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Heterotrimeric G-protein Signaling Is Critical to Pathogenic Processes in Entamoeba histolytica

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Abstract
Heterotrimeric G-protein signaling pathways are vital components of physiology, and many are amenable to pharmacologic manipulation. Here, we identify functional heterotrimeric G-protein subunits in Entamoeba histolytica, the causative agent of amoebic colitis. The E. histolytica Gα subunit EhGα1 exhibits conventional nucleotide cycling properties and is seen to interact with EhGBP dimers and a candidate effector, EhRGS-RhoGEF, in typical, nucleotide-state-selective fashions. In contrast, a crystal structure of EhGα1 highlights unique features and classification outside of conventional mammalian Gα subfamilies. E. histolytica trophozoites overexpressing wildtype EhGα1 in an inducible manner exhibit an enhanced ability to kill host cells that may be wholly or partially due to enhanced host cell attachment. EhGα1-overexpressing trophozoites also display enhanced transmigration across a Matrigel barrier, an effect that may result from altered baseline migration. Inducible expression of a dominant negative EhGα1 variant engenders the converse phenotypes. Transcriptional studies reveal that modulation of pathogenesis-related trophozoite behaviors by perturbed heterotrimeric G-protein expression includes transcriptional regulation of virulence factors and altered trafficking of cysteine proteases. Collectively, our studies suggest that E. histolytica possesses a divergent heterotrimeric G-protein signaling axis that modulates key aspects of cellular processes related to the pathogenesis of this infectious organism.

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Introduction
GTP-binding proteins (G-proteins) are important transducers of cellular signaling [1]. Heterotrimeric G-proteins are composed of three distinct subunits (Gα, Gβ, and Gγ) and typically coupled to seven-transmembrane domain, G-protein coupled receptors (GPCRs). Gα binds guanine nucleotide while Gβγ form an obligate heterodimer [1]. Conventionally, Gα forms a high-affinity binding site for Gβγ when Gα is in its inactive GDP-bound state. Activated receptor acts as a guanine nucleotide exchange factor (GEF) for Gα, releasing GDP and allowing subsequent GTP binding. The binding of GTP causes a conformational change in three flexible “switch” regions within Gα, resulting in Gβγ dissociation. Gα-GTP and freed Gβγ independently activate downstream effectors, such as adenyl cyclase, phospholipase C isoforms, and Rho-family guanine nucleotide exchange factors (RhoGEFs) to modulate levels of intracellular second messengers [1,2]. ‘Regulator of G-protein signaling’ (RGS) proteins generally serve as inhibitors of Gα-mediated signaling [3]; however, one class of RGS protein, the RGS-RhoGEFs, serve as positive “effectors” for activated Gα signal transduction [2,4].

Heterotrimeric G-protein signaling has provided a wealth of targets amenable to pharmacologic manipulation, most prevalent being the GPCR itself [5]. Heterotrimeric G-proteins in mammals regulate processes as diverse as vision, neurotransmission, and vascular contractility [1,5]. Heterotrimeric G-proteins in non-mammalian organisms also exhibit a wide range of functions; for example, pheromone and nutrient sensing in yeast [6], hydrophobic surface recognition in the rice blast fungus [7], and cellular proliferation and chemical gradient sensing in the slime mold Dictyostelium discoideum [8,9].

Entamoeba histolytica causes an estimated 50 million infections and 100,000 deaths per year worldwide [10]. E. histolytica infection is endemic in countries with poor barriers between drinking water and sewage; however, outbreaks also occur among travelers and susceptible subpopulations in developed countries [11]. Upon cyst ingestion, the amoeba may colonize the human colon. Although the majority of infections are asymptomatic (e.g. ref [12]), a fraction results in symptomatic amoebic colitis. Migratory E. histolytica...
Entamoeba histolytica causes an estimated 50 million intestinal infections and 100,000 deaths per year worldwide. Here, we identify functional heterotrimeric G-protein subunits in Entamoeba histolytica, constituting a signaling pathway which, when perturbed, is seen to regulate multiple cellular processes required for pathogenesis. Like mammalian counterparts, EhGα1 forms a heterotrimer with EhGβγ that is dependent on guanine nucleotide exchange and hydrolysis. Despite engaging a classical G-protein effector, EHRG-S-RhOGEF, EhGα1 diverges from mammalian Gα subunits and cannot be classified within mammalian Gα subfamilies, as highlighted by distinct structural features in our crystal structure of EhGα1 in the inactive conformation. To identify roles of G-protein signaling in pathogenesis-related cellular processes, we engineered trophozoites for inducible expression of EhGα1 or a dominant negative mutant, finding that G-protein signaling perturbation affects host cell attachment and the related process of contact-dependent killing, as well as trophozoite migration and Matrigel transmigration. A transcriptomic comparison of our engineered strains revealed differential expression of known virulence-associated genes, including amoebapores and cytotoxic cysteine proteases. The expression data suggested, and biochemical experiments confirmed, that cysteine protease secretion is altered upon G-protein overexpression, identifying a mechanism by which pathogenesis-related trophozoite behaviors are perturbed. In summary, E. histolytica encodes a vital heterotrimeric G-protein signaling pathway that is likely amenable to pharmacologic manipulation.

Results

Identification of E. histolytica heterotrimeric G-protein subunits

By a BLAST sequence similarity search with human Gαi1 (E-value cutoff of 10^-30), we identified a single gene in E. histolytica encoding a putative Gα subunit (EhGα1; AmoebaDB EHI_140550) also present in the related E. dispar, E. monodii, E. moshkovskii, and E. terrapinae. One Gβ subunit was also identified (AmoebaDB EHI_000240) by sequence similarity to human Gβ1 (E-value cutoff of 10^-30), termed EhGβ1 (Fig. S1B). As Gβ subunits form obligate heterodimers with short Gγ polypeptides or Gγ-like (GGL) domains [3], we also searched for putative Gγ-encoding genes. Based on sequence similarity with S. cerevisiae Ste18 and D. discoideum gpGγA, together with alignment of candidate protein sequences and identification of key functional residues, we identified two putative Gγ-encoding genes named EhGγ1 and EhGγ2; these two open-reading frames (in the NCBI E. histolytica genomic contigs AAFLB02000029.1 and AAFLB02000157.1, respectively) each possess a C-terminal CAAX-box that specifies isoprenylation in conventional Gγ subunits [33].

To determine whether these G-protein subunits are expressed in E. histolytica, we amplified trophozoite mRNA using quantitative RT-PCR. Transcripts of EhGα1, EhGβ1, and EhGγ1 were all detected, along with the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH; AmoebaDB EHI_167320) (Fig. S2).

