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(57) Abstract: Cloning and characterization of a TgIF2 α kinase from *Toxoplasma gondii* designated TgIF2K-D illustrates that this protein is related to GCN2, an eIF2 α kinase known to respond to nutrient starvation in other organisms. TgIF2K-D is present in the cytosol of both intra- and extracellular *Toxoplasma* and facilitates translational control through TgIF2 α phosphorylation in extracellular parasites. Both a TgIF2K-D knockout parasite and a parasite harboring the TgIF2 α mutant (S71A substitution) exhibited loss of eIF2 α kinase activity which manifested itself as significant fitness defect. Accordingly, eIF2 α phosphorylation and translational control are an important mechanism by which vulnerable extracellular parasites protect themselves while searching for a new host cell. TgIF2K-D is an excellent target for the development of compounds and therapies that can be used to treat infections caused by *Toxoplasma* and other eukaryotic parasites, especially parasites that have high homology or identity to TgIF2K-D.



**MUTANT PARASITES FOR USE AS VACCINES AND PLATFORMS FOR
SCREENING FOR COMPOUNDS**

PRIORITY CLAIM

[0001] This application claims the benefit of U. S. provisional patent application serial number 61/351,389 filed on June 4, 2010 which is incorporated herein by reference in its entirety.

STATEMENT OF GOVERNMENTAL RIGHTS

[0002] This invention was made with government support under A1084031 and GM049164 awarded by the National Institutes of Health. The U.S. government has certain rights in the invention.

FIELD OF THE INVENTION

[0003] Aspects of the invention relate to selecting for and using novel strains of *Toxoplasma* parasites that include nonphosphorylatable TgIF2 α and TgIF2 α and homologous proteins to identify compounds that can be used to treat and/or control infections caused by organisms such as *Toxoplasma gondii*.

BACKGROUND

[0004] Parasites that have adapted to live and replicate within another cell benefit from abundant resources, protection from host immunity, and shelter from therapeutic agents. As the demands of the parasites exceed what the host cell can supply, the parasites must find a new host cell, a journey that can leave them vulnerable to nutrient deprivation and environmental stresses. Protozoa in the phylum Apicomplexa are parasites that require a eukaryotic host cell in order to replicate. *Toxoplasma gondii* is one such obligate intracellular parasite, capable of using virtually all warm-blooded vertebrates as host organisms (Hill *et al.*, 2005). Acute *Toxoplasma* infection can cause spontaneous abortion or congenital birth defects, as well as severe disease in immunocompromised patients. This disease is widespread in the developing world and poses a significant risk to both humans and animals.

[0005] Currently, there is no effective vaccine for treating humans and only some partially effective vaccines available to treat animals. Current treatments consist of anti-folates that are problematic due to toxicity issues; therefore there is an urgent need to develop novel therapies to treat this parasitic infection. There is a dearth of effective treatments for infected humans and

animals. Accordingly, there is a pressing need for compounds to vaccinate vulnerable populations and to treat infected individuals. Some aspects of the instant invention seek to address these needs.

SUMMARY

[0006] Some embodiments of the invention include methods of identifying a compound, comprising the steps of: providing at least one translation initiation factor that is phosphorylated by a kinase and identified in a singled celled eukaryotic parasite. In some methods the factor and its kinase are found in a single celled parasite selected from the genera consisting of *Toxoplasma*, *Plasmodium*, and *Cryptosporidium*. In some embodiments the translation initiation factor is phosphorylated by an eIF2 α kinase selected from the group consisting of: TgIF2K-A, TgIF2K-B, TgIF2K-C, and TgIF2K-D.

[0007] In some embodiments the eIF2 α kinase that serves as the target for the compound search has greater than or equal to about 90 percent homology to TgIF2K-D identified in the parasite *Toxoplasma gondii*. In some embodiment the target has about 95 percent homology to TgIF2K-D. In some embodiments the target has about 85 percent or greater identity to TgIF2K-D. In some embodiments the target has about 95 percent or greater identity to TgIF2K-D. In still other embodiments, the target has about 85 percent or greater identity to TgIF2K-D as identified in *Toxoplasma gondii*. And in still other embodiments, the target has about 95 percent or greater identity to TgIF2K-D identified in *Toxoplasma gondii*. In yet more embodiments the target kinase is TgIF2K-D identified in *Toxoplasma gondii*.

[0008] In some embodiments compounds are screened for their ability to bind to either the translation initiation factor for example a protein with at least 90 percent homology to the translation initiation factor TgIF2 α identified in the parasite *Toxoplasma gondii*. In some embodiments the target is a protein having at least 90 percent homology to the translation initiation factor TgIF2 α identified in *Toxoplasma gondii*. In some embodiments the target has at least 95 percent homology to TgIF2 α identified in *Toxoplasma gondii*. In some embodiments the target has between 80 to 95 percent identity to TgIF2 α identified in *Toxoplasma gondii*.

[0009] Some aspects of the invention include methods for reducing the infectivity of a singled celled eukaryotic parasite, comprising the steps of: contacting a single celled eukaryotic parasite with at least one compound that interferes with the activity of at least one eIF2 α kinase

associated with the parasite, or of genetically engineering a parasite to exhibit a reduction or a loss of activity caused by altering or knocking out at least one protein having eIF2 α activity.

[0010] Still other embodiments include methods for reducing the infectivity of a single celled eukaryotic parasite, comprising the steps of: providing a single celled eukaryotic parasite, wherein the parasite includes at least one eIF2 α kinase and at least one translation initiation factor, wherein the at least one kinase phosphorylates the at least one translation factor; and contacting the parasite with at least one compound that interferes with or eliminates the phosphorylation of the translational initiation factor.

[0011] In some embodiments a compound acts by interfering or preventing the phosphorylation of at least one translation initiation factor such as TgIF2 α identified in *Toxoplasma gondii*. In some embodiments the translation initiation factor that binds to the compounds has between 75 to 95 percent identity to TgIF2 α identified in *Toxoplasma gondii*. In some embodiments the phosphorylated translation initiation factor has at least 90 to 95 percent homology to TgIF2 α identified in *Toxoplasma gondii*.

[0012] In some embodiments translation initiation is reduced by altering the activity of at least one eIF2 α kinase associated with the parasite. In some embodiments the compounds act by binding to, and interfering with, at least one kinase selected from the groups consisting of: TgIF2K-A, TgIF2K-B, TgIF2K-C, and TgIF2K-D.

[0013] In some embodiments the compounds act by binding to at least one kinase having at least 90 percent homology to TgIF2K-D identified in the parasite *Toxoplasma gondii*.

[0014] In some embodiments the eIF2 α kinase that binds to the compound has greater than or equal to about 90 percent homology to TgIF2K-D identified in the parasite *Toxoplasma gondii*. In some embodiment the target has about 95 percent homology to TgIF2K-D. In some embodiments the target has about 85 or greater identity to TgIF2K-D. In some embodiments the kinase that binds to the inhibitor has about 95 percent or greater identity to TgIF2K-D. In still other embodiments, the kinase has about 85 percent or greater identity to TgIF2K-D as identified in *Toxoplasma gondii*. And in still other embodiments the kinase has about 95 percent or greater identity to TgIF2K-D identified in *Toxoplasma gondii*. In yet more embodiments the target kinase is the same TgIF2K-D identified in *Toxoplasma gondii*.

[0015] In some embodiments of the invention the parasitic infection that is treated by interfering with the translation initiation factor is selected the genera consisting of; *Toxoplasma*,

Plasmodium and *Cryptosporidium*. In some embodiments the compound acts by reducing or eliminating the activity of at least one protein that has at least 90 percent homology to TgIF2 α identified in *Toxoplasma gondii*.

[0016] Some aspects of the invention provide methods for vaccinating a patient, comprising the steps of: providing a mutant of a single celled organism selected from a group of parasites consisting of *Toxoplasma*, *Plasmodium*, and *Cryptosporidium*, wherein said mutant is deficient in eIF2 α kinase activity; and contacting a mammal with said mutant form. In some embodiments the parasite is *Toxoplasma gondii*. In some embodiments the mutant eIF2 α kinase has greater than or equal to about 95 percent homology to at least one kinase selected from the group consisting of TgIF2K-A, TgIF2K-B, TgIF2K-C, and TgIF2K-D. In some embodiments the mutant parasite lacks virtually all TgIF2K-D activity.

[0017] In some embodiments the parasite used is either alive or killed in the vaccine and includes an eIF2 α kinase having greater than or equal to about 75 percent identity to TgIF2K-D; in some embodiments it has about 85 percent identity to TgIF2K-D and in some embodiments it is TgIF2K-D. In some embodiments the organism is a knockout of TgIF2K-D or of a similar protein having at least 90 percent homology or at least 75 percent identity to TgIF2K-D identified in *Toxoplasma gondii*.

[0018] In some embodiments that parasite used is either alive or killed in the vaccine and includes a translation factor that cannot be phosphorylated by wild type kinase having greater than or equal to about 85 percent identity to TgIF2 α -S71A. In some embodiments it has about 85 percent identity to TgIF2 α -S71A, while in other embodiments the translation factor has greater than 90 or 95 percent identity to TgIF2 α -S71A. In some embodiments the patient that is either vaccinated against or treated for an infection caused by a single celled eukaryotic parasite is an animal selected from the group of animals consisting of: humans, ovines, felines, and bovines. While seeking a new host cell, obligate intracellular parasites, such as the protozoan *Toxoplasma gondii*, must be able to endure the extracellular environment. The mechanisms *Toxoplasma* utilizes to remain viable while deprived of a host cell are not well understood. Phosphorylation of eukaryotic initiation factor-2 alpha is a well conserved stress response. A *Toxoplasma* harbouring a point mutation (S71A) in eIF2 α cannot be phosphorylated, as in the wild-type, was generated. Experiments with the mutant organism demonstrate that TgIF2 α phosphorylation is critical for parasite viability as TgIF2 α -S71A mutants are ill-equipped to

cope with life outside the host cell. For example organisms bearing the TgIF2 α -S71A mutation demonstrate a significant delay in the onset of acute toxoplasmosis *in vivo*. These results indicate that the phosphorylation of TgIF2 α plays a crucial role during the lytic cycle of these organisms. Phosphorylation of TgIF2 α may help to ameliorating some of the stress imposed on the parasite when it is exposed to the extracellular environment while it searches for a new host cell to invade.

