development in type I and type III strain parasites, we expressed CDA1 ectopically in HeLa cells and assessed parasite growth and induced expression of cyst wall protein at 12-h intervals to 48 h postinfection (Figure 4A). It was evident that induced CDA1 was able to inhibit type III VEG strain parasite replication and reduce average parasites per vacuole beginning at 36 h postinfection, and by 48 h, parasites in CDA1-transformed HeLa cells, which had not been induced with tetracycline, had undergone nearly five division cycles relative to only three complete replication cycles in cells where CDA1 was induced by tetracycline (compare 22 parasites per vacuole with seven at 48 h postinfection, Figure 4A). Concurrently, 47% and 50% of all parasite vacuoles in CDA1-transformed cultures that were maintained in tetracycline expressed and assembled bradyzoite cyst wall protein (Figure 4A; 36 and 48 h postinfection, respectively). This was close to the maximum levels of cyst wall expression we observed for Compound 1 induction in these cells (ranges from 64% to 88%). The number of cyst wall positive vacuoles in HeLa cells transformed with the CDA1 plasmid but not induced with tetracycline was slightly higher than the spontaneous levels we observed in non-transformed HeLa cells (8% spontaneous) or in HeLa cells transformed with a tetracycline-regulated *YFP* gene (9% with or without tetracycline), suggesting that there is some leaky expression of CDA1 from this plasmid. No induction of cyst wall expression beyond spontaneous levels was seen in VEG-infected HeLa cells treated with tetracycline alone. Collectively, these results demonstrate that CDA1 alone was able to slow parasite growth and induce expression of a bradyzoite-specific antigen similar to that observed for Compound 1 treatment. In contrast, but as expected, primary type I GT-1 parasites (less than 12 passages) were unresponsive to the overexpression of CDA1 in the host cell and were able to grow normally (Figure 4B)—an observation consistent with the observed growth of type I strains in the Compound 1-altered cell.

Summary

It is widely accepted that treatments to induce bradyzoite development act specifically on the parasite [21,29,42,43], although host cell influences on this process cannot be ruled out and may, in fact, be evident in the clinical pathologies where tropisms for specific tissues of the central nervous system and the eye are prevalent [6,44,45]. The results presented here are consistent with this view as Compound 1

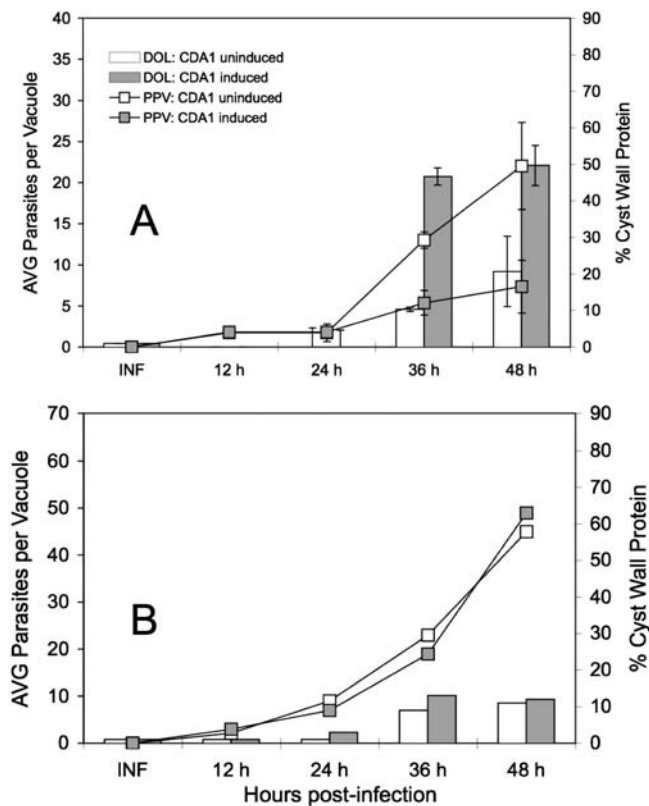


Figure 4. Overexpression of CDA1 in Infected Cells Slows Parasite Growth and Induces Bradyzoite Development