Functional assessments of E. histolytica G-protein subunits

To determine whether the identified EhGα1, EhGβ1, EhGγ1, and EhGγ2 subunits form conventional heterotrimeric (Gβγ) and heterotrimeric (Gα-GDP/Gβγ) complexes, bimolecular fluorescence complementation and co-immunoprecipitation assays were performed (Fig. 1A, B). The N-terminal half of yellow fluorescent protein (YFPN) was fused to EhGγ1 and EhGγ2 open reading frames while the C-terminus of YFP (YFP C) was fused to EhGβ1. Only when YFP N and YFP C are fused to interacting proteins will functional assessments of E. histolytica G-protein subunits be expressed in E. histolytica, we amplified trophozoite mRNA using quantitative RT-PCR. Transcripts of EhGα1, EhGβ1, and EhGγ1 were all detected, along with the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH; AmoebaDB EHI_167320) (Fig. S2).

Heterotrimeric G-proteins Modulate Pathogenesis
ref. [37]). Exposure to the activating reagent AlF$_4^-$ and magnesium (AMF) increases tryptophan fluorescence (Fig. 2C), and thus EhG$\alpha_1$ appears to assume a similar, activated switch conformation as conventional G$\alpha$ subunits. Since the measured rates of EhG$\alpha_1$ nucleotide exchange (0.27 min$^{-1}$ at 30°C) and hydrolysis (0.21 min$^{-1}$ at 20°C) were on the same order of magnitude, we tested whether hydrolysis was rate-limiting, as seen for the A. thaliana G$\alpha$ protein, AtGPA1 [38]. While EhG$\alpha_1$ assumes an activated conformation upon exposure to the non-hydrolyzable GTP analog, GppNHp, as indicated by intrinsic tryptophan fluorescence, addition of hydrolyzable GTP was insufficient to activate EhG$\alpha_1$ (Fig. 2D). Thus, nucleotide

Figure 1. E. histolytica G-protein subunits form a heterotrimer in a nucleotide-dependent manner. Interactions between G$\beta$ and G$\gamma$ subunits were detected with split-YFP protein complementation in COS-7 cells. (A) Human G$\beta_1$ heterodimerized with human G$\beta_2$, but not with E. histolytica G$\gamma$ subunits. (B) EhG$\beta_1$ interacts with EhG$\gamma_1$ or EhG$\gamma_2$ when co-expressed with EhG$\alpha_1$. (C) G-protein heterotrimer formation in the presence of excess GDP ("D") or the non-hydrolyzable GTP analog, GTP$_{\gamma}$S ("T"), was examined with co-immunoprecipitation. EhG$\gamma_1$ and EhG$\gamma_1$ or EhG$\gamma_2$ interacted selectively with EhG$\alpha_1$ in its GDP-bound, inactive state. Error bars represent standard error of the mean for three experiments. * represents statistically significant difference from zero, as determined by 95% confidence intervals excluding zero.

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exchange is the rate-limiting step in the steady-state nucleotide cycling of EhGα1, as for mammalian Gα subunits, indicating that activation likely relies on GEF-stimulated exchange.

EhGα1 functional mutants
To further characterize EhGα1 activation properties and provide tools for probing G protein function in E. histolytica trophozoites, we mutated presumed key residues of the nucleotide-cycling function of EhGα1. Glu-189 in switch 2 (Fig. S1A) is predicted to coordinate the critical nucleophilic water responsible for γ-phosphoryl group hydrolysis [39]. Mutation of this residue to leucine in mammalian Gα subunits results in inability to hydrolyze GTP even in the presence of GTPase-accelerating proteins [35]. The corresponding EhGα1(Q189L) mutation abolished the ability of EhGα1 to hydrolyze GTP (Fig. 2E), suggesting a conserved role for the switch 2 Glu-189 residue in orienting the nucleophilic...
negative behavior due to disrupted GTP/Mg
a
mutating Ser-37 to cysteine. The EhG
could create an EhG
presented with GDP or GTP analogs. We hypothesized that we
identified as constitutively binding G
no appreciable GTP
c
either GDP or GTP
mammalian G
RhoGEF) with distant homology to the RGS-RhoGEF effectors of
containing RhoGEF (AmoebaDB EHI_010670; named EhRGS-
resonance, immobilized EhRGS-RhoGEF protein was found to
subfamilies (G
a
subunits of
showed sequence similarity
respectively [6]. The
A. thaliana
latter with roles in pheromone response and nutrient sensing,
transpiration and cellular proliferation in response to
A. thaliana
expression plasmids [47] encoding either wildtype EhG
1 and an array of human G
subunits based upon multiple sequence alignments. In calibrating this method, the five known
subunits of
showed sequence similarity patterns allowing facile classification into each of the G
subfamilies (G
, G
, G
, G
) (Fig. S3A); however, both
EhG
1 and GPA1 from
exhibited low sequence similarities to each of the human G
subfamilies (Fig. S5B). EhG
1 exhibits the lowest similarity to each mammalian G
tested, implying a likely early evolutionary departure from an
ancestral G
.

The
E. histolytica
genome was found to encode an RGS domain-containing
RhoGEF (AmoebaDB EHI_010670; named EhRGS-
RhoGEF) with distant homology to the RGS-RhoGEF effectors of
mammalian G
subunits; no other canonical G
effector proteins, such as adenylyl cyclases or phospholipase C
b
molecules that interact with the
haploidy motifs. EhG
also harbors a unique 16-residue insert in the
Ras-like domain following the
GoLoco motifs. EhG
also has similarity to
A. thaliana GPA1 and the yeast G
subunits, GPA1 and GPA2, the
latter with roles in pheromone response and nutrient sensing,
estimated [42,43]. We also calculated sequence similarity between
EhG
1 and an array of human G
subunits upon based upon multiple sequence alignments. In calibrating this method, the five known
subunits of
Drosophila melanogaster showed sequence similarity patterns allowing facile classification into each of the G
subfamilies (G
, G
, G
, G
) (Fig. S3A); however, both
EhG
1 and GPA1 from
exhibited low sequence similarities to each of the human G
subfamilies (Fig. S5B). EhG
1 exhibits the lowest similarity to each mammalian G
tested, implying a likely early evolutionary departure from an
ancestral G
.