[0019] Some aspects of the invention include, mutant eukaryotic single cell parasites, comprising a mutant form of a translation factor, wherein the mutant form of the translation factor includes at least one amino acid substitution, wherein the amino acid substitution results in a form of TgIF2 having a different phosphorylation pattern than a wild type isoform of the translation factor, wherein the translation factor is TgIF2. In some embodiments, the parasite is *Toxoplasma*, for example, *Toxoplasma gondii*. In some embodiments, the parasite is selected from the group consisting of *Plasmodium* and *Cryptosporidium*. In some embodiments, a mutation in the parasite's translation factor reduces the parasite's ability to exist outside of a host cell and decreases the virulence of the parasite in the mouse model of infection. In some embodiments, a mutation is in the translation factor TgIF2, referred to as TgIF2 α -S71A. In some of these mutants, the single cell eukaryotic parasite harbouring the mutant form of the translation factor TgIF2 is similar to a parasite harbouring wild type TgIF2 in motility, invasion, replication, or egress but not its ability to survive outside of a host cell.

[0020] Still other aspects of the invention include methods for vaccinating a patient, comprising the steps of: providing a mutant form of a single cell eukaryotic parasite according to claim 1; supplying a mammal; and contacting the mammal with said mutant form of a single cell eukaryotic parasite. In some embodiments, the mammal is selected from the group consisting of: ovines, felines, and bovines and some embodiments the mammal is a human. In some embodiments, the single cell eukaryotic parasite is *Toxoplasma gondii*.

[0021] Still other aspects of the invention include methods for controlling a single celled eukaryotic parasite, comprising the steps of: identifying a single celled eukaryotic parasite, wherein the parasite has a translation complex and the translation complex has a wild type phosphorylation pattern; and altering the phosphorylation pattern of the translation complex in the single celled eukaryotic parasite. In some embodiments, the single eukaryotic parasite to be altered or controlled is selected from the group consisting of: *Toxoplasma*, *Plasmodium*, and

Cryptosporidium. In some embodiments, altering the parasite includes introducing a change into the genome of the parasite. In still other embodiments, altering the parasite may include providing at least one compound that interferes with the phosphorylation pattern of at least one component of the translation complex, e.g. contacting at least one component of the translation complex with a compound that inhibits either phosphorylation or de-phosphorylation. In some embodiments the compound is salubrinal.

BRIEF DESCRIPTION OF THE SEQUENCES

- [0022] SEQ. ID NO. 1 provides primer 5' S71A for + *NotI*.
- [0023] SEQ. ID NO. 2 provides primer 5' S71A rev + *BamHI*.
- [0024] SEQ. ID NO. 3 provides primer 3' S71A for + *BclI*.
- [0025] SEQ. ID NO. 4 provides primer 3' S71A rev + *BclI*.
- [0026] SEQ. ID NO. 5 provides primer TgIF2 α S71A quikchange 1F.
- [0027] SEQ. ID NO. 6 provides primer TgIF2 α S71A quikchange 1R.
- [0028] SEQ. ID NO. 7 provides primer S71A screen for.
- [0029] SEQ. ID NO. 8 provides primer S71A screen rev.
- [0030] SEQ. ID NO. 9 provides primer TgIF2 α Taqman for.
- [0031] SEQ. ID NO. 10 provides primer TgIF2 α Taqman rev.
- [0032] SEQ. ID NO. 11 provides primer FAM-WT TgIF2 α .
- [0033] SEQ. ID NO. 12 provides primer VIC-TgIF2 α -S71A.
- [0034] SEQ. ID NO. 13 provides the cDNA sequence for TgIF2KD.
- [0035] SEQ. ID NO. 14 provides an alignment of TgIF2K-D protein kinase domain with other eIF2 α kinases.
- [0036] SEQ. ID NO. 15 provides an alignment of TgIF2K-C protein kinase domain with other eIF2 α kinases.
- [0037] SEQ. ID NO. 16 provides an alignment of NcIF2K-D protein kinase domain with other eIF2 α kinases.
- [0038] SEQ. ID NO. 17 provides an alignment of Pflk1 protein kinase domain with other eIF2 α kinases.
- [0039] SEQ. ID NO. 18 provides an alignment of Pf14_0264 protein kinase domain with other eIF2 α kinases.

- [0040] SEQ. ID NO. 19 provides an alignment of PbeIK1 protein kinase domain with other eIF2 α kinases.
- [0041] SEQ. ID NO. 20 provides an alignment of TbeIF2K1 protein kinase domain with other eIF2 α kinases.
- [0042] SEQ. ID NO. 21 provides an alignment of AtGCN2 protein kinase domain with other eIF2 α kinases.
- [0043] SEQ. ID NO. 22 provides an alignment of DmGCN2 protein kinase domain with other eIF2 α kinases.
- [0044] SEQ. ID NO. 23 provides an alignment of ScGCN2 protein kinase domain with other eIF2 α kinases.
- [0045] SEQ. ID NO. 24 provides an alignment of MmGCN2 protein kinase domain with other eIF2 α kinases.
- [0046] SEQ. ID NO. 25 provides an alignment of HsGCN2 protein kinase domain with other eIF2 α kinases.
- [0047] SEQ. ID NO. 26 provides an alignment of TgIF2K-D with RWD domains from other GCN2 protein kinases.
- [0048] SEQ. ID NO. 27 provides an alignment of NcIF2K-D with RWD domains from other GCN2 protein kinases.
- [0049] SEQ. ID NO. 28 provides an alignment of Pf14_0264 with RWD domains from other GCN2 protein kinases.
- [0050] SEQ. ID NO. 29 provides an alignment of MmGCN2 with RWD domains from other GCN2 protein kinases.
- [0051] SEQ. ID NO. 30 provides an alignment of DmGCN2 with RWD domains from other GCN2 protein kinases.
- [0052] SEQ. ID NO. 31 provides an alignment of ScGCN2 with RWD domains from other GCN2 protein kinases.
- [0053] SEQ. ID NO. 32 provides an alignment of the C-terminal homology region of TgIF2K-D with other predicted GCN2-like protein kinases from parasites.
- [0054] SEQ. ID NO. 33 provides an alignment of the C-terminal homology region of NcIF2K-D with other predicted GCN2-like protein kinases from parasites.

- [0055] SEQ. ID NO. 34 provides an alignment of the C-terminal homology region of Cmu_027700 with other predicted GCN2-like protein kinases from parasites.
- [0056] SEQ. ID NO. 35 provides an alignment of the C-terminal homology region of PF14_0264 with other predicted GCN2-like protein kinases from parasites.
- [0057] SEQ. ID NO. 36 provides an alignment of the C-terminal homology region of Pb_101620 with other predicted GCN2-like protein kinases from parasites.
- [0058] SEQ. ID NO. 37 provides an alignment of the C-terminal homology region of PkH_113740 with other predicted GCN2-like protein kinases from parasites.
- [0059] SEQ. ID NO. 38 provides an alignment of the C-terminal homology region of Pvx_085120 with other predicted GCN2-like protein kinases from parasites.
- [0060] SEQ. ID NO. 39 provides the TglF2K-D primer 1.
- [0061] SEQ. ID NO. 40 provides the TglF2K-D primer 2.
- [0062] SEQ. ID NO. 41 provides the TglF2K-D primer 3.
- [0063] SEQ. ID NO. 42 provides the TglF2K-D primer 4.
- [0064] SEQ. ID NO. 43 provides the TglF2K-D primer 5.
- [0065] SEQ. ID NO. 44 provides the TglF2K-D primer 6.
- [0066] SEQ. ID NO. 45 provides the TglF2K-D primer 7.
- [0067] SEQ. ID NO. 46 provides the TglF2K-D primer 8.
- [0068] SEQ. ID NO. 47 provides the TglF2K-D primer 9.
- [0069] SEQ. ID NO. 48 provides the TglF2K-D primer 10.
- [0070] SEQ. ID NO. 49 provides the TglF2K-D primer 11.
- [0071] SEQ. ID NO. 50 provides the TglF2K-D primer 12.
- [0072] SEQ. ID NO. 51 provides the TglF2K-D primer 13.
- [0073] SEQ. ID NO. 52 provides the TglF2K-D primer 14.
- [0074] SEQ. ID NO. 53 provides the TglF2K-D primer 15.
- [0075] SEQ. ID NO. 54 provides the TglF2K-D primer 16.
- [0076] SEQ. ID NO. 55 provides the TglF2K-D primer 17.
- [0077] SEQ. ID NO. 56 provides the TglF2K-D primer 18.
- [0078] SEQ. ID NO. 57 provides the TglF2K-D primer 19.
- [0079] SEQ. ID NO. 58 provides the TglF2K-D primer 20.
- [0080] SEQ. ID NO. 59 provides the TglF2K-D primer 21.

- [0081] SEQ. ID NO. 60 provides the TglF2K-D primer 21N.
[0082] SEQ. ID NO. 61 provides the TglF2K-D primer 22.
[0083] SEQ. ID NO. 62 provides the TglF2K-D primer 22N.
[0084] SEQ. ID NO. 63 provides the TglF2K-D primer 23.
[0085] SEQ. ID NO. 64 provides the TglF2K-D primer 23N.
[0086] SEQ. ID NO. 65 provides the TglF2K-D primer 24.
[0087] SEQ. ID NO. 66 provides the TglF2K-D primer 25.
[0088] SEQ. ID NO. 67 provides the TglF2K-D primer 26.
[0089] SEQ. ID NO. 68 provides the TglF2K-D primer 27.
[0090] SEQ. ID NO. 69 provides the TglF2K-D primer 28.
[0091] SEQ. ID NO. 70 provides the TglF2K-D primer 29.

BRIEF DESCRIPTION OF THE FIGURES

- [0092] FIG. 1A. Diagram depicting the relevant portion of the TglF2 α genomic locus and the TglF2 α -S71A allelic replacement vector.
[0093] FIG. 1B. Agarose gel showing wild type and S71A alleles measured in mutation.
[0094] FIG. 1C. Western blot of WT and S71A lysates showing that S71A cannot be phosphorylated.
[0095] FIG. 2A. Diagram of parasite fitness assay.
[0096] FIG. 2B. Bar graph of the percentage of WT and S71A parasites in co-culture at days 0, 3, 6 and 9.
[0097] FIG. 3. Results of attachment and invasion assays run on WT and S71A parasites.
[0098] FIG 4A. Photo micrograph of trails measured with WT and S71A mutants (right); graph of the percentage of parasites with trails (left).
[0099] FIG. 4B. Graph of % egress versus time (mins.) for both wild type and S71A parasites.
[00100] FIG 5A. Coomassie Brilliant Blue Stained plaques created with either wild type or S71A parasites (Top); measured 8, 16, 24 and 32 hrs. post inoculation (bottom).
[00101] FIG. 5B. Graph of parasites/vacuole measured for both WT and S71A parasites at three different times.
[00102] FIG. 5C. Western blot of lysates.