(A) HeLa cells expressing the tetracycline repressor protein transfected with the pcDNA4 TO-CDA1 plasmid inhibit parasite growth and induce cyst wall protein when grown in media containing tetracycline to induce expression of the CDA1 transgene. Compare parasite growth in transfected HeLa cells cultured without tetracycline (growth measured as average parasites per vacuole, PPV, open box with lines) with that of parasite growth in media containing tetracycline to induce CDA1 expression (shaded box with lines). Concurrent with the observed inhibition of parasite replication, cyst wall protein was also expressed. Compare background levels of cyst wall protein (light bars) with levels induced by overexpression of CDA1 (dark bars) at 36 and 48 h. Treatment of HeLa cells with tetracycline alone, transfection of HeLa cells with the pcDNA4 TO expression vector alone, or transfection of HeLa cells with the pcDNA4 TO vector and induced to expressed the coding sequence for yellow fluorescent protein (YFP) were all unable to slow parasite growth or induce the expression of cyst wall protein (unpublished data). (B) Early passage type I GT-1 parasites (fewer than 12 passages from the oocyst) do not slow growth or initiate the expression of bradyzoite-specific cyst wall protein in a cell expressing CDA1 under control of the tetracycline-inducible promoter. Compare parasite growth and cyst wall protein measured by immunofluorescence in uninduced cells (open boxes, light bars) with growth and cyst wall protein in cells ectopically expressing the CDA1 protein (shaded boxes, dark bars). DOI: 10.1371/journal.ppat.0020105.g004

induces changes in host cell transcription that directly contribute to a molecular environment that causes the parasite to initiate bradyzoite development. Other published methods to trigger bradyzoite formation may also act through the host as we have found that long-term preconditioning of HFF cells in alkaline media can induce some bradyzoite gene expression in parasites that invade this altered environment but are then maintained under physiological pH. Parasite growth and the expression of acknowledged bradyzoite-specific BAG1 and cyst wall mRNA and proteins in the Compound 1 and pH-altered HFF environment are consistent with elevated levels of these mRNAs in in vivo cysts [46] and

during sporozoite initiated development [47] and suggest we have described an authentic bradyzoite development pathway.

It is intriguing that type I RH and GT-1 parasites were resistant to the Compound 1-altered host cell environment and were unable to slow growth significantly, express BAG1, or assemble a cyst wall. It is unclear whether this resistance is related to reported differences in development among strain types [21,23–32], but it is possible that type I strains respond to different stimuli. Alternatively, the distinct developmental phenotype of type I strains may be the result of a dominant mutation that lessens the response of these strains to a host environment that otherwise slows growth and initiates parasite development in type II and type III strains. Phenotypic differences between strains when assessing inherent growth rate, virulence, tissue migration, and the induction of cytokines appear to be inherited [32]; such that differences in persistence are likely to also have a genetic basis. Consistent with this concept, we have found that F1 progeny from a type I × type III genetic cross show development phenotypes that are similar to one or the other parent (Michael White, Montana State University, and L. David Sibley, Washington University, unpublished data), and we have recently transferred Compound 1 resistance to type III CTG parasites using genetic complementation with a type I-specific RH strain cosmid library (Jay Radke and Michael White, Montana State University, unpublished data). Combined, these observations suggest a genetic mechanism may underlie observed developmental differences in type I strains.

We have used a novel multivariate approach using microarray hybridizations to successfully identify host cell pathways most relevant to the observed Compound 1-induced bradyzoite development. Through the use of siRNA knockdown methods, we have confirmed that increased expression of at least one of the host genes altered by Compound 1 is required to arrest the parasite cell cycle and induce bradyzoite gene expression. The inhibition of parasite growth and the induction of cyst wall protein in cells where CDA1 is overexpressed suggest an important, although unknown, role for CDA1 in the host-parasite interaction leading to bradyzoite development. These results are congruent with the discovered effects of the *HIF1* hypoxia response gene required for growth of tachyzoites in a low oxygen environment (3%, [48]) and suggest that other host cell proteins may play critical roles in the pathogenesis of *Toxoplasma* infection. Along with CDA1, the final gene list from Table S1 includes various other factors with demonstrated roles in the proliferative state of the host cell suggesting the growth state of the cell may help define a host environment as more (or less) suitable for parasite development. How the parasite biochemically registers the growth status of the cell is unknown but fits the observation that parasite tissue cysts are primarily distributed in differentiated, long-lived cells such as mature skeletal muscle and brain neurons [45]. Further investigation of these host pathways with respect to variation in strain response will increase our understanding of the potential links between the cell cycle state of the host cell and parasite development.