A crystal structure of EhG
1
To gain better insight into the distant homology of EhG
1 versus other G
subunits, we determined a crystal structure of EhG
1 bound to GDP by single-wavelength anomalous dispersion (SAD) using data to 2.6 Å resolution (Table S1; Fig. S6). To obtain high-quality diffracting crystals, we modified EhG
1 by removing its extended N-terminal helix (a.a. 1–22) and subjecting it to reductive lysine methylation. Neither alteration perturbed the nucleotide cycle or activation kinetics of EhG
1 (Fig. 2B,C). EhG
1 features the highly conserved Ras-like and all-helical domain structure and nucleotide-binding pocket characteristic of G
subunits (Fig. 4A). The three switch regions are ordered in one of the two monomers in the asymmetric unit, likely due to crystal contacts (Fig. S6B). EhG
1 exhibits a highly conserved mode of nucleotide interaction, including the dispositions of residues Ser-37 and Gln-189 (Fig. 4B). The guanine ring is embraced by the conserved NKxD motif (residues 254–257; Fig. S7), with the hydrophobic portion of Lys-255 packing against the planar guanine ring. The phosphate-binding loop (P-loop) forms numerous polar contacts with the α- and β-phosphoryl groups of GDP [39].

Unique to EhG
1 is the absence of an αB helix in the all-helical domain (Fig. 4A). Although the segment between αA and αC (αA–
αC loop) could be affected by crystal packing, five prolines scattered throughout this loop (positions 84, 89, 99, 103, and 106; Fig. S1A) suggest this region likely also lacks helical structure in solution. GoLoco motif-containing proteins are one of the few molecules that interact with the
haploidy helix (e.g. ref. [37]); not surprisingly, given the lack of a structurally-conserved binding site on EhG
1, the
E. histolytica
genome does not seem to encode any GoLoco motifs. EhG
also harbors a unique 16-residue insert in the
Ras-like domain following the
haploidy helix (Figs. 4A, S1A). A portion of this insert forms a short β-strand (here termed β7) that extends the six-stranded β-sheet common to all heterotrimeric and Ras-family GTPases [44,45], followed by a 15-residue loop that is disordered in our crystal structure. This region of G
is critical for interaction with GPCRs as seen, e.g., in the crystal structure of the
B adrenergic receptor/Gs complex [46]. Because this region is important for receptor coupling and/or specificity, the existence of this insert in EhG
1 suggests a potentially unique GPCR-coupling mechanism in
E. histolytica, but no receptor has yet been identified (see Discussion).

G-protein signaling perturbation modulates trophozoite migration, Matrigel transmigration, and host cell attachment and killing
To determine roles of heterotrimeric G-protein signaling in pathogenesis-related behaviors of
E. histolytica, HM-1:IMSS trophozoites were stably transfected with tetracycline-inducible expression plasmids [47] encoding either wildtype EhG
1 or the dominant negative EhG
1S37C (Fig. 5A). A strain expressing the constitutively active EhG
1L134F could not be established, potentially due to cellular toxicity; however, overexpression of wildtype EhG
1 is expected to result in a moderately higher basal level of signaling to downstream components. Overexpression of signaling components is subject to limitations, including the possibility that supra-physiological expression levels and/or protein mislocalization result in toxicity or other cellular effects not typically mediated by endogenous signaling. However, this approach is useful to suggest cellular processes that may be regulated by heterotrimeric G-protein signaling and to mimic the gross perturbation that may be achieved with pharmacological agents acting on this pathway. Immunofluorescence of overex-
pressed EhG1 revealed a diffuse, cytoplasmic cellular distribution
that did not differ significantly between the wild type and S37C
mutant strains (Fig. S8A). Endogenous EhG1 was not assessed
due to a current lack of specific antibodies. To assess potential
effects of Gα subunit overexpression on trophozoite growth and
viability, growth curves were assessed for the parent HM-1:IMSS,
EhG1wt, and EhG1S37C strains in the presence and absence of
tetracycline. No significant differences in growth or viability
(>90%) at all time points) were observed over three days, although
trophozoites expressing EhG1S37C displayed a trend toward
slower growth at day 3 (Fig. S8B). All subsequent cellular
experiments were conducted following growth with or without
tetracycline for 24 hours.

Trophozoite motility is related to the pathogenesis of amoebic
colitis, likely contributing to tissue invasion [48,49]. Tetracycline-
induced EhG1wt overexpression increased migration in the
absence of a serum stimulus while EhG1S37C expression reduced
migration in the presence or absence of serum in Transwell
migration assays (Fig. 5B), suggesting that perturbation of
heterotrimeric G-protein signaling may regulate motility at
basal level and potentially in response to serum factor stimuli.
However, the reduced migration of the EhG1S37C strain in the
presence of serum may be due to the lower baseline trophozoite
motility, as observed in the absence of a serum stimulus, rather
than due to specific heterotrimeric G-protein involvement in a
signaling response to serum factors. Tetracycline treatment had
no measurable effect on the migration of the HM-1:IMSS parent
strain or trophozoites transfected with an empty expression vector
(Fig. S9A).

E. histolytica invades the intestinal mucosa, giving rise to ulcers
and, in rare cases, systemic amoebiasis [50,51]. To assess
migration across a barrier, transfected trophozoite strains were
profiled by a Transwell assay, with upper and lower chambers
separated by Matrigel. Induced expression of EhG1wt enhanced,
but EhG1S37C reduced, Matrigel transmigration relative to
uninduced controls (Fig. 5C), revealing a potential regulatory role
for heterotrimeric G-protein signaling. Tetracycline treatment had
no effect on the transmigration of HM-1:IMSS or empty vector-
transfected trophozoites (Fig. S9B). The effects of EhG1wt and
EhG1S37C overexpression on Matrigel transmigration displayed the
same trends seen for migration in the absence of serum
(Fig. 5B). Thus, differential baseline migration rates may account
for part or all of the observed differences in Matrigel transmigration.

E. histolytica trophozoites also attach to and kill host cells,
including intestinal epithelium and responding immune cells. Host
cell attachment, achieved primarily through a galactose-inhibi-
table lectin [13,52], is required for subsequent cell killing.
Trophozoites expressing EhG1wt displayed greater attachment
to CHO cell monolayers than uninduced controls, and the
opposite effect was seen in the EhG1S37C strain (Fig. 6A, S10).
EhG1wt overexpression enhanced Jurkat cell killing, as assessed
with a membrane integrity assay, while trophozoites expressing the
dominant negative EhG1S37C were less cytotoxic (Fig. 6B).
Tetracycline treatment had no effect on host cell attachment or
killing by HM-1:IMSS or empty vector-transfected trophozoites
(Fig. 5C, D). Thus, perturbation of heterotrimeric G-protein
signaling also regulates host cell killing by E. histolytica. Similar
patterns were observed in host cell attachment and cell killing
assays; different degrees of attachment upon expression of
EhG1wt or EhG1S37C may be partially or wholly responsible for
the observed changes in contact-dependent cell killing.