- [00103] **FIG. 5D.** SDS-PAGE autoradiograph [³⁵S]met/cys. translation rates.
- [00104] **FIG. 6.** The domain structure of TgIF2K-D.
- [00105] **FIG. 7A.** TgIF2K-D_{3xHA} protein was detected by western blotting by probing parasite lysates with anti-HA antibody.
- [00106] **FIG. 7B.** Western blot of TgIF2K-D_{3xHA} parasites with antibodies specific for total TgIF2 α or phosphorylated TgIF2 α (TgIF2 α -P) during 0, 4, or 8 hours extracellular stress.
- [00107] **FIG. 7C.** Immunofluorescence analysis using a rat monoclonal HA-antibody and an anti-rat Alexa488 conjugate (green) was performed to show localization of TgIF2K-D_{3xHA} protein in intra- and extracellular parasites.
- [00108] **FIG. 8A.** The *TgIF2K-D* genomic locus, depicted with 18 exons, was replaced by a minigene conferring resistance to pyrimethamine (DHFR*) using homologous recombination in $\Delta Ku80$ RH strain parasites.
- [00109] **FIG. 8B.** Genomic PCR assays used gDNA harvested from WT or $\Delta IF2K-D$ parasites and the indicated primers to validate replacement of the *TgIF2K-D* genomic locus.
- [00110] **FIG. 8C.** The absence of *TgIF2K-D* mRNA in the $\Delta IF2K-D$ parasites was confirmed by RT-PCR.
- [00111] **FIG. 9A.** Wild-type (WT), TgIF2K-D_{2xDD} (DD), and $\Delta IF2K-D$ parasites were exposed for 0 or 8 hours (h) to the extracellular environment.
- [00112] **FIG. 9B.** WT and $\Delta IF2K-D$ tachyzoites were treated with 5 μ M calcium ionophore A23187 for 30 minutes and then analyzed for TgIF2 α -P by immunoblotting.
- [00113] **FIG. 9C.** WT, TgIF2 α -S71A and $\Delta IF2K-D$ were physically released from host cells and incubated for 1 or 8 h in DMEM culture medium.
- [00114] **FIG. 10A.** TgIF2K-D contributes to the fitness of *Toxoplasma* tachyzoites Schematic of the “head-to-head” fitness assay.
- [00115] **FIG. 10B.** Map of primers (arrows) used to distinguish between wild-type (WT) and $\Delta IF2K-D$ parasites.
- [00116] **FIG. 11A.** 500 wild-type (WT), TgIF2K-D_{2xDD} (DD), $\Delta IF2K-D$, or TgIF2 α -S71A (S71A) parasites physically released from host cells were incubated extracellularly in DMEM culture medium for the designated times before being allowed to infect HFF monolayers in 12-well plates.
- [00117] **FIG. 12.** cDNA sequence *TgIF2K-D*.

- [00118] **FIG. 13.** Oligonucleotides used to determine the start site of the *TgIF2K-D* gene.
- [00119] **FIG. 14.** Alignment of TgIF2K-D protein kinase domain with other eIF2 α kinases.
- [00120] **FIG. 15.** Alignment of TgIF2K-D with RWD domains from other GCN2 protein kinases.
- [00121] **FIG. 16.** Alignment of the C-terminal homology region of TgIF2K-D with other predicted GCN2-like protein kinases from parasites.
- [00122] **FIG. 17.** SDS-PAGE and normalized for western blotting using a monoclonal anti-HA antibody.

DESCRIPTION

[00123] For the purposes of promoting an understanding of the principles of the novel technology, reference will now be made to the preferred embodiments thereof, and specific language will be used to describe the same. It will nevertheless be understood that no limitation of the scope of the novel technology is thereby intended; such alterations, modifications, and further applications of the principles of the novel technology being contemplated as would normally occur to one skilled in the art to which the novel technology relates are within the scope of the claimed invention.

[00124] Unless clearly stated or implied otherwise the term "about" as used herein means plus or minus 10 percent. For example, the term about 1.0 encompasses a range of values from 0.9 to 1.1.

[00125] The lytic cycle of *Toxoplasma* tachyzoites can be described as comprising three discrete stages: adherence to a host cell, invasion, replication, exit from host cell (egress), and movement to a new host cell (Black and Boothroyd, 2000). Tachyzoites remain viable for only a limited time outside of the host cell; the ability of freshly egressed parasites to infect a new host cell monolayer drops significantly between 6-12 hrs of exposure to the extracellular environment (Khan *et al.*, 2009). The mechanisms that the parasite may invoke in order to cope with the extracellular environment while it searches for a new host cell are not well known.

[00126] One well characterized stress response pathway conserved in many eukaryotic cells types involves translational control by virtue of the phosphorylation of the alpha subunit of eukaryotic initiation factor-2 (eIF2 α) (Sonenberg and Hinnebusch, 2009; Wek *et al.*, 2006). The protein eIF2-GTP escorts Met-tRNA_i to the translational machinery for eventual placement into the P-site of ribosomes (Sonenberg and Hinnebusch, 2009); however, in many organisms when it

is phosphorylated at a regulatory serine (serine-51), eIF2 becomes an inhibitor of its guanine nucleotide exchange factor, eIF2B. Consequently, global translation initiation is reduced when eIF2 α is phosphorylated which, in turn decrease the synthesis of the current proteome. Globally decreasing the amount of protein synthesised by the cell enables the cell to conserve energy and it may aid the cell in reprogramming its gene translation pattern as necessary to address the conditions that are stressing the cell (Wek and Cavener, 2007a). Four eIF2 kinases have been identified in mammals that phosphorylate eIF2 α in response to stress (Wek and Cavener, 2007b; Wek *et al.*, 2006): HRI (EIF2KA1), which responds to heme-deficiency and oxidation stress; PKR (EIF2KA2), which is involved in anti-viral defences; PEK/PERK (EIF2KA3), which is activated by endoplasmic reticulum (ER) stress; and GCN2 (EIF2KA4), which responds to nutrient deprivation. An eIF2 α orthologue, as well as four TgIF2 α kinases (TgIF2K-A through -D) were identified in *Toxoplasma* (TgIF2 α). (Narasimhan *et al.*, 2008a; Sullivan Jr. *et al.*, 2004a). While TgIF2K-C and -D are most closely related to GCN2, TgIF2K-A in the parasite is localized to its ER where it likely mediates the activation of the unfolded protein response (UPR) analogous to PEK/PERK (Narasimhan *et al.*, 2008a). TgIF2K-B is a novel eIF2 kinase that is not compartmentalized and likely responds to cytoplasmic stresses. Homologues of GCN2 (PfeIK1) and TgIF2K-A (PfPK4) have also been described in *Plasmodium* (malarial) parasites (Fennell *et al.*, 2009; Mohrle *et al.*, 1997).

[00127] The importance of eIF2 α phosphorylation in general and gene-specific translation in the adaptive processes to stress have been determined in yeast and in some mammalian systems by allelic gene replacement involving, for example, the substitution of alanine for the serine-51 the site of phosphorylation in eIF2 α (S51A) (Dever *et al.*, 1992; Hinnebusch, 2005; Scheuner *et al.*, 2001; Schroder and Kaufman, 2005b). As disclosed herein, a mutant parasite incapable of phosphorylating eIF2 α was engineered by substituting alanine for the regulatory serine (Ser-71) in TgIF2 α and characterized. TgIF2 α -S71A mutant parasites have decreased viability *in vitro* and are less virulent in a mouse model of infection. The underlying mechanism for the growth defect does not appear to involve parasite attachment, invasion, replication, egress, or motility, but rather it is due to the impaired ability of the parasites to manage the stress caused by their exposure to the extracellular environment. These results provide significant new insights into how intracellular parasites survive while they attempt to locate a new host cell.

Generation of mutant *Toxoplasma* incapable of phosphorylating TgIF2 α

[00128] The *Toxoplasma* eIF2 α orthologue (TgIF2 α), possesses a conserved regulatory serine residue (Ser-71) that is phosphorylated during cellular stress (Sullivan Jr. *et al.*, 2004b was previously characterized). In order to access the impact of TgIF2 α phosphorylation in *Toxoplasma* tachyzoites, a mutant parasite line in which the Ser-71 residue of TgIF2 α was changed to alanine was generated. The TgIF2 α -S71A mutation was created by allelic replacement using homologous recombination in RH Δ Ku80 parasites (Fox *et al.*, 2009; Huynh and Carruthers, 2009). Referring now to **FIG. 1A**, the dark grey box denotes the beginning of the TgIF2 α genomic locus and the black box represents ~2.4kb of upstream sequence. The serine-71 codon (AGC) was mutated to an alanine codon (GCC) which created a unique *MscI* restriction site within the mutant allele. RH Δ Ku80 parasites were transfected with a 'knock-in' construct that included a point mutation encoding the S71A substitution along with a mini-gene that encoded chloramphenicol resistance. The point mutation generated a unique *MscI* restriction site in the TgIF2 α genomic locus, which was used as a means to select for true allelic replacements among the chloramphenicol-resistant clones. Screening was performed by amplifying a fragment of the first exon in the TgIF2 α genomic locus and cutting the amplicon with *MscI*.

[00129] Referring now to **FIG. 1B**, the first exon of the TgIF2 α gene was amplified from WT and TgIF2 α -S71A parasites (PCR primers shown in grey in **FIG. 1A**). The resulting PCR product was digested with *MscI* and resolved on 1% agarose gel. In a true allelic replacement, two products (340 and 220 bp) would be visualized instead of one (560 bp). The *MscI*-digested PCR product from parental RH Δ Ku80 parasites (hereafter referred to as "wild-type" (WT)) yields a single DNA fragment of 560 bp; however, two fragments of 340 and 220 bp are produced after allelic replacement. Two independent clones containing the S71A knock-in within the endogenous TgIF2 α genomic locus were identified, and both exhibited similar phenotypic properties as described below.