Materials and Methods

Parasite culture in human foreskin fibroblasts. Tachyzoites of VEG (type III strain), ME49 (type II strain), and RH and GT-1 strains (both type I) were maintained separately by serial passage in HFF, and these cells were cultured in Dulbecco's modified Eagle medium (DMEM;

GIBCO BRL, Grand Island, New York, United States) supplemented with 1% (v/v) newborn calf serum (Hyclone Laboratories, Logan, Utah, United States) as previously described [8]. To harvest tachyzoites, infected HFF monolayers were scraped from culture flasks, needle-passed, and filtered using a 3- μ m nucleopore membrane to separate parasites from host cell debris. These parasites were then pelleted (15 min at 2,500 rpm; Sorvall ST-6000), resuspended in fresh media, counted, and diluted for the experimental infections described below.

BAG1 immunofluorescence and parasite growth assays. Parasite BAG1 expression was evaluated by IFA in HFF cells cultured in eight-well slides as previously described [2,8]. Confluent monolayers were cultured in 1% DMEM containing concentrations of Compound 1 ranging from 0.5 to 10 μ M for 3 h and then washed extensively with normal media prior to tachyzoite infection. Parasite BAG1 expression was measured by IFA at 12-h intervals to 72-h postinfection using monoclonal antibody against the antigen [42]. Fluorescence was evaluated using an epifluorescence microscope (Eclipse TE300; Nikon, Melville, New York, United States), and images were collected with a digital camera (SPOT; Dynamic Instruments, Sterling Heights, Michigan, United States). Percent BAG1⁺ parasites was determined by counting the BAG1⁺ vacuoles in 100 randomly selected fields. Staining of cyst wall protein was completed using antibody against *Dolichos* lectin as previously described [49]. VEG strain tissue cysts were induced in vitro using (1) 48-h growth in alkaline media (pH 8.2) [26] or, separately, (2) 72-h treatment postinfection in 3 μ M Compound 1. In all analyses where parasite growth was monitored, growth rate was evaluated by parasites per vacuole using the average number of parasites in 100 vacuoles, calculated at 12-h intervals postinfection [8].