Regulation of transcription by perturbed heterotrimeric
G-protein signaling

To gain insight into potential mechanisms by which perturba-
tion of EhG1 expression controls pathogenesis-related behaviors
in E. histolytica, RNA-seq was performed on mRNA isolated from
trophozoites expressing EhG1wt, EhG1S37C, and uninduced
controls. To emphasize highly transcribed genes and eliminate
potential transcriptional effects of tetracycline treatment,
transcripts with a Fragments Per Kilobase of exon per Million fragments mapped
(FPKM) value less than 10 and transcripts that were up- or down-
regulated (in the same direction) in both EhG1wt and EhG1S37C
samples (24 hour tetracycline treatment at 5 μg/mL relative to
uninduced [tetracycline-free] trophozoites were excluded. Twenty-
one genes were differentially transcribed in opposite directions
upon expression of either EhG1wt or EhG1S37C (Fig. 7A).
Transcriptional changes of multiple genes were verified over a
24 hour time course by RT-PCR (Fig. S11). For instance, EhGB1
was found to be more highly expressed in trophozoites expressing
EhG1S37C. Analysis of putative functions for the differentially
transcribed genes revealed a diversity of responses to altered
heterotrimeric G-protein signaling (Fig. 7B). Stress response-
related transcripts, such as those encoding heat shock proteins,
were exclusively down-regulated upon EhG1wt expression and
up-regulated in the dominant negative EhG1S37C strain;
conversely, numerous metabolic enzymes were selectively up-
regulated following expression of EhG1S37C, suggesting that
heterotrimeric G-protein signaling may be involved in sensing and
responding to vital extracellular nutrients.

Genes with known effects on E. histolytica pathogenesis were also
differentially transcribed, as measured by RNA-seq. (Table S2).
For example, the host cell lytic factor amoebapore C was up-
regulated upon EhG1wt expression, while the amoebapore A
precursor was down-regulated by EhG1S37C (Table S2), consis-
tent with the higher or lower cell killing efficiencies, respectively, of
each strain (Fig. 6B) [14,15,48]. Down-regulation of amoebapore A
upon expression of EhG1S37C was confirmed by RT-PCR at
the transcriptional level, and by western blot at the protein level
(Fig. S11; anti-amoebapore A was a gift from Dr. M. Lippke, U. of
Kiel, Germany). A number of cysteine proteases, known factors in
both host cell killing and Matrigel transmigration [53], were
differentially transcribed following expression of EhG1S37C
(Table S2). The down-regulation of one cysteine protease
(EHI_006920) was confirmed by RT-PCR (Fig. S11). Ten Rab
family GTPases, known to regulate vesicular trafficking and
cysteine protease secretion [28], as well as other putative
secretion/trafficking proteins, were also differentially transcribed.
Specifically, four cysteine protease binding factors (CBPFs),
recently shown to modulate cysteine protease secretion [54], were

Heterotrimeric G-proteins Modulate Pathogenesis

Figure 3. Evolutionary relationship of Gα subunits and identification of EhRGS-RhoGEF as a putative effector for activated EhG1. (A) Gα subunit protein sequences from E. histolytica, D. discoideum (D.d.), A. thalassio (A.t.), S. cerevisiae (S.c.), D. melanogaster (D.m.), and H. sapiens (H.s.) were aligned and a bootstrapping consensus phylogram created using MEGA5 [41]. Bootstrap values are indicated at each branch point. EhG1
is distantly related to metazoan Gα subunits, specifically the adenyl cyclase stimulatory Gαs, adenyl cyclase inhibitory Gαi, phospholipase Cβ
and RGS-RhoGEF activating Gα12/13 subfamilies. (B) Recombinant EhRGS-RhoGEF protein was immobilized on a surface plasmon
resonance chip and EhG1 protein flowed over in one of two nucleotide states. The EhRGS-RhoGEF biosensor bound EhG1 selectively in the
activated, GDP-AlF4- bound state (AMF).

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down-regulated in trophozoites expressing EhGα1S37C (Table S2). These transcriptional effects suggested that altered cysteine protease activity and/or secretion may be a mechanism by which perturbation of heterotrimeric G-protein signaling modulates Matrigel transmigration and host cell killing (Figs. 5C & 6B). To test this hypothesis, intracellular and secreted cysteine protease activities were each measured in the EhGα1wt and EhGα1S37C strains. EhGα1wt expression increased extracellular and decreased intracellular cysteine protease activity, likely reflecting more efficient vesicular trafficking and secretion (Fig. 7C). In contrast, EhGα1S37C expression resulted in a trend toward more intracellular protease activity, although not statistically significant (p = 0.07), and significantly less extracellular protease activity relative to uninduced control trophozoites, correlating with...
Heterotrimeric G-protein signaling positively regulates *E. histolytica* attachment to host cells as well as host cell killing. (A) Trophozoites attach to CHO cell monolayers, primarily through a galactose-inhibitable lectin. Overexpression of EhGα1^wt^ enhanced monolayer attachment, while expression of EhGα1^S37C^ reduced attachment. Parent strain HM-1:IMSS trophozoites were unaffected by tetracycline treatment and were indistinguishable from non-induced EhGα1^wt^ and EhGα1^S37C^. Attached trophozoites quantities were obtained by multiplying detached cell concentrations by a dilution factor. * indicates a statistically significant difference (p < 0.05) between quadruplicate experiments. Error bars represent standard error of the mean. * indicates statistical significance by an unpaired, two-tailed Student’s t-test (p < 0.05) for four independent experiments. (B) Amoebae overexpressing EhGα1^wt^ or EhGα1^S37C^ displayed enhanced or reduced abilities to kill Jurkat (human T-lymphocyte) cells, respectively, as measured by LDH release in a membrane integrity assay. Cell killing by HM-1:IMSS trophozoites was not altered by tetracycline treatment. 0.5% Triton X-100 was added to Jurkat cells to define 100% host cell lysis. Tetracycline treatment was 5 μg/mL over 24 hours. Error bars represent standard error of the mean. * indicates statistical significance by an unpaired, two-tailed Student’s t-test (p < 0.05) for three independent experiments, with four technical replicates each.