[00130] In order to confirm that the TgIF2 α -S71A clone could not be phosphorylated under normal physiological condition, a Western blot of lysates from parasites treated with the ionophore A23187 was carried out using antisera that specifically recognizes TgIF2 α which is phosphorylated at ser-71 or total TgIF2 α protein (Narasimhan *et al.*, 2008b; Sullivan Jr. *et al.*, 2004b). Referring now to **FIG. 1C**, equal amounts of protein lysates from WT or TgIF2 α -S71A tachyzoites were resolved on a 4-12% polyacrylamide gel and transferred to a membrane for

immunoblotting with antibody that specifically recognizes TgIF2 α phosphorylated at ser-71 (TgIF2 α -P) or total TgIF2 α protein. The ionophore A23187 produces ER stress, and consistent with a previous study, this ionophore is a potent inducer of TgIF2 α phosphorylation in wild-type parasites (Narasimhan *et al.*, 2008b). By comparison, phosphorylation is absent in the TgIF2 α -S71A mutant parasites that have been exposed to the ionophore A23187 (**FIG. 1C**). These results confirm that a mutant version of *Toxoplasma* that cannot phosphorylate eIF2 α was created.

TgIF2 α -S71A parasites exhibit reduced growth *in vitro*

[00131] Observational analysis suggests that the TgIF2 α -S71A parasites take longer to lyse monolayers of host cells than do wild-type parasites. In order to directly assess whether the mutant parasites were less fit for survival than were the comparable to wild-type organisms, “head-to-head” competition assays were carried out (Fohl and Roos, 2003). Referring now to **FIG. 2A**, equal numbers of WT and TgIF2 α -S71A parasites (shown as white and black, respectively) were grown in mixed culture in a T25-flask (gray). Every three days the parasites were removed from the host cells by physically scraping the monolayer. Recovered parasites were then passed onto a fresh monolayer. On the day of passage, a sample was collected for genomic DNA (gDNA) isolation. The gDNA was used as a template for a TaqMan-based fitness assay designed to distinguish WT and mutant parasites. This assay used two probes, WT-TgIF2 α -FAM (shown in white) was used to detect the WT allele, while S71A-TgIF2 α -VIC (shown in black) was used to identify the mutant (S71A) allele. The S71A mutants were created according to the methods disclosed herein. An essentially equal number of wild-type and TgIF2 α -S71A parasites were inoculated into the same culture flask. The standard comparative fitness assay was modified in order to take advantage of TaqMan probes and real-time PCR as a means of distinguish between the wild-type (WT-TgIF2 α -FAM) and mutant (S71A-TgIF2 α -VIC) alleles of the TgIF2 α gene.

[00132] Referring now to **FIG. 2B**, the percentage of the WT and S71A mutant allele was determined using SDS software version 1.2.1 (Applied Biosystems) and plotted for each day sampled (white bars, WT; black bars, S71A). $C 10^4$ parasites were cultured in 12-well plates that had confluent HFF monolayers. The percentage of host cell lysis was evaluated at days 5, 6, and 7 respectively by washing each well with PBS buffer and staining the remaining host cells with Coomassie Brilliant Blue. A digital image of each well was recorded and analyzed using Alpha

Innotech software to determine the percent of the monolayer disrupted by either WT or TgIF2 α -S71A mutant parasites, represented respectively by either white or black bars. Genomic DNA was collected and examined at the time of inoculation (day 0), and then sampled again every three days. At day 0, the PCR analysis showed that equal numbers of TgIF2 α -S71A and wild-type parasites were present in the mixed culture. However, by day three the mutants were significantly outgrown by the wild-type organisms. And by day nine, TgIF2 α -S71A mutant parasites could no longer be detected in the culture.

[00133] Referring now to **FIG. 2C**, WT and S71A mutant parasites were allowed to adhere/invoke a monolayer of HFF cells for 30 min. Adhered (extracellular) parasites were visualized with a mouse anti-Sag1 immune sera (green parasites). Following permeabilization, both intracellular (invaded) and extracellular parasites were visualized with a rabbit anti-Sag1 antibody (red parasites). In the merged image, adhered parasites appear yellow and invaded parasites are red. The number of adhered (yellow) and invaded (red) parasites per microscope field were plotted for both WT and S71A parasites (bottom panel). A standard parasite growth assay that examined the extent of host cell monolayer disruption over time was also performed. Six days after inoculation about 90 % of wild-type parasites lyse out of the host cell monolayer but in the same six day period an equal number of TgIF2 α -S71A parasites had only destroyed ~18% of the host cells in the monolayer. It took the mutant parasites until day to destroyed ~90% of the host cell monolayer, a full 24 hours slower than wild-type parasites. Together, these results indicate that TgIF2 α phosphorylation is important in order for *Toxoplasma* tachyzoites to progress normally through host cell cultures.

TgIF2 α -S71A parasites are defective in adapting to the extracellular environment

[00134] In order to try and identify the cause of the growth retardation in the TgIF2 α -S71A mutant, each step in the tachyzoite lytic cycle was examined. Referring now to **FIG. 3**, WT and S71A mutant parasites were allowed to adhere/invoke a monolayer of HFF cells for 30 min. Adhered (extracellular) parasites were visualized with a mouse anti-Sag1 immune sera (green parasites). Following permeabilization, both intracellular (invaded) and extracellular parasites were visualized with a rabbit anti-Sag1 antibody (red parasites). In the merged image, adhered parasites appear yellow and invaded parasites are red. The number of adhered (yellow) and invaded (red) parasites per microscope field were plotted for both WT and S71A parasites

(bottom panel). Using a standard red/green attachment and invasion assay, no detected difference was observed in the ability of TgIF2 α -S71A mutants to adhere or penetrate host cells.

[00135] Referring now to **FIG. 4A**, the WT and S71A mutant parasites were allowed to adhere and glide along a glass coverslip for 30 min. The parasites and surface protein ‘trails’ were detected with mouse anti-Sag1. The percentage of parasites with ‘trails’ was plotted for both WT parasites (grey bar) and the S71A mutant (black bar). No defect in gliding motility was found nor was any detected. Referring now to **FIG. 4B**, WT and S71A parasites were cultured overnight in a monolayer of HFF cells. In order to induce egress, the infected monolayers were exposed to 2 μ M for A23187 for 0, 0.5, 1, 2, 3, or 4 min. Following each time interval a sample was taken and the parasites in the samples were fixed with cold methanol. Finally, using the parasite counts that were obtained the percentage of egress was calculated for the WT (black boxes) and S71A parasites (white circles).

[00136] The ability of TgIF2 α -S71A parasites to exit from host cells upon ionophore-induced egress was not compromised. With no measurable defects in their ability to either enter or exit host cells, two not mutually exclusive possibilities remain which may help to explain why WT parasites overgrow TgIF2 α -S71A parasites, there are: 1) the TgIF2 α -S71A mutants are be defective in asexual replication, and 2) exposure to the extracellular environment produces a stress that TgIF2 α -S71A mutants are ill-equipped to withstand. In order to test these possibilities, two independent types of parasite growth measurements were carried out: plaque assays and doubling assays. In order to obtain a homogenous population of parasites that had nominal exposure to the extracellular environment, intracellular parasites were physically separated from their host cells.

[00137] Referring now to **FIG. 5A**, intracellular WT and TgIF2 α -S71A parasites were physically removed from host cells and then incubated without host cells in DMEM plus 1% FBS at 37°C in 5% CO₂ for 0, 2, 4, or 8 hr. Following each time point, 5x10² parasites were passed onto a fresh HFF monolayer in a 12-well plate. Upon plaque formation, each well was washed with PBS and stained with Coomassie Brilliant Blue (upper panel). The number of plaques formed by the WT (diamonds) or TgIF2 α -S71A mutants (squares) were counted using Alpha Innotech imaging software and plotted on a line graph (lower panel). The freed tachyzoites were immediately passed onto a fresh HFF monolayer (0 h), or deprived of host cells by incubating in extracellular medium for 2, 4, or 8 hr. Without appreciable exposure to the

extracellular environment consisting of DMEM plus 1% FBS (0 hr), wild-type and TgIF2 α -S71A mutants formed a near identical number of plaques, suggesting that the inability to phosphorylate TgIF2 α has no appreciable impact on parasite replication. However, TgIF2 α -S71A mutants were much more sensitive to being deprived of their host cells. Compared to wild-type, TgIF2 α -S71A mutants showed a significant reduction in plaque numbers following an 8 hour exposure to the extracellular environment.

[00138] Referring now to **FIG. 5B**, using the same method above, WT and TgIF2 α -S71A mutants were deprived of host cells for 0, 4, or 8 hours and then allowed to infect a fresh HFF monolayer. *Toxoplasma* growth was quantitated using a standard parasite doubling assay. The average number of parasites/vacuole is displayed for each counting period (8, 16, 24, and 32 hours post-inoculation). Next the doubling time of wild-type and TgIF2 α -S71A mutant parasites was measured. The parasites were immediately passed into fresh host monolayers (0 hours of extracellular exposure). Both grew at a similar rate during the 32 hour time course. As observed in the plaque assay, the inability to phosphorylate TgIF2 α compromises the ability of the parasite to recover from the stress of the extracellular exposure. Wild-type parasites deprived of host cells for 8 hours have an average number of 12 parasites/vacuole 32 hours post-inoculation, whereas TgIF2 α -S71A mutants only achieve an average of 7 parasites per vacuole.

[00139] These studies suggest that following egress, TgIF2 α becomes phosphorylated in order to help the parasite cope with the extracellular environment until it invades a new host cell. Referring now to **FIG. 5C**, protein lysates were generated from RH and Pru parasites deprived of the host cell for 0, 4, or 8-hr. A Western blot was carried out using equal amounts of protein lysate with antibodies that specifically recognize phosphorylated TgIF2 α or total TgIF2 α protein. Consistent with this idea, it was found that phosphorylation of TgIF2 α increases during increased exposure to the extracellular environment. Interestingly, TgIF2 α phosphorylation was more significantly induced in type I (RH) parasites compared to a type II (Pru) strain following exposure to the extracellular environment.

Translation by incubating extracellular wild-type and TgIF2 α -S71A tachyzoites in medium containing [^{35} S] Met/Cys radiolabel.