Western analyses. Western blot analyses were performed essentially as previously described [50] for (1) detection of phosphorylated p38 MAPK and associated downstream targets, (2) the detection of the BAG1 protein in parasites grown in HFF cells treated with Compound 1 or alkaline pH (pH 8.2), and (3) assessment of Lamin A/C protein control in the siRNA knockdown of CDA1. (1) For assessment of MAPK and associated proteins, lysates from cell monolayers pretreated with Compound 1, 202190, 506126, or alkaline media (pH 8.2) and co-activated with anisomycin, LPS, or PMA/ionomycin were fixed using M-PER (product No. 78501; Pierce Chemical, Rockford, Illinois, United States) containing protease inhibitors and according to the manufacturer's protocol. Protein concentration was assessed using bicinchoninic acid assay (BCA assay, product No. 23225; Pierce Chemical), and 10 to 50 μ g of total protein per lane was resolved using 7% to 15% SDS-PAGE. Separated proteins were transferred to nitrocellulose, and the transferred blot was then blocked for 1 h at room temperature (RT) using 5% milk in Tris-buffered saline (pH 7.4) containing 0.01% Tween-20 (TBST). The blot(s) was incubated overnight at 4 °C separately with one of several primary antibodies against p38, phospho-38, phospho-MAPKAP2, phospho-ATF-2, or phospho-Elk1 (product No. 9913; Cell Signaling Technology, Danvers, Massachusetts, United States), diluted 1:1,000 in 0.5% milk in TBST. Blots were washed three times in TBST for 15 min and then incubated 4 h at RT with anti-rabbit IgG-horseradish peroxidase conjugate (HRP) diluted 1:2,500 in 0.5% milk in TBST. Protein bands were detected via enhanced chemiluminescence (ECL) reagent and x-ray film. (2) To detect parasite BAG1, tachyzoites were obtained from infected HFF monolayers grown in alkaline pH (pH 8.2) by scraping and needle-passing to disrupt host cells and then purified by filtration through a 3.0- μ m Nucleopore filter. Parasite proteins were extracted by adding NP-40 to a final concentration of 2% (v/v). The resulting lysate was then incubated on ice for 30 min, followed by centrifugation for 10 min at 12,000 \times g. The supernatant was mixed with nonreducing buffer, and proteins were resolved by SDS-PAGE and transferred to nitrocellulose. The transferred blot was blocked for 1 h in 5% (w/v) nonfat dry milk in 50 mM Tris (pH 8.0) and 150 mM NaCl and then probed for at least 1 h with the primary monoclonal antibody against BAG1 [42] diluted 1:500 in 0.5% milk as described above. The blot was washed three times for 15 min in blocking buffer, and proteins were visualized by incubating the blots in anti-mouse IgG alkaline phosphatase-conjugated antibody for 1 h, washing as above, and protein visualized by conversion of 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium with localized antibody-alkaline phosphate conjugate according to standard methods (product No. 2209; Invitrogen, Carlsbad, California, United States) [51]. (3) We assessed Lamin A/C control protein in the siRNA knockdown using 3 μ g of total protein resolved via 10% SDS-PAGE and transferred to nitrocellulose. The transferred blot was blocked overnight at 4 °C in TBST containing

10% horse sera and probed 3 h in blocking buffer at RT with primary anti-mouse IgG monoclonal antibody against Lamin A/C (1:1,000; BD Biosciences, Woburn, Massachusetts, United States). The blot was washed three times for 5 min in TBST, and blots were incubated for 2 h in blocking solution with secondary anti-mouse IgG alkaline phosphatase conjugate (1:10,000; Promega, Madison, Wisconsin, United States). Blots were washed and protein visualized using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium methods as described above.

Microarray analysis of Compound 1-induced gene expression. Total RNA was isolated from HFF cells using Qiagen RNeasy spin columns according to standard protocols (Qiagen, Valencia, California, United States). Cells were treated using Compound 1 treatment regimens known to induce parasite BAG1 but also using alternate p38 MAPK inhibitors known to antagonize the Compound 1 effects on BAG1 expression, as well as treatment with inhibitors of RNA polymerase II. Total RNA was evaluated using the Agilent Bioanalyzer (Agilent Technologies, Palo Alto, California, United States), and mRNA levels were characterized by hybridizations with the Human Oligo 1A and 1B microarrays (Agilent Technologies; ServiceXS, Leiden, the Netherlands). Total RNA (5 μ g) was used to prepare double-stranded cDNA using T7-(dT)24 primer and MMLV reverse transcriptase according to standard protocols (Agilent Technologies), and cDNA was transcribed independently in the presence of Cy5- and Cy3-labeled UTP and CTP and labeled cRNA purified using Qiagen RNeasy Kit. Purified cRNA was fragmented to less than 200 bases by incubation for 30 min at 60 °C in fragmentation buffer and hybridized with the Agilent Human Oligo 1A and 1B slide arrays for 17 h at 60 °C. The Human Oligo 1A and 1B slide arrays contain 47,000 sequences selected from GenBank, dbEST, and RefSeq and the clusters created from the UniGene database. Arrays contain 60-bp oligonucleotides and most probe sets are duplicated. Each array contains internal probe sets that serve as controls for monitoring RNA integrity. The protocol includes two posthybridization washes, staining, and a post-stain wash. Arrays were scanned twice, and hybridization intensities were collected from scanned images. Gene expression data were extracted with GeneSpot and Gene Spring (Silicon Genetics, San Diego, California, United States) expression analysis software. For each treatment to induce parasite development, the treatment itself, e.g., Compound 1, DRB, SB202190, etc., the annotated sequence name for each spot on the array, and the induction (or not) of parasite BAG1 expression were stored separately in a unique field within a MySQL relational database. A single MySQL database contained all hybridization results. The database was then queried for mRNAs that were changed 3-fold or greater as a result of the treatment and whose regulation was coincident with observed inhibition of parasite growth and BAG1 expression in the parasite.