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Figure 7. Heterotrimeric G-protein signaling alters *E. histolytica* transcription to modulate cysteine protease secretion. (A) 96 genes or 394 genes were differentially transcribed upon overexpression of EhGα 1<sub>wt</sub> or EhGα 1<sub>337C</sub>, respectively, when compared to uninduced controls as determined by RNA-seq. 21 transcripts were oppositely regulated in trophozoites expressing EhGα 1<sub>wt</sub> vs EhGα 1<sub>337C</sub>. (B) Differentially transcribed
Heterotrimeric G-proteins Modulate Pathogenesis

genes were categorized by putative function based on prior studies, homology to genes of known function, or predicted protein domains of known function. "Virulence/encystation" category includes genes known to modulate E. histolytica pathogenesis, such as cysteine proteases [48]. (C) Both intracellular and secreted cysteine protease activities were assessed with an azo-collagen assay. EhGα1 overexpression enhanced cysteine protease secretion, while EhGα1 expression resulted in less extracellular (E), despite higher intracellular (I), cysteine protease activity, suggesting that transcriptional responses downstream of heterotrimeric G-protein signaling modulate E. histolytica pathogenic processes in part by regulating cysteine protease secretion. Tetracycline treatment in all experiments was 5 µg/ml over 24 hours. * = statistical significance by an unpaired, two-tailed Student’s t-test (p<0.05) for four independent experiments.

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Discussion

Here we demonstrate that functional heterotrimeric G-protein subunits are encoded by the pathogen Entamoeba histolytica, including single Gα and Gβ subunits, and two Gγ subunits. Like their mammalian counterparts, EhGα1, EhGβ1, and EhGγ1/2 form a nucleotide state-dependent heterotrimer. EhGα1 binds and hydrolyzes GTP and its switch regions undergo a conserved conformational change. When in an activated state, EhGα1 is seen to engage a putative effector protein, namely an RGS domain-containing RhoGEF (EhRhoGEF). EhRGS-RhoGEF likely represents a functional signaling link between heterotrimeric G-proteins and Rho family GTPases in E. histolytica. Indeed, Rho GTPases and other Dih family RhoGEFs in E. histolytica have been implicated in multiple processes important for pathogenesis-related processes such as actin reorganization during chemotaxis, surface receptor capturing, cell killing, phagocytosis, and tissue destruction [24,25,26,27,55].

The sequence of EhGα1 diverges from each of the mammalian Gα subunit subfamilies, including the Gα12/13 subfamily that couples to RGS-RhoGEFs. Thus EhGα1 likely represents an early evolutionary departure from the metazoan Gα/RGS-RhoGEF signaling axis, or possibly a signaling pathway of similar function with an independent evolutionary origin. A search of publicly available genome sequences using SMART [56] identified the RGS and DH-PH domain combinations exclusively in metazoan species, with the only exception being the amoebazoa. This lack of RGS-RhoGEF related proteins in non-metazoan species suggests an independent origin of the E. histolytica Gα/RGS-RhoGEF interaction; however, we cannot rule out the possibility that a Gα/RGS-RhoGEF interaction arose early in evolutionary history, such as an ancestral Unikonta supergroup member (e.g. [57]), and was later lost in fungal species, but retained in metazoans and amoebae. Among the species compared in this study, EhGα1 was found to be most similar in sequence to the D. discoideum Gα9, followed more distantly by S. cerevisiae Gα1 and Gα2, as well as A. thalassae Gα1. This set of Gα subunits is only loosely related by function, with D. discoideum Gα9 regulating cellular proliferation [8], while yeast Gα1 and Gα2 transduce signals in response to pheromones and nutrients, respectively [6]. A variety of downstream signaling machinery is utilized as well, with S. cerevisiae pheromone signaling occurring predominantly through Gβγ subunit effectors, while S. cerevisiae Gα2 engages an adenylyl cyclase effector [6]. The current study clearly differentiates EhGα1 from these relatively similar Gα subunits on the sequence level, demonstrating interaction with an RGS-RhoGEF effector and no significant effect on cellular proliferation, but apparent roles in multiple pathogenesis-related processes of E. histolytica.

Perturbation of heterotrimeric G-protein signaling in E. histolytica trophozoites was observed to modulate migration, Matrigel transmigration, and host cell attachment and killing. Notably, trophozoite Matrigel transmigration is dependent on general migration to some degree, and host cell killing is dependent on attachment. Thus, the effects of heterotrimeric G-protein perturbation on Matrigel transmigration and host cell killing may be partially or wholly due to the alterations in migration and attachment, respectively. Induced expression of the dominant negative EhGα1 impaired these pathogenic processes, suggesting that antagonizing G-protein signaling may reduce E. histolytica virulence. The complete mechanisms by which heterotrimeric G-proteins are linked to specific trophozoite behaviors remain to be elucidated. For instance, it is presently unclear which signaling cascades are utilized to effect transcriptional changes in response to perturbed EhGα1 expression. EhGα1 likely engages its RGS-RhoGEF effector, leading to activation of specific Rho GTPases, some of which are known to regulate cytoskeletal dynamics required for such processes as migration and Matrigel transmigration [24,27,55,58]. EhGβ may also engage as yet unidentified effectors, like its homologs in other species, leading to changes in pathogenic processes [1].

It is presently unclear how heterotrimeric G-protein signaling is activated in E. histolytica. Since nucleotide exchange is the rate-limiting step in the nucleotide cycle of EhGα1, an exchange factor, such as a GPCR, is likely required for high levels of EhGα1 activation. At this time, the only putative GPCR described is the Rab GTPase-binding protein EhGPCR-1 [59]. While it would be compelling to demonstrate receptor-mediated nucleotide exchange on EhGα1, our own bioinformatic analysis revealed that EhGPCR-1, while containing seven-transmembrane spanning regions, is more likely a conserved Wnt-binding factor required for Wnt secretion (as seen in C. elegans) [60]. Identification of a bona fide GPCR/ligand pair or other heterotrimeric G-protein activation mechanism in E. histolytica will provide powerful tools for further probing of the roles of heterotrimeric G-protein signaling in trophozoites.