[00140] Referring now to **FIG. 5D**, Extracellular WT or TgIF2 α -S71A tachyzoites were incubated in the presence of [^{35} S] Met/Cys for 30 min at 37°C with 5% CO₂. Radiolabeled

proteins were resolved on SDS-PAGE for autoradiography. Asterisks (*) denotes P-value < 0.05. Radiolabeled parasite proteins were then resolved by SDS-PAGE and visualized by autoradiography. Wild-type parasites had lower levels of radiolabeled protein than did mutant parasites that harboured mutant TgIF2 α -S71A. It is noted that this translation measurement was carried out in parasites cultured in the extracellular medium in the absence of an added stressing agent. This finding suggests that extracellular *Toxoplasma* tachyzoites experience a stress that elicits translational control. Parasites that have the TgIF2 α -S71A mutant form of the protein are unable to readily reduce the rate at which they produce protein when stressed by exposure to the extracellular environment and this renders them less fit than parasites that have the wild type form of this protein.

Reduced virulence of TgIF2 α -S71A parasites *in vivo*

[00141] Since *Toxoplasma* that are incapable of phosphorylating eIF2 α exhibit reduced fitness *in vitro*, the TgIF2 α -S71A mutant was tested to determine if it had decreased virulence *in vivo*. In order to test this idea, 10 or 100 wild-type or TgIF2 α -S71A mutant parasites were injected into female BALB/c mice immediately after the organisms egressed from human cell hosts. In this model for acute toxoplasmosis, hypervirulent RH strain *Toxoplasma* typically produces a moribund mouse within 7 days (168 hr). In this study, mice infected with 10 wild-type parasites become moribund at 167 hr, but mice infected with 10 TgIF2 α -S71A parasites showed a 28 hour delay, not becoming moribund until 195 hours (**Table I**). The same delay in the course of infection occurred with a dosage of 100 parasites (**Table I**), leading the conclusion that TgIF2 α phosphorylation and translation control enhances the virulence of *Toxoplasma in vivo*.

Table 1:

GROUP	SURVIVAL TIME, hr
WT (10 parasites)	167
S71A (10 parasites)	195
WT (100 parasites)	147
S71A (100 parasites)	174

Characterization of the GCN2-like kinase TgIF2K-D

[00142] The predicted gene TgME49_119610 (ToxoDB.org) was previously designated TgIF2K-D and is suggested to be an orthologue of GCN2 (24), the eIF2 α kinase that is well-documented as a responder to nutrient starvation stress in other species, RT-PCR was used to

identify and characterize the full-length *TgIF2K-D* cDNA. This analysis revealed a predicted *TgIF2K-D* product consisting of 2,729 amino acid residues (GenBank JF827031), which modifies the predicted sequence for TgME49_119610 due to a discrepancy at the exon3/intron3 boundary. Referring now to **FIG. 12**, *TgIF2K-D* cDNA contains an open reading frame (upper case letters) of 8,190 nucleotides and a 5'- and 3'-UTR (lower case letters) of 2,151 and 1,000 nucleotides, respectively. Start and stop codons are indicated by bold letters. The in-frame stop codon upstream of the start codon is underlined. The predicted start codon for the *TgIF2K-D* ORF matches the consensus sequence for translation initiation in *Toxoplasma* (35) and is preceded by an in-frame stop codon. RACE analyses indicated a 5'-untranslated region (UTR) of 2,151-bp, which is consistent with the transcriptional start site (TSS) derived from the Full-parasites database and ChIP-Chip data available in the ToxoDB, and a 3'UTR of ~1,000 bp.

[00143] Referring now to **FIG. 13**, the 5'-UTR was further validated by RT-PCR using primers flanking the TSS. RT-PCR analysis to delineate the transcriptional start site of the *TgIF2K-D* gene. Oligonucleotide primers complementary to sequences upstream (#21) and downstream (#22) of the transcriptional start site (arrow), as well as downstream of the start codon (#23) were designed to verify the 5'-end of the *TgIF2K-D* mRNA as determined by 5'RACE. PCR assays were carried out using oligonucleotides primers #21 and #23, or #22 and #23. These PCRs were then nested with oligonucleotides #21N and #23N, or #22N and #23N, respectively. PCR products were analyzed by electrophoresis using an agarose gel. Sizes of DNA markers are indicated in kilobases (kb).

[00144] Referring now to **Fig. 14**, alignment of *TgIF2K-D* protein kinase domain with other eIF2 α kinases. The *TgIF2K-D* protein kinase domain comprises 12 regions indicated by solid bars and roman numbers (I-XII), including an insert between motifs IV and V that is characteristic of eIF2 α kinases. This insert, as well as other sequences that would create gaps in the alignment, were replaced by the number of omitted amino acid residues. Sequence identities shared among the eIF2 α kinases are highlighted in black, while gray indicates residues with similar chemical properties. The invariant lysine residue located in the active site is marked by an asterisk. Numbers on the right indicate the residue position in the following GCN2-related sequences: Tg: *Toxoplasma gondii*, Nc: *Neospora caninum*, Pf: *Plasmodium falciparum*, Pb: *Plasmodium berghei*, Tb: *Trypanosoma brucei*, At: *Arabidopsis thaliana*, Dm: *Drosophila melanogaster*, Sc: *Saccharomyces cerevisiae*, Mm: *Mus musculus*, Hs: *Homo sapiens*. An

alignment between TgIF2K-D and the eIF2 α kinases from multiple species was compiled using BLAST and CLUSTALW. TgIF2K-D (residues 1,318 to 1,630) has the central features characteristic of eIF2 α kinases, including an insert between subdomains IV and V (Figs. 1 and S3). As judged by BLAST analyses, this portion of TgIF2K-D is most closely related to putative eIF2 α kinases from parasites *Plasmodium falciparum* (AAN37036; 4e-14) and *Trypanosoma brucei* (XP_828792.1; 6e-10) followed by characterized GCN2 orthologues from *Arabidopsis thaliana* (CAD30860; 6e-32); *Drosophila melanogaster* (AAC13490; 8e-27); *Schizosaccharomyces pombe* (AAU11313; 2e-25); and *Saccharomyces cerevisiae* (AAA34636; 1e-22). Another hallmark feature of GCN2 is an RWD domain, which is present between residues ~800 and ~1,000 of TgIF2K-D, with a significance of 4e-6 as determined by the motif search program Pfam. Finn, R. D., J. Mistry, J. Tate, P. Coghill, A. Heger, J. E. Pollington, O. L. Gavin, P. Gunasekaran, G. Ceric, K. Forslund, L. Holm, E. L. Sonnhammer, S. R. Eddy, and A. Bateman. 2010.

[00145] Referring now to **FIG. 15**, the TgIF2K-D RWD domain contains the YPXXXP motif, indicated by the solid bar, which is conserved among RWD domains of GCN2-related protein kinases. Sequence identities shared among these eIF2 α kinases are highlighted in black, while gray indicates residues with similar chemical properties. Numbers on the right indicate the residue position in the following GCN2-related sequences: Tg: *Toxoplasma gondii*, Nc: *Neospora caninum*, Pf: *Plasmodium falciparum*, Dm: *Drosophila melanogaster*, Mm: *Mus musculus*, Sc: *Saccharomyces cerevisiae*. The Pfam protein families database. Nucleic acids research **38**:D211-222. The RWD in Gcn2 from *S. cerevisiae* was reported to directly bind to the activator protein GCN1, and residue changes in Gcn2 that blocked this binding, or abolition of the Gcn2/Gcn1 association by Gcn1 binding with another RWD-containing protein Yih1, blocked Gcn2 phosphorylation of eIF2 α in yeast depleted for amino acids. *Toxoplasma* also has a predicted GCN1 orthologue (TGME49_031480) and a Yih1-related protein (TGME49_112350), supporting the idea that this network functions to regulate a GCN2-related eIF2 α kinase in this parasite.

[00146] The sequences of the histidyl-tRNA synthetase (HisRS) domain, which stimulates eIF2 α kinase activity by binding to uncharged tRNAs accumulating during nutrient deprivation (13), appears to be less well conserved in the protozoan GCN2-like kinases. Analysis of the sequences flanking the C-terminal end of the protein kinase domain (residues 1,750-2,360)

identified the PRGGRVY²²⁹⁹ sequence as the closest match to the Histidine B sequence (AAGGRYD), which is characteristic for the HisRS-related domains. **Sood, R., A. C. Porter, D. Olsen, D. R. Cavener, and R. C. Wek.** 2000. *A mammalian homologue of GCN2 protein kinase important for translational control by phosphorylation of eukaryotic initiation factor -2a.* *Genetics* **154**:787-801. This weaker conservation of the HisRS-related sequences is a feature shared with other GCN2-related protein kinases from apicomplexans, including *P. falciparum*. TgIF2K-D also lacks the pseudokinase domain found in mammalian and yeast GCN2s, which is thought to contribute to the eIF2 α kinase activity. The C-terminus of Gcn2 is important for dimerization and ribosome association and this region in TgIF2K-D (residues 2,436 – 2,499) is rich in hydrophobic and basic residues, which are suggested to contribute to these regulatory processes in this eIF2 α kinase.

[00147] Referring now to **FIG. 16**, alignment of the C-terminal homology region of TgIF2K-D with other predicted GCN2-like protein kinases from parasites. Sequence identities shared among the C-terminal homology regions of the GCN2-like protein kinases are highlighted in black. Gray highlights indicate residues with similar chemical properties. Numbers on the right indicate the residue position in the following GCN2-related sequences: Tg: *Toxoplasma gondii*, Nc: *Neospora caninum*, Pf: *Plasmodium falciparum*, Pb: *Plasmodium berghei*, PkH: *Plasmodium knowlesi* and Pvx: *Plasmodium vivax*. Numbers listed on the left of the sequences indicate the GeneID from the corresponding databases. Interestingly, this region shares sequence identity with GCN2-like kinases encoded in apicomplexans *Neospora caninum* (NCLIV_010550, 3e-30), *Cryptosporidium muris* (CMU_027700; 0.011), *Plasmodium falciparum* (PF14_0264; 9e-08), *Plasmodium berghei* (PBANKA_101620, 4.8e-08), *Plasmodium knowlesi* (PKH_113740, 1.1e-07) and *Plasmodium vivax* (PVX_085120; 2e-07). Designated herein as the conserved region as the C-terminal homology (CTH) region (**FIG. 6**).