Gene knockdown using siRNA. HFF cells (4×10^4) were transfected in 150- μ l suspensions (1% DMEM without FBS) per well of an eight-well slide with 25 to 150 nM siRNA in Opti-MEM (Invitrogen) and LipofectAMINE 2000, according to the manufacturer's instructions (Invitrogen). The siRNA against CDA1 was a Smartpool of four distinct siRNAs against alternate segments of the coding sequence (Dharmacon). siRNAs against Lamin A/C [52] were used to demonstrate siRNA activity in primary HFF (positive control) and also to ensure that transfection of siRNAs unrelated to Compound 1-induced parasite growth inhibition and BAG1 expression was not able to induce this parasite phenotype alone (negative control). To transfect siRNAs into cells, HFF cells were incubated for 6 h at 37 °C (5% CO₂) with 25 to 150 nM siRNAs in LipofectAMINE and Opti-DMEM (without serum or antibiotics, total volume 150 μ l per well of an eight-well slide; Invitrogen). After 6 h, maximum transfection was complete and FBS was added back to a final concentration of 10%. Transfected cells could be incubated up to 96 h at 37 °C (5% CO₂) without appreciable loss of siRNA. Transfection of the siRNA was completed 6 h immediately prior to parasite infection, and at 3 h preinfection, the Compound 1 was added (3 μ M final concentration; 6 h total time for transfection of siRNA and Compound 1 treatment). Parasites were then allowed to invade 2 h, and then parallel eight-well slides were fixed at 24-h intervals to 72 h for analysis of BAG1 expression by IFA as described above. Parasite growth was also evaluated in these same slides using average parasites per vacuole. To evaluate mRNA levels for CDA1 and GAPDH and mRNA and protein levels for Lamin A/C, total RNA or cell lysates were isolated from 24-well plates infected in concert with the eight-well slides used for BAG1 expression and analysis of parasite growth.

RT-PCR analyses. RT-PCR was used (1) to assess mRNA levels for GAPDH, CDA1, and Lamin A/C during siRNA knockdown of CDA1 in the Compound 1-induced host cell and (2) to assess the level of

parasite BAG1 and LDH2 mRNAs following exposure to Compound 1. (1) Total RNA from a single well of a six-well plate was isolated using an RNeasy spin column according to the manufacturers protocol (Qiagen) and first-strand cDNA synthesis completed using less than 1 µg of total RNA and SuperScript reverse transcriptase according to the manufacturer's protocol (Invitrogen). PCR amplification of gene-specific target sequences was done using Platinum Taq (Invitrogen) and primers specific to GAPDH (forward 5'-tcacatgacaaacttggatcg-3', reverse 5'-gagcttgacaagaatggctgtga-3' [53]), Lamin A/C (forward 5'-aacttcaggatgagatgctgcg-3', reverse 5'-gtccagaagctcctgactctcg-3'), and CDA1 mRNAs (forward 5'-aatttcacattttgatcaaccgacg-3', reverse 5'-aggattctcgtctgcagat-3'). GAPDH transcripts were amplified using 30 rounds of temperature cycling, each for 30 s at 94 °C, 30 s at 58 °C, and 1 min at 72 °C, followed by a single extension for 10 min at 72 °C. CDA1 and Lamin A/C transcripts were amplified using annealing temperatures of 52 °C and 55 °C, respectively. Amplification products were resolved using agarose gel electrophoresis and ethidium bromide staining. (2) We also used semiquantitative RT-PCR [54,55] to assess mRNA levels for BAG1 and LDH2 in cells treated with Compound 1. Following first-strand cDNA synthesis from 1 µg of total RNA as described above, PCR amplification of gene-specific target sequences was done using Platinum Taq (Invitrogen) and primers specific to α -tubulin (forward 5'-tcgacaacagggccatctacacatctgcc-3', reverse 5'-acca tagccctctcttcaccttcaccttc-3'), BAG1 (forward 5'-gctttgcccctgaatcctcgacttga-3', reverse 5'-gtaagccgcttctcctgctactaccct-3'), and LDH2 (forward 5'-gggaagagtgcagaaggagtgagcagaac-3', reverse 5'-ccaccacgtcatcaactgactctgaaac-3') target sequences. To enable semiquantitative assessment of these mRNA levels, 4-fold serial dilutions of the first-strand cDNA were completed prior to amplification. α -Tubulin, BAG1, and LDH2 amplifications were all completed from the same cDNA, made from total RNA from either untreated parasites or from parasites grown in Compound 1. BAG1 and α -tubulin transcripts were amplified using 25 rounds of temperature cycling, each for 30 s at 94 °C, 30 s at 62 °C, 1 min at 72 °C, followed by a single extension for 10 min at 72 °C. For LDH2, the annealing temperature was lowered to 55 °C and extension for 1.5 min at 72 °C in each round of amplification. α -Tubulin mRNA is expressed equally in tachyzoites and bradyzoites and was used in these studies as a PCR control.