Materials and Methods

Cloning of E. histolytica G-protein subunits

The open reading frame (ORF) of EhGα1 was amplified from E. histolytica genomic DNA (Dr. M. Vargas, Center of Investigation and Advanced Studies, Mexico City) by polymerase chain-reaction (PCR) using Phusion polymerase (New England BioLabs) and Invitrogen primers. Amplicons were subcloned using ligation-independent cloning [61] into a Novagen pET vector-based prokaryotic expression construct (“PET-His-LIC-C”) to form N-terminal tobacco etch virus (TEV) protease-cleavable, hexahistidine-tagged fusions. Mutations were made using QuickChange site-directed mutagenesis (Stratagene). ORFs of EhGα1, EhGβ1, EhGγ1, and EhGγ2, codon-optimized for mammalian cells, were obtained from Geneart (Regensburg, Germany); EhGα1 with an internal FLAG epitope, DYKDDDDK inserted after His-83, was also obtained for co-immunoprecipitations. Sequences for EhGγ1 and EhGγ2, identified in genomic shotgun sequences were MSQQQLIRLLQEKERLMKNFERSKNLMKVSEACSDLVeFQFQKSFvDPFSEKFDSNPDWKNNCGCCALV and MSQQQLIRLLQEKERLMKNFERSKNLMKVSEACSFNLVPFTK-NKIDPFSEFKDTNPWDDKSNAGCCSLM, respectively.
Protein purification, crystallization, and structure determination

See the Supplementary Methods for details.

Fluorescence complementation and co-immunoprecipitation

Yellow fluorescent protein (YFP) bimolecular fluorescence complementation was performed as described [62] with modifications below. Codon-optimized ORFs of EhGα1 and EhGα2 isoforms were subcloned as HA-tagged fusions to the N-terminal 159 amino acids of YFP-venus (pCDNA3.1-YFP<sub>N</sub>; Dr. Nevin Lambert, MCG). The EhGβ1 ORF was subcloned as an HA-tagged fusion with a C-terminal fragment (residues 159–239) of YFP-venus (pCDNA3.1-YFP<sub>C</sub>; also obtained from Dr. Lambert, along with control YFP<sub>N</sub>-human Gβ1 and YFP<sub>C</sub>-human Gβ1 fusions). 200,000 COS-7 cells per well in 6-well dishes were transfected with 1 μg DNA using FuGENE-6 as per manufacturer’s directions. Empty pCDNA3.1 DNA was used to maintain a constant amount of total DNA per well. Forty-eight hours post-transfection, epifluorescence was observed using an Olympus IX70 microscope with Hamamatsu monochrome CCD camera. Digital images were imported into MATLAB 2007a and quantified as previously described [62]. Pixels with greater than 40 units of intensity were considered to be fluorescent, and the percentage of positive pixels was quantified. All experiments were repeated three times. Co-immunoprecipitation was performed using the YFP-fusion proteins as previously described [62].

Nucleotide binding, hydrolysis, and EhGα1 activation

Spontaneous GDP release, measured by [35S]GTP<sup>γ</sup>S incorporation, and [γ-<sup>32</sup>P]GTP hydrolysis by single turnover assays were both quantified as previously described [37]. For GTPase acceleration assays, increasing concentrations of purified EhRGS-RhoGEF were added along with the hydrolysis-initiating magnesium. Real-time monitoring of EhGα1 tryptophan fluorescence (excitation 280 nm; emission 350 nm) was conducted as described for G<sub>α</sub>1 [37].

Evolutionary analysis

The protein sequences of Gα subunits from humans, S. cerevisiae, A. thaliana, D. melanogaster, and D. discoideum were aligned and an unrooted phylogram derived using T-coffee [63]. Percent amino acid sequence similarities of EhGα1 and S. cerevisiae GPA1 were calculated relative to each human Gα subunit, using a multiple sequence alignment, as described previously [64]. The Gα family of Drosophila melanogaster served as a positive control for subfamily classification.

Surface plasmon resonance

Optical detection of protein binding was conducted as described previously [65]. Brieﬂy, His<sub>6</sub>-tagged EhRGS-RhoGEF was immobilized on an NTA chip surface and increasing concentrations of wildtype EhGα1 and mutants were flowed over at 10 μL/s in various nucleotide states.

Trophozoite stable transfection

EhGα1 and EhGα1<sup>C63S70C</sup> were subcloned with internal FLAG epitope tags into a tetracycline-inducible expression vector, described previously [47]. Asexual cultures were transfected by lipofection as previously described [66]. Brieﬂy, amoebae at ~5×10<sup>7</sup>/mL were suspended in medium 199 (Sigma) supplemented with 5.7 mM cysteine, 1 mM ascorbic acid, 25 mM HEPES (pH 6.9), 15 μg of DNA, and 30 μL of Superfect (Qiagen). After 3 hours at 37°C, trophozoites were transferred to TYI-S-33 medium overnight and selected for stable transfection with 10 μg/mL hygromycin over 3 weeks.

Trophozoite migration and Matrigel transmigration

Trophozoite migration assays were performed essentially as described previously [67]. Brieﬂy, amoebae were grown in the presence or absence of 5 μg/mL tetracycline for 24 hours, harvested in log growth phase, suspended in serum free TYI growth medium, and 50,000 cells loaded in the upper chamber of a Transwell migration chamber (Costar, 8 μm pore size). The lower chamber contained growth medium with or without 15% adult bovine serum. Transwell plates were incubated at 37°C for 2 hr under anaerobic conditions (GasPak EZ, BD Biosciences). Matrigel transmigration assays were performed in similar fashion, except that Matrigel was first diluted to 5 mg/mL in serum free TYI growth medium, layered on the Transwell porous filter, and allowed to gel for 6 hr prior to assay initiation. Incubation time was also extended to 16 hr to allow penetration. Migrated trophozoites attached to the lower chamber wall were detached on ice, ﬁxed, and counted. Each experiment was performed in triplicate and statistical signiﬁcance among four independent experiments was determined by an unpaired, two-tailed Student’s t-test.

Host cell attachment

Attachment of E. histolytica trophozoites to epithelial monolayers was assessed as previously described [68]. Chinese hamster ovary (CHO) cells were grown to confluency in 24-well plates, washed, and fixed in 4% paraformaldehyde for 30 minutes. Trophozoites (3×10<sup>4</sup> cells) grown in the presence or absence of 5 μg/mL tetracycline for 24 hours were added to the ﬁxed monolayers in medium 199 supplemented with 5.7 mM cysteine, 1 mM ascorbic acid, and 25 mM HEPES (pH 6.9). After incubation at 37°C for 30 minutes, each well was washed gently two times with warm PBS to remove unattached trophozoites. Monolayer-attached trophozoites were detached on ice and quantitated by counting with an inverted microscope. In similar experiments, trophozoites were labeled with carboxyﬂuorescein diacetate succinimidyl ester (CFDA-SE). Attached ﬂuorescent trophozoites were counted in three microscopic ﬁelds at 10× magniﬁcation. Each experiment was performed in quadruplicate and statistical signiﬁcance determined by an unpaired two-tailed Student’s t-test.