[00148] Referring now to **FIG. 6**, TgIF2K-D contains a protein kinase domain (black boxes) with an insert (I) characteristic of eIF2 α kinases, and signature regulatory regions including the RWD domain (dark gray) and a proposed HisRS-related region (light gray). The conserved C-terminal homology (CTH) domain is denoted with a mottled box. The numbers below the diagram demarcate the amino acid residues for each of the domains of TgIF2K-D. Based on the presence of sequences related to the eIF2 kinases juxtaposed to the signature RWD domain, a

putative Histidine B-like sequence, and a C-terminus rich in hydrophobic and basic residues, the TgIF2K-D is suggested to be a parasite orthologue of GCN2.

TgIF2K-D is expressed in intra- and extracellular parasites

[00149] Using RHΔ*Ku80* parasites engineered to have greater frequencies of homologous recombination TgIF2K-D was tagged with three HA epitopes (3xHA) at the C-terminus. Immunoblot analyses of total protein lysate using anti-HA antibody identified three clustered protein bands with a molecular weight similar to the deduced 289 kDa for TgIF2K-D (**FIG. 7A**). These proteins were not present in parental parasites referred to as wild-type, WT. Referring now to **FIG. 7A**, the faster migrating TgIF2K-D_{3xHA} variants diminished when parasites were subjected to extracellular stress for 4 or 8 hours. Samples were normalized in the immunoblot analysis using antibody specific for *Toxoplasma* tubulin. Referring now to **FIGs. 7A** and **7B**, it was also observed that upon extracellular incubation for up to 8 hours, a condition that induces high levels of TgIF2α phosphorylation the levels of the faster migrating TgIF2K-D_{3xHA} variants diminished while the slower migrating protein increased. The difference between these variants of TgIF2K-D may be attributable to post-translational modification(s), such as protein phosphorylation, or alternative mRNA splicing, which may contribute to TgIF2K-D activation by stress. While an alternative mRNA splice products were not detected during this analysis of the *TgIF2K-D* cDNAs, alternative mRNA splicing was reported in earlier studies of mammalian GCN2, although their biological significance has not been confirmed.

[00150] Referring now to **FIG. 7C**, nuclear DNA was co-stained with 4,6-diamidino-2-phenylindole (DAPI, blue). TgIF2K-D_{3xHA} does not colocalize with nuclear DNA, indicating a cytoplasmic localization in the parasite. In order to identify the cellular location of TgIF2K-D, immunofluorescence microscopy was also carried out. The HA-tagged TgIF2K-D localized to the parasite cytosol in both intra- and extracellular parasites. A cytosolic localization is consistent with reports on GCN2 in other species. *Wek, R. C., 2006*.

TgIF2K-D facilitates TgIF2α phosphorylation and translational control in extracellular parasites

[00151] Generation of a conditional TgIF2K-D knockdown. Extracellular stress is a potent inducer of TgIF2α phosphorylation and loss of translational control in the TgIF2α-S71A mutant

reduced parasite viability. *Joyce, B. R., 2010*. In order to address whether TgIF2K-D is required to manage extracellular stress, knockdown and knockout parasite clones were generated in the RH Δ Ku80 background. Referring now to **FIG. 17**, a genetic fusion of a destabilization domain (DD) causes degradation of endogenously expressed TgIF2K-D. Cell culture medium was supplemented with 500 nM Shield-1 for up to 24 hours to stabilize TgIF2K-D_{2xDD}. Vehicle was used a negative control. Equal amounts of protein lysate were resolved via SDS-PAGE and normalized for western blotting using a monoclonal anti-HA antibody. Levels of TgIF2 α protein were probed as a loading control. The knockdown of *TgIF2K-D* involved an in frame-fusion of two HA tags and a 12 kDa destabilization domain (DD) at the C-terminus of the endogenous *TgIF2K-D* in the RH Δ Ku80 strain (Fig. S6). The parasite clone, designated TgIF2K-D_{2xDD}, allowed tunable expression of the TgIF2K-D protein. In the absence of the stabilizing ligand Shield-1, DD-tagged proteins are rapidly degraded. TgIF2K-D_{2xDD} parasites cultured without Shield-1 had no detectable levels of TgIF2K-D protein as assayed by western blot analysis.

[00152] Referring now to **FIG. 8A**, the numbered arrows indicate the positions of primers used to screen genomic DNA from transfected pyrimethamine-resistance clones and parental (WT) parasites. Primer sequences are listed in the 2. The knockout of *TgIF2K-D* eliminated the entire genomic locus through homologous recombination and allelic replacement with a modified DHFR-TS minigene, which confers resistance to pyrimethamine (**FIG. 8A**). The Δ IF2K-D was verified by PCR analyses of genomic DNA purified from pyrimethamine-resistant clones (**FIG. 8B**). In addition, total RNA from the parental strain and a Δ IF2K-D knockout clone was isolated for RT-PCR analysis of the *TgIF2K-D* transcript. This parasite clone represents the first knockout of an eIF2 α kinase in *Toxoplasma*.

[00153] Referring now to **FIG. 8C**, the absence of *TgIF2K-D* mRNA in the Δ IF2K-D parasites was confirmed by RT-PCR analysis using primers upstream and downstream of the encoded protein kinase domain (primer #11 and #12). *Toxoplasma* actin mRNA was amplified as a positive control (primers #13 and #14). A no-template control (Ø) was included in all PCRs. While *TgIF2K-D* mRNA was amplified from parental parasites, the corresponding transcript was not detected in Δ IF2K-D parasites.

Oligonucleotide #	5'-3' sequence
(SEQ ID NO:39) 1	GAAATAGCGGCCGCGTCACTGACCAATGAGCTTTGG
(SEQ ID NO:40) 2	GAACAACTAGTAGAACGAAGGGAGGAGAGTTCCG

(SEQ ID NO:41) 3	GTTCAAAGCTTTGGAAGAGACCGACGCTGAACG
(SEQ ID NO:42) 4	GTAGTTGGGCCCCGCTTCGTTGAGTGATGTGAGACG
(SEQ ID NO:43) 5	CTTCAGCAATTTGTGGGAGGAAATGC
(SEQ ID NO:44) 6	GAGCGGCTCGTCGCTCGTCGTTGAGG
(SEQ ID NO:45) 7	TCCTCCACTTCCAATTTTAGCGCGTCGGTCTCTCCACCTCTGCCTGG
(SEQ ID NO:46) 8	TACTTCCAATCCAATTTAATGCATGTTCCGTAGGCGGTGATCCCTCGTGG
(SEQ ID NO:47) 9	CCACAGTTTCGTTGCTCTCTTGG
(SEQ ID NO:48) 10	CTGTATGCCGCTAGAGTGCTGG
(SEQ ID NO:49) 11	GAAACGGGATCCGCGAAGAAACAGAGCGACAGCGGTGCTGG
(SEQ ID NO:50) 12	GCAGAGTCTAGATCACGCGGGAGAGTCAGAAGTACATTTCTGTGG
(SEQ ID NO:51) 13	ACGTATGATGCGCGAGAAAA
(SEQ ID NO:52) 14	GGGCGTTTCATGACCTAAA
(SEQ ID NO:53) 15	GCTTAACGGGTACGGCGTTT
(SEQ ID NO:54) 16	GCGGTTAATCCAGCGTATGC
(SEQ ID NO:55) 17	CAGGGCCGTACGAGAACGT
(SEQ ID NO:56) 18	GCCCACGACAGCAGACAACT
(SEQ ID NO:57) 19	CACTCGGTTCTGTGCTTTCT
(SEQ ID NO:58) 20	CCGTCACGCCACTACAACAG
(SEQ ID NO:59) 21	CTCTGCATGCAGCTGGTCGTTGG
(SEQ ID NO:60) 21N	GCAGCCGATTGATTTTCATCGGATGG
(SEQ ID NO:61) 22	CACCGTGTGAGCTGCCTCCAAGG
(SEQ ID NO:62) 22N	CTTTCTGTCTGTCCTCGCTGACTGG
(SEQ ID NO:63) 23	CCACCAGTGTCCAAGATCCATCG
(SEQ ID NO:64) 23N	GATCCATCGTCTCCGTCCTCTTCG
(SEQ ID NO:65) 24	CACTCGGTTCTGTGCTTTCT
(SEQ ID NO:66) 25	CCGTGACGCCACTACAACAG
(SEQ ID NO:67) 26	GCTTAACGGGTACGGCGTTT
(SEQ ID NO:68) 27	GCGGTTAATCCAGCGTATGC
(SEQ ID NO:69) 28	CAGGGCCGTACGAGAACGT
(SEQ ID NO:70) 29	GCCCACGACAGCAGACAACT

[00154] Next addressed was whether TgIF2K-D is required for induced TgIF2 α phosphorylation when the parasite is outside of the host cell. Referring now to **FIG. 9A**, TgIF2 α phosphorylation was analyzed by separating cell lysates via denaturing SDS-PAGE, followed by western blotting using antibodies to total TgIF2 α or phosphorylated TgIF2 α (TgIF2 α -P). Parental WT parasites showed robust TgIF2 α phosphorylation after 8 hours of incubation in the

extracellular environment. By comparison, there was minimal TgIF2 α phosphorylation in the TgIF2K-D_{2xDD} knockdown or Δ IF2K-D knockout parasites following extracellular exposure. In order to test the specificity of TgIF2K-D in responding to extracellular stress, WT and Δ IF2K-D parasites were exposed to the calcium ionophore A23187, a known inducer of ER stress and TgIF2 α phosphorylation. As shown in **FIG. 9B**, the Δ IF2K-D parasites were not defective for TgIF2 α phosphorylation in response to ER stress. These results support the model that each TgIF2 α kinase in *Toxoplasma* recognizes distinct stress arrangements, and TgIF2K-D is central for inducing TgIF2 α phosphorylation when parasites are outside the host cell.

[00155] Under stress conditions, eIF2 α phosphorylation represses general translation as part of the cellular stress response. In order to compare translational control in WT versus Δ IF2K-D parasites, the incorporation of radiolabeled Cys/Met in parasites subjected to extracellular stress for 1 and 8 hours was measured. Referring now to **FIG. 9C**, one hour prior to harvesting, the parasites were incubated in the presence of [³⁵S]-Cys/Met. Lysates were prepared and equal amounts of protein were precipitated with TCA. Levels of incorporation of radiolabeled amino acids were determined via scintillation counting. Three experiments were performed and incorporation of the radiolabel is represented as a percentage of that measured for parasites subject to 1 h of stress. Error bars indicate the standard error and significance indicates $p < 0.05$. Protein synthesis was repressed by greater than 90% in WT parasites subjected to 8 hours of extracellular stress; however, in the Δ IF2K-D and TgIF2 α -S71A mutant parasites, protein synthesis was only diminished by about 40%. These data strongly suggest that TgIF2K-D is the primary eIF2 α kinase that mediates translational control in response to extracellular stress.