Regulated expression of the CDA1 transgene in HeLa cells. The sequence encoding the human CDA1 mRNA was obtained from American Type Culture Collection (the American Type Culture Collection [http://www.atcc.org] sequence encoding the human CDA1 mRNA was gb VC024270 [ATCC designation = 7520908, the I.M.A.G.E. = 2819684], and the T-REX tetracycline-inducible "tet-on" expression system was used according to manufacturer's protocol (Invitrogen). A Gateway (Invitrogen) recombination expression plasmid was first created by cloning a reading frame cassette into the EcoRV site of the pcDNA4/TO TET-inducible plasmid vector to prepare a destination vector for recombinational cloning of the coding sequence (Invitrogen). The sequence encoding CDA1 mRNA was amplified from the plasmid vector using the primers (attB1 CDA1 forward 5'-ggggacaagttgtacaaaaagcaggctcgcacatggaccgccag-3' and attB2SE20-4 reverse 5'-ggggaccacttgtacaa-gaaagctgggtcttaccggtttccctcttc-3') and PFU and the product cloned by BP recombination into pDONR221 to create an entry clone. LR recombination with the destination vector generated the pcDNA4/TO expression vector containing the complete CDA1 coding sequence under control of the tetracycline operator sequences and the cytomegalovirus promoter sequence (Invitrogen). HeLa cells expressing the tetracycline repressor protein (7×10^6 , T-Rex; Invitrogen) were combined in 400 µl of Opti-MEM with 17.5 mg of plasmid and 12.5 mg of salmon sperm DNA and electroporated at 850 µF, 260 V, and 720 Ω in a 4-mm gap cuvette using a BTX ECM630 electroporator (BTX Instrument, Holliston, Massachusetts, United States). Cells were cultured overnight in 10% TET-free DMEM at 37 °C in 5% CO₂ and then washed with HBSS, lifted from plastic with 1× trypsin, and counted. Under these conditions, more than 80% of host cells are transfected with the plasmid based on parallel transfection with a tetracycline-regulated YFP plasmid and greater than 15-fold induction of CDA1 by quantitative RT-PCR. Approximately 60,000 HeLa cells were plated onto each well of an eight-well slide and cultured 6 h prior to infection with 100,000 tachyzoites (type III VEG or CTG and type I GT-1 or RH). Parasites were allowed to invade for 1 h, and remaining free parasites were washed from the monolayer. Expression of the CDA1 transcript was immediately induced by addition of tetracycline (1 µg/ml) to the growth media. To maintain tetracycline-induction,

media containing this antibiotic were refreshed at 24-h intervals throughout the 48-h time course.