Cell killing

Killing of mammalian cells (Jurkat) was assessed using the CytoTox-ONE membrane integrity assay (Promega). In 96-well plates, 5×10<sup>5</sup> Jurkat cells and/or 2.5×10<sup>4</sup> trophozoites, grown with or without 5 μg/mL tetracycline for 24 hours, were incubated at 37°C in 200 μL of medium 199 (Sigma) supplemented with 5.7 mM cysteine, 0.5% BSA, and 25 mM HEPES pH 6.8. After 2.5 hr, 50 μL of medium from each well was incubated with Cytotox reagent and a colorimetric measure of extracellular lactate dehydrogenase activity was obtained after 10 min. 0.3% Triton X-100 was used to deﬁne 100% host cell death. Each experiment was performed with five replicates and statistical signiﬁcance among three independent experiments was determined by an unpaired two-tailed Student’s t-test.

Whole transcriptome shotgun sequencing

Total RNA from 10<sup>6</sup> trophozoites each of the tetracycline-induced (5 μg/mL tetracycline for 24 hours) EhGα1<sup>1wt</sup> and EhGα1<sup>C63S70C</sup> strains, as well as a tetracycline-free control, was...
isolated using an RNeasy Mini Kit (Qiagen) per manufacturer’s instructions. Duplicate RNA purifications and sequencing were obtained for each condition.

Quality of total RNA from each sample was estimated by automated electrophoresis (Bioanalyzer, Agilent). Libraries were constructed using TruSeq RNA library preparation kits (Illumina) according to manufacturer’s recommendations; molarity was estimated by analysis of DNA concentration from fluorometer detection and DNA fragment size. Prepared libraries with equal molarity were pooled and used for multiplex sequencing reactions. Libraries were sequenced using 57 cycles in a single end Illumina flowcell v.5 on a HiSeq2000 instrument (Illumina) at the UNC High Throughput Sequencing Facility. Primary data analysis and demultiplexing was performed using a standard Illumina pipeline 1.8.2.

Resulting mRNA sequence reads were mapped to the annotated Entamoeba histolytica genome (AmoebaDB.org) using Bowtie v0.12.7 [69]. Between 12 × 10^6 and 32 × 10^6 reads were aligned for each sample. Aligned reads were further analyzed with Cufflinks v1.3.0 [70] and visualized using the Integrative Genomics Viewer (www.broadinstitute.org/igv/). Cuffdiff was used to determine differential expression by comparing relative transcript abundances between pairs of duplicate experiments: EhGz1^{1wt} expression vs tetracycline-free control, EhGz1^{1S37C} expression vs tetracycline-free control, and EhGz1^{1wt} vs EhGz1^{1S37C} expression. Genes exhibiting statistically significant differential transcription were compiled and corresponding annotations retrieved using software from Dr. Chung-Chau Hon (Institut Pasteur) [71]. Transcripts that were either up- or down-regulated in both the induced EhGz1^{1wt} and EhGz1^{1S37C} strains were excluded from further analysis, because of potential transcriptional modulation due to tetracycline treatment. Functions of the associated proteins were inferred from prior E. histolytica studies, by similarity to mammalian protein families, or from conserved domains of known function. All encoded proteins without annotated conservation and those with domains of unknown function were classified as “unknown”.

Cysteine protease activity

Intracellular cysteine protease activity in amoebic lysates was assayed essentially as described previously [72]. Crude extracts of 10^7 trophozoites, grown with or without 5 μg/mL tetracycline for 24 hours, were obtained by lysing with 5 cycles of freeze-thaw. Total protein concentration was quantified by Bradford’s method. Libraries were sequenced using 57 cycles in a single end Illumina flowcell v.5 on a HiSeq2000 instrument (Illumina) at the UNC High Throughput Sequencing Facility. Primary data analysis and demultiplexing was performed using a standard Illumina pipeline 1.8.2.

Acquisition numbers for proteins used in this study

EhGz1, AmoebaDB EHI_140350; EhGβ1, AmoebaDB EHI_000240; glyceraldehyde-3-phosphate dehydrogenase, AmoebaDB EHI_167320; EhRGS-RhoGEF, AmoebaDB EHI_010670. EhGγ1, identified within the NCBI genomic contig AAFB02000029.1; EhGγ2, identified within the NCBI genomic contig AAFB02000157.1; amoebapore A, AmoebaDB EHI_159480; cysteine protease, AmoebaDB EHI_006920.

Supporting Information

Figure S1 The genome of Entamoeba histolytica encodes heterotrimeric G-protein subunits. (A) A multiple sequence alignment of EhGz1 with selected Gα subunits from other species (Dd = Dictyostelium discoideum, Sc = Saccharomyces cerevisiae, Hs = Homo sapiens). The secondary structure information above the aligned sequences reflects the crystal structure of EhGz1 (this study), with naming adapted from human transducin (PDB 1TND). Residues mutated in this study are marked with black arrowheads, and gray bars indicate relative sequence identity. A 110-residue insert within Sc GZα, based on the sequence of the amino terminus of EhGz1, is likely that this protein is myristoylated on its second residue (glycine) and palmitoylated on its third residue (cysteine) [33]. (B) EhGβ1 is aligned with selected Gβ subunits in a fashion identical to panel A with secondary structure elements as found in transducin Gβγ (PDB 1TBG).

Figure S2 Heterotrimeric G-protein signaling components are expressed in E. histolytica. qRT-PCR amplification of RNA isolated from HMI E. histolytica trophozoites (a kind gift of Dr. William Petri, Jr.) confirmed transcription of EhGz1, EhGβ1, EhGγ1, and EhRGS-RhoGEF genes. The basally expressed housekeeping gene GAPDH was used as a control. AC reflects the difference in threshold cycle relative to reactions lacking reverse transcriptase, used as a control for DNA contamination. Error bars represent standard error of the mean.

Figure S3 Example bimolecular fluorescence complementation micrographs. YFP fluorescence was detected microscopically in COS-7 cells expressing heterotrimeric G-protein subunits. YFP complementation was observed when EhGz1 was co-expressed with EhGβ1 and EhGγ1 (A, B) or EhGγ2 (C, D). The human subunits Gβ1 and Gγ2 exhibited complementation, while the expressed N- and C-terminal fragments of YFP did not (E, F). For a quantification of fluorescence, see Figure 1.