Parasites lacking TgIF2K-D exhibit a fitness defect

[00156] TgIF2 α -S71A mutants are outcompeted by wild-type parasites when placed in a “head-to-head” competition assay, as the mutant struggles to cope with the extracellular environment experienced while finding a new host cell. Given that Δ IF2K-D failed to phosphorylate TgIF2 α in response to extracellular stress (**FIG. 9A**), Δ IF2K-D parasites were tested to determine if they too would be outcompeted by parental wild-type parasites using the head-to-head fitness assay.

[00157] Equal numbers of WT and Δ IF2K-D parasites were premixed and transferred into the same culture flask containing a confluent monolayer of HFF cells (**FIG. 10A**). Samples were taken prior to infection and after day 6 for genomic DNA isolation. The relative amount of WT

and $\Delta IF2K-D$ parasites was determined using a SYBR Green-based quantitative PCR assay and primers specific for WT or $\Delta IF2K-D$ parasites. Referring now to **FIG. 10B**, relative levels of WT and $\Delta IF2K-D$ parasites were determined using a SYBR green assay with primers #26 and #27, or #28 and #29, as indicated. Samples were normalized for the amplification of a DNA fragment encoding the 5'-UTR, which is conserved between WT and $\Delta IF2K-D$ (primers #24 and #25). Error bars indicate standard error and significance was determined using two-tailed Student's t-test, with $p < 0.01$, as indicated by the asterisk. Primers that amplify DNA from both strains were used to ensure normalization between the samples. WT parasites outgrew the mutant parasites by day 6 (**FIG. 10C**), establishing that parasites lacking TgIF2K-D exhibit reduced fitness in the parasite lytic cycle.

TgIF2K-D promotes the viability of extracellular tachyzoites

[00158] These data suggest that parasites deficient for TgIF2K-D suffer a loss in viability due to an inability to respond appropriately to the extracellular stress experienced while outside host cells. To further address the role of TgIF2K-D in the extracellular stress response, WT and the different mutant parasites (TgIF2 α -S71A, $\Delta IF2K-D$, and TgIF2K-D_{2xDD} without Shield) were incubated outside of host cells in DMEM between 0 and 10 hours prior to applying them to a fresh host cell monolayer. After 7 days, the infected host cells were fixed and stained to determine the degree of host cell lysis. With increased periods of extracellular stress, $\Delta IF2K-D$ parasites showed sharply reduced infection and lysis of host cells that was similar to that measured for the TgIF2 α -S71A mutants (**FIG. 11A**). This defect was more pronounced in the $\Delta IF2K-D$ parasites compared to the TgIF2K-D_{2xDD} knockdown, suggesting that there are residual levels of functional TgIF2K-D despite the absence of Shield.

[00159] In order to further characterize the role of translational control in the resistance to extracellular stress the doubling rate of the $\Delta IF2K-D$ parasites was also measured. $\Delta IF2K-D$ parasites proliferated at a similar rate relative to WT when allowed to infect a new host cell monolayer immediately upon release from their initial host cells (**FIG. 11B**, 0 hr. extracellular stress). Still referring to **FIG. 11B**, consistent with the plaque assay, extracellular stress led to a significant reduction in the proliferation of $\Delta IF2K-D$ parasites. WT parasites subjected to extracellular stress for 10 hours grew to an average of ~17 parasites/vacuole, but $\Delta IF2K-D$ parasites only to ~10 parasites/vacuole. This reduction in doubling time was also observed when the TgIF2 α -S71A mutants were subjected to extracellular stress prior to infection of the HFF

cells. Collectively, these studies establish that TgIF2K-D is critical for promoting survival of extracellular tachyzoites through translational control mediated by the phosphorylation of TgIF2 α .

[00160] Eukaryotic cells have evolved mechanisms to tolerate stresses encountered in their environments. A well characterized stress response pathway conserved from yeast to humans centers on the phosphorylation of eIF2 α , which reduces translation initiation and provides the cell with time to reprogram its genome in order to address the new challenges caused by a change in conditions. This stress response has been found to be conserved in early-branching protozoa, including parasitic species such as the Apicomplexa and kinetoplastids (Mohrle *et al.*, 1997; Moraes *et al.*, 2007; Sullivan Jr. *et al.*, 2004b).

[00161] As described herein, one function for eIF2 α phosphorylation in the obligate intracellular protozoan, *Toxoplasma*, centers on the ability of the parasite to survive without the resources and shelter supplied by its host cell. Upon egress tachyzoites have evolved mechanisms to help them to survive in the extracellular environment long enough for them to invade a new host cell. This study shows that without eIF2 α phosphorylation, tachyzoites have a diminished capacity to remain virulent when they are deprived of host cells. The decrease in virulence as a result of not being able to phosphorylate eIF2 α is also seen when the mutant parasites are used in the mouse model of acute toxoplasmosis. These findings indicate that intracellular parasites are stressed when deprived of host cells, and that a key part of managing this “extracellular stress” involves translation control mediated by the phosphorylation of eIF2 α .

[00162] There are four eIF2 kinases encoded in the *Toxoplasma* genome, designated TgIF2K-A through D. TgIF2K-A possesses a transmembrane domain and was found to be localized to the parasite ER (Narasimhan *et al.*, 2008b). Upon treatment with ER stress agents, TgIF2K-A is released from its association with BiP, suggesting shared activation mechanisms with those described in yeast and mammals (Schroder and Kaufman, 2005a). TgIF2K-B is a cytosolic eIF2 kinase lacking homology to previously characterized eIF2 kinases (Narasimhan *et al.*, 2008b). TgIF2K-C and -D are less well characterized, but resemble GCN2, an eIF2 kinase that is documented to respond to nutrient deficiency. The nature of the extracellular stress could be the absence of the host cell protecting or buffering the parasite from deficiencies in nutrients or metabolites, and/or a whole range of damaging environmental insults that confront the parasite when it is not in a host cell.

[00163] In other species, the phosphorylation of eIF2 α causes a general depression in global protein synthesis, but a subset of mRNAs encoding master regulator transcription factors (such as yeast GCN4 or mammalian ATF4) are preferentially translated. *Apicomplexa* lack most conventional transcription factors conserved from yeast to mammals, but possess an expanded lineage of plant-like transcription factors with APETELA-2 domains, or ApiAP2 (Balaji *et al.*, 2005). Whether ApiAP2 mRNAs are preferentially translated is not yet known, but the existence of DNA-binding transcription factors in *Toxoplasma* makes it likely that these primitive eukaryotes adhere to a similar stress response paradigm. Identification of the genes preferentially translated may provide more information explaining how TgIF2 α phosphorylation enables parasites to survive while locating a new host cell.

[00164] In addition to the rapid proliferation of tachyzoites, pathogenesis of *Toxoplasma* also involves the ability of the tachyzoites to differentiate into bradyzoites. Bradyzoites form a tissue cyst that can remain in the host organism for life. During immunosuppressive conditions, the bradyzoites can reemerge as an acute tachyzoite infection. Consistent with their quiescent nature, latent bradyzoites maintain TgIF2 α in its phosphorylated state, and it was suggested that this stress response may also function to lower protein synthesis in the dormant parasitic cyst (Narasimhan *et al.*, 2008b). The TgIF2 α -S71A mutant was created in the hypervirulent RH type I strain of *Toxoplasma*; a strain that is well suited for studies the proliferate stage of the parasite, this strain has largely lost its ability to develop into bradyzoites *in vitro* and *in vivo* (Khan *et al.*, 2009). In order to further examine the role of TgIF2 α phosphorylation in bradyzoites, it will be useful to generate the TgIF2 α -S71A mutant in type II strain *Toxoplasma*. While type II strains grow more slowly and are less genetically amenable than type I strains there is every reason to believe that what is now known about TgIF2 α phosphorylation in type I strains will be broadly applicable to type II strains of the parasite.

[00165] In addition to previous observations that phosphorylated TgIF2 α accumulates in latent bradyzoites, data presented herein demonstrates that TgIF2 α phosphorylation is also important during the tachyzoite lytic cycle, specifically during the critical time when the parasite is without a host cell. Therefore, pharmacological targeting of the TgIF2 α stress response pathway promises to have multiple benefits in treating or perhaps preventing both acute and chronic forms of toxoplasmosis. It would be of great interest to assess if eIF2 phosphorylation

and translation control also contribute to the survival of other intracellular pathogens during the times in their life cycle when they are outside of host cells.

[00166] The *TgIF2K-D* knockout showed reduced TgIF2 α phosphorylation and translational control in response to extracellular stress, along with reduced viability when outside the host cell (**FIGS. 9 and 11**). This phenotype was also observed for the TgIF2 α -S71A mutant, supporting the idea that induced TgIF2K-D phosphorylation of TgIF2 α is central for *Toxoplasma* to persist in the extracellular environment (**FIG. 11**). Intracellular tachyzoites proliferate within a parasitophorous vacuole membrane that operates as a molecular sieve and regulates the acquisition of nutrients from the host cell. Upon exit from their host cell, the tachyzoites must find a new host cell in order to survive and replicate. The extracellular environment is likely to be reduced in essential nutrients that are available to the parasite, and/or the tachyzoites may not be equipped with the uptake mechanisms needed to acquire them. The data presented herein suggests that TgIF2 α phosphorylation serves to protect the parasite during this period of vulnerability. One possible explanation for this observation is that global translation control enables the tachyzoites to conserve energy and nutrients, and may also induce preferential translation of key proteins required for extracellular survival (e.g. membrane transporters or a new array of metabolic enzymes). Such preferential translation of transcripts, such as *ATF4* in mammals and *Gcn4* in *S. cerevisiae*, during eIF2 α phosphorylation are central for ameliorating nutrient stress. *Wek, R. C., H. Y., 2006*. The importance the parasite overcoming extracellular stress has significance in pathogenesis as demonstrated by our prior report that the TgIF2 α -S71A mutant has reduced virulence when inoculated into mice. *Toxoplasma* strains differing in virulence are also suggested to differ in their ability to initiate translational control; hypervirulent strains are able to phosphorylate TgIF2 α faster and more robustly than hypovirulent strains during extracellular stress.

[00167] The mechanisms employed by tachyzoites to overcome the dramatic changes in its extracellular environment are poorly understood, but have recently emerged as a new area of intensive research. Microarray analyses have revealed significant changes in the transcriptome between intra- and extracellular tachyzoites. *Lescault, P. J., A. B. Thompson, V. Patil, D. Lirussi, A. Burton, J. Margarit, J. Bond, and M. Matrajt. 2010. Genomic data reveal Toxoplasma gondii differentiation mutants are also impaired with respect to switching into a novel extracellular tachyzoite state. PLoS One 5:e14463*. Generally, intracellular parasites favor

expression of genes involved in metabolism and DNA replication, while *Toxoplasma* in the extracellular environment activate genes focused on invasion, motility and signal transduction.

[00168] Coincident with the reprogramming of the transcriptome, extracellular parasites form a novel plant-like vacuole/vacuolar compartment (PLV/VAC). The PLV/VAC may protect parasites from osmotic or ionic stresses encountered outside host cells, or mediate the proteolytic maturation of proproteins targeted to micronemes, a cellular compartment important for the parasite invasion into host cells. **Francia, M. E., 2011.** Several studies have also shown that extracellular parasites undergo a metabolic shift from oxidative phosphorylation to glycolysis in order to generate the ATP required for gliding motility and invasion. **Lin, S. S., M. Blume, N. von Ahsen, U. Gross, and W. Bohne. 2011.** *Extracellular Toxoplasma gondii tachyzoites do not require carbon source uptake for ATP maintenance, gliding motility and invasion in the first hour of their extracellular life. International journal for parasitology.* Collectively, these studies suggest that tachyzoites undergo extensive changes in their morphology, metabolism, and transcriptome when transitioning to the extracellular environment.

[00169] Translational control through TgIF2 α phosphorylation provides an additional mechanism that can modulate *Toxoplasma* gene expression that is designed to facilitate extracellular survival. The data discussed herein support this model, illustrating that parasites lacking the GCN2-like TgIF2K-D are significantly impaired in their ability to survive outside of host cells. In addition to TgIF2K-D, *Toxoplasma* is suggested to express three other eIF2 α kinases that are each proposed to respond to unique stress arrangements or environmental cues. TgIF2K-A resides in the parasite endoplasmic reticulum and is suggested to function analogous to mammalian PEK/PERK. TgIF2K-B is a parasite-specific eIF2 α kinase likely to respond to a cytosolic stress. **Narasimhan, J., B. R. Joyce, A. Naguleswaran, A. T. Smith, M. R. Livingston, S. E. Dixon, I. Coppens, R. C. Wek, and W. J. Sullivan, Jr. 2008.** *Translation Regulation by Eukaryotic Initiation Factor-2 Kinases in the Development of Latent Cysts in Toxoplasma gondii. J Biol Chem* **283**:16591-16601. Finally, TgIF2K-C is another GCN2-like protein kinase present in the *Toxoplasma* genome. However, this putative eIF2 α kinase appears to lack an RWD that was reported to be essential for Gcn2 activity in the yeast model system. The data presented herein clearly demonstrates that deletion of TgIF2K-D alone is sufficient to disrupt the translational control required for the parasite to cope with the extracellular environment.

Accordingly, TgIF2K-D and the eIF2 α kinase stress response pathway is clearly a therapeutic target.

[00170] The tandem arrangement of GCN2-related eIF2 α kinases is also found in the related parasite, *Plasmodium falciparum*. Conservation of multiple GCN2-related kinases may indicate that each phosphorylates eIF2 α in response to distinct stress conditions. The *P. falciparum* PF14_0264 product is most closely related to TgIF2K-D and contains an RWD domain, while PfeIK1 appears to lack an RWD domain and has recently been reported to respond to amino acid starvation during the intraerythrocyte ring stage. This observation suggests that the RWD/GCN1 regulatory network may not be essential for invoking translational control during certain nutritional deficiencies.

[00171] GCN2-like protein kinases lacking the RWD domain are not restricted to Apicomplexa. Three GCN2-related kinases have been described in *Dictyostelium* (IFKA through C) but only IFKC possesses a RWD domain. *Dictyostelium* is capable of developing a fruiting body, a process that is induced upon nutrient starvation. Although involved in regulating *Dictyostelium* development, neither IFKA nor IFKB appear to represent the initial sensor for this stress, supporting the idea that different GCN2 isoforms sense distinct stress conditions. The role of IFKC in this process has not yet been studied.

MATERIALS AND METHODS

Allelic replacement vector for TgIF2 α -S71A

[00172] To generate the allelic replacement construct, two fragments (-2600 bp to -1411 bp and -1238 bp to +440 bp) were amplified and inserted on opposing ends of the CAT minigene cassette within the pminCAT/HXGPRT⁺ vector, as illustrated in **FIG. 1A** (Roos *et al.*, 1994). The ~1.2 kb 5' TgIF2 α flanking sequence (-2600 bp to -1411 bp) was inserted between the *NotI* and *BamHI* sites using the oligonucleotides "5'S71A for + *NotI*" and "5'S71A rev + *BamHI*". Sequences for all primers used for this study are listed in **Table 3**. The 3' TgIF2 α flanking fragment (-1238 bp to +440 bp), which includes the entire first exon of TgIF2 α as well as ~1.2 kb of upstream sequence, was inserted into the *BclI* site of the construct described above. A point substitution was generated to change Ser71 to Ala71 using the QuikChange XL mutagenesis kit (Stratagene) and the oligonucleotides "TgIF2 α S71A quikchange 1F" and "TgIF2 α S71A quikchange 1R".

Table 3. List of some of the primers used in this study

SEQ ID NO.	Primer Name	Sequence
SEQ ID NO. 1	5' S71A for + <i>NotI</i>	gcgcgcggccgcGCCGGGTCGTTTTGTATAG
SEQ ID NO. 2	5' S71A rev + <i>BamHI</i>	ggcgggatccCGCTTCACCTGTCGGTTTCC
SEQ ID NO. 3	3' S71A for + <i>BclI</i>	gcgctgatcaCTCACCGCCTCGTCGCTGTGT
SEQ ID NO. 4	3' S71A rev + <i>BclI</i>	gcgctgatcaGGTGTCGGATGTCAGGTGGCGG
SEQ ID NO. 5	TgIF2 α S71A quikchange 1F	TCTCATGAGTGAAGTGGCCAAACGGCGGTTCCGC
SEQ ID NO. 6	TgIF2 α S71A quikchange 1R	GCGGAACCGCCGTTTGGCCAGTTCACTCATGAGA
SEQ ID NO. 7	S71A screen for	CCTGCGCGAGTCTGTGAG
SEQ ID NO. 8	S71A screen rev	GGTGTCGGATGTCAGGTGGCGG
SEQ ID NO. 9	TgIF2 α Taqman for	CAACAACATGGAAGGCATGATT
SEQ ID NO. 10	TgIF2 α Taqman rev	GATCGACGCGGAGAACCA
SEQ ID NO. 11	FAM-WT TgIF2 α	FAM-CATGAGTGAAGTGAAGCA
SEQ ID NO. 12	VIC-TgIF2 α -S71A	VIC-ATGAGTGAAGTGGCCAA

Generation of TgIF2 α -S71A mutant parasites

[00173] Twenty-five micrograms of the TgIF2 α -S71A allelic replacement vector was linearized with *NotI* and transfected into RH strain Δ Ku80 parasites (Fox *et al.*, 2009; Huynh and Carruthers, 2009) as previously described (Roos *et al.*, 1994). Parasites were cultivated in confluent monolayers of human foreskin fibroblasts (HFF) under standard conditions (DMEM plus 1% FBS (Invitrogen) in a humidified incubator at 37°C with 5% CO₂). Transgenic parasites were selected in 20 μ M chloramphenicol and cloned by limiting dilution in 96-well plates. Parasite clones were screened by immunoblot analysis of the phosphorylation status of TgIF2 α phosphorylation following stress with 10 μ M tunicamycin for 1 hour (Narasimhan *et al.*, 2008b). Positive clones were confirmed independently using a PCR-based approach: replacement of the Ser71-encoding nucleotides with those that encode Ala71 creates a unique restriction site, *MscI* (FIG. 1A). Genomic DNA was isolated from parasite clones using the Qiagen DNeasy kit, and the designated portion of the TgIF2 α genomic locus was amplified by PCR using oligonucleotides “S71A screen for” and “S71A screen rev” (FIG. 1A). The resulting amplicon

was gel-purified and digested with MscI. Clones with the S71A substitution yield two bands of 340 and 220 bp, whereas the parental strain yields a single 560 bp band.

TgIF2 α phosphorylation detection and protein radiolabelling

[00174] Phosphorylation of TgIF2 α was monitored by Western blotting performed in accordance with standard methods. Intracellular parasites were released from HFF host cells by physical disruption (scrapping and/or syringe passage) and purified by filtration through 3 micron polycarbonate filters (Roos *et al.*, 1994). The purified extracellular parasites were incubated in DMEM containing 1% FBS at 37°C under 5% CO₂ for the designated length of time. Fifty μ g of protein lysates were separated by electrophoresis using a 10% Bis-Tris acrylamide gel (Invitrogen). Proteins were transferred to nitrocellulose membranes and probed with either rabbit anti-TgIF2 α antibody (diluted 1:10,000) or phospho-specific (Ser71) TgIF2 α antibody (diluted 1:500) followed by an anti-rabbit IgG-horseradish peroxidase conjugate (GE Healthcare) (Narasimhan *et al.*, 2008b; Sullivan Jr. *et al.*, 2004b). Total and phospho-TgIF2 α was visualized using an ECL Western blotting substrate (Pierce).

[00175] In experiments in which parasites were radiolabeled, equal numbers of extracellular tachyzoites were resuspended in labeling media, Dulbecco's modified Eagle's medium without L-methionine, L-cysteine, L-glutamine, or sodium pyruvate (Invitrogen #21013-024) supplemented with 5% fetalbovine serum, 1 mM L-glutamine, 0.5 mM sodium pyruvate. 0.145 mCi of Express Protein Label Mix containing [³⁵S]methionine and [³⁵S]cysteine (PerkinElmer Life Sciences) was added to the sample and incubated for 1h. Samples were washed twice in PBS, and a portion was counted to determine similar uptake of the radiolabel. Parasites were resuspended in lysis buffer and