Supporting Information

Figure S1. The Induction of BAG1 mRNA and BAG1 and Cyst Wall Proteins Was Similar in Cells Treated with Compound 1 and Alkaline pH

(A) Compound 1 and alkaline pH (pH 8.2) have similar effects on type III CTG tachyzoites when measuring cyst wall protein by immunofluorescence assay at 40 h postinfection. Both alkaline pH and Compound 1 were significantly less able to induce cyst wall protein in the type I GT-1 strain (compare cyst wall expression between type I GT-1 and type III CTG strains under Compound 1 and pH 8.2 induction).

(B) Similar results were observed when comparing BAG1 protein levels induced by Compound 1 or alkaline pH (pH 8.2) in type III strain CTG and type II Prugniald with the type I GT-1 strain parasites at 48 and 72 h postinduction.

(C) RT-PCR from 1 µg of total RNA was used to compare levels of BAG1 and LDH2 mRNA in type I GT-1 and type III CTG strain parasites grown in Compound 1. Products were resolved via 1% agarose gel electrophoresis and visualized by ethidium bromide staining. BAG1 or LDH2 mRNAs were undetectable in type I GT-1 parasites exposed to Compound 1, while these mRNAs are clearly induced in the type III CTG parasites. Compare RT-PCR products for BAG1 mRNA amplification in lanes 1 to 4 (untreated cells) with lanes 1 to 5 (3 h Compound 1 treatment) for CTG and GT-1 parasites. Lanes 1 to 4 and lanes 5 to 8 represent 4-fold serial dilutions of the first-strand cDNA from untreated and Compound 1-treated parasites. α -Tubulin mRNA is expressed equally in tachyzoites and bradyzoites and is presented here as a PCR control.

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Figure S2. Assessment of p38 MAPK Activation in Compound 1-Induced Parasite Development

(A) LPS (100 ng/ml) and PMA (20 ng/ml and ionomycin 0.5 µg/ml) were unable to induce significant phosphorylation of p38 MAPK in HFF as measured by anti-phospho-p38 antibodies and Western blot. Compare untreated cell lysates, labeled C, with those from cells treated from 10 min to 24 h. In contrast, anisomycin (10 µg/ml) was able to induce significant levels of phosphorylated p38 in these cell types, and as early as 15 min post-treatment. Parasite infection alone was unable to induce phosphorylation of p38 (compare lysates from the uninfected control, labeled C, with lysates from infected cells [P], and also with lysates from treatment with LPS [100 ng/ml, L]). The phosphorylation state of p38 was unaffected by co-treatment of parasite-infected cells, or LPS-treated cells (100 ng/ml), and the p38 MAPK inhibitor SB202190 (30 nM, compare P or L with lysates labeled P⁺ and L⁺, respectively).

(B) HFF cells treated with anisomycin effectively phosphorylate p38 MAPK (compare untreated control cell lysates, labeled C, with lysates from cells treated 15 min with anisomycin, labeled A). Note that 3-h treatment with Compound 1 (labeled C1) or the known inhibitors of p38 MAPK SB202190 (labeled 20) and 506126 (labeled 50) prior to activation with anisomycin was unable to effect the observed induction of p38 phosphorylation. Significantly, anisomycin-treated cells were unable to mediate phosphorylation of the known p38 MAPK targets MAPKAP2 and Elk1, and while ATF-2 was phosphorylated, co-treatment with SB202190, SB506126, or Compound 1 was unable to alter or reduce the level of phosphorylation.

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Table S1. Fold-Change in mRNA Levels as Measured by Hybridization Signal Intensities during Host Cell Treatment with Compound 1, the p38 α MAPK Inhibitors 506126 and 202190, and DRB

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Accession Numbers

The GenBank (http://www.ncbi.nlm.nih.gov/Genbank) accession numbers for CDA1 (also known as SE20-04) is NM_022117 and for Lamin A/C is NM_005572.

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Author contributions. JRR, RGD, MEJ, MSB, PL, and MWW conceived and designed the experiments. JRR, AE, MEJ, and MSB performed the experiments. JRR, RGD, AE, MEJ, MSB, PL, and MWW analyzed the data. JRR, RGD, AE, PL, and MWW contributed reagents/materials/analysis tools. JRR, RGD, AE, and MWW wrote the paper.

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