Figure S4 The inactive EhGz1(S37C) constitutively binds to EhGβ1γ2, while the constitutively active EhGz1(Q189L) mutant does not. Co-immunoprecipitations of EhGz1 and mutants with EhGβ1 and EhGγ2 were conducted as in Figure 1. As predicted, the dominant negative S37C mutant remains bound to EhGβ1γ2, even in excess GTPγS. The constitutively active, GTPase-deficient Q189L mutant does not bind EhGβ1γ2 in either nucleotide state.

Figure S5 Mammalian Gα subfamily homology analyses. Sequence similarity to human Gα subunits was plotted for the
Figure S6 Structural comparison of EhGtl with Hs transducin and switch 2 crystal contacts. (A) The two EhGtl molecules in the asymmetric unit are highly similar, although switch 2 of chain B (wheat) is partially disordered. (B) Crystal contacts between the ordered switch 2 of chain A (blue) and a neighboring molecule (orange) likely account for the structural differences between the two molecules in the asymmetric unit. The non-polar Trp-196 and N-dimethyl lysine-195 (MLY-195) interface with a hydrophobic patch on a neighboring molecule. Switch 2 may be drawn away from the nucleotide pocket, accounting for the absence of AlF4− (see discussion below). (C, D) The model of EhGtl is superposed with human transducin in two nucleotide states (slate blue, AMF, PDB 1TAD; teal, GDP, PDB 1TADG). EhGtl lacks an zB helix seen in transducin and all other Gt subunits and contains a unique z4–z6 insert (orange). Switch 2 of EhGtl (chain A) adopts a distinct conformation from both the active and inactive forms of transducin, likely due to crystal contacts with a neighboring molecule. (EPS)

Figure S7 Electron density map of guanine nucleotide binding pocket of EhGtl. A region of the 2Fo-Fc electron density map is shown in stereo view from the structure of EhGtl (yellow sticks) bound to GDP (purple sticks). The nucleotide binding pocket is highly similar to mammalian Gt subunits, featuring a conserved phosphate binding loop (P-loop; Glu-33 shown) and an NXSxD motif (residues 254–257). Switch one also directly contacts the nucleotide, and Arg-163 forms polar contacts with the P-loop Glu-33. (EPS)

Figure S8 Expression of EhGtlWM or EhGtlS37C does not significantly alter trophozoite proliferation. (A) The cellular distribution of overexpressed FLAG-EhGtlWM and FLAG-EhGtlS37C were assessed by immunofluorescence with a Cy3 anti-FLAG conjugate. Both wild type and mutant EhGtl exhibited similar diffuse cytoplasmic localizations following induced expression by treatment with 5 µg/mL tetracycline for 24 hr. Nuclei were stained with DAPI. (B) Trophozoites from the parent HM-1:IMSS, EhGtlWM, and EhGtlS37C strains were seeded in TYI medium with or without 5 µg/mL tetracycline and cell numbers assessed over 3 days. Cell viability was >90% at each measurement, as determined by trypan blue dye exclusion. No significant differences in growth were identified among the strains, although trophozoites induced to express EhGtlS37C tended toward slower growth at day 3. Error bars represent standard error of the mean for three independent experiments. (EPS)

Figure S9 E. histolytica transfected with empty vector is not affected by tetracycline treatment. HM-1:IMSS trophozoites were stably transfected with empty tetracycline-inducible expression vector. (A) Transwell migration and (B) Matrigel transmigration of parent strain and vector-transfected trophozoites did not differ significantly upon tetracycline treatment of 24 hours prior to the assay. Similarly, transfection with empty vector and tetracycline treatment had no significant effect on host cell attachment (C) or host cell killing (D). Error bars represent standard error of the mean for four independent experiments in panels A–C and three independent experiments in panel D. Statistical significance was tested using an unpaired, two-tailed Student’s t-test. (EPS)

Figure S10 Microscopic analysis of perturbed E. histolytica attachment to host cells upon overexpression of EhGtlWM or EhGtlS37C. (A) Trophozoites grown in the presence or absence of 5 µg/mL tetracycline were fluorescein-labeled with CFDA and allowed to attach to fixed, confluent layers of CHO cells. Phase contrast (upper panels) and epifluorescence (lower panels) images were obtained of attached trophozoites. (B) Attachment was quantified by counting trophozoites in three microscopic fields (10×). Overexpression of EhGtlWM enhanced monolayer attachment, while expression of EhGtlS37C reduced attachment. Parent strain HM-1:IMSS trophozoites were unaffected by tetracycline treatment and were indistinguishable from non-induced EhGtlWM and EhGtlS37C. Error bars represent standard error of the mean. * represents statistical significance by an unpaired, two-tailed Student’s t-test (p<0.05) for three independent experiments. (EPS)

Figure S11 RT-PCR analysis of differentially transcribed genes and altered expression of amoebapore A protein. (A) qRT-PCR amplification of RNA isolated from HM1 E. histolytica trophozoites confirmed differential transcription of EhGtl1, EhGβ1, amoebapore A, and a cysteine protease (EH1_006920) upon tetracycline treatment of the parent HM-1:IMSS, EhGtl1WM, or EhGtl1S37C strains over 24 hours. * indicates statistically significant difference from time zero (no tetracycline exposure), using an unpaired, two-tailed Student’s t-test for two technical duplicates of two independent experiments. EhGtl1 expression was significantly up-regulated in the EhGtl1WM and EhGtl1S37C strains, while EhGβ1 was up-regulated and amoebapore A and cysteine protease (EH1_006920) were down-regulated upon expression of EhGtl1S37C. (B) Trophozoite lysates were subjected to western blotting with anti-amoebapore A (kind gift of M. Leippe, U. of Kiel, Germany), with actin serving as a loading control. Amoebapore A protein expression is reduced in parallel with its transcriptional downregulation upon overexpression of EhGtl1S37C. (EPS)

Table S1 Data collection and refinement statistics for lysine-methylated selenomethionine EhGtl1. (PDF)

Table S2 Genes differentially transcribed in E. histolytica trophozoites expressing EhGtl1 or EhGtl1S37C with known roles in pathogenesis or putative vesicular trafficking functions. (PDF)

Text S1 Supplementary materials and methods. (DOC)

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Author Contributions
Conceived and designed the experiments: DEB AJK REM MM BRST. Analyzed the data: DEB AJK REM MM BRST. Performed the experiments: DEB AJK REM MM BRST. Analyzed the data: DEB AJK REM MM BRST. Conceived and designed the experiments: DEB AJK FSW DPS. Performed the experiments: DEB AJK REM MM BRST. Wrote the paper: DEB AJK DPS.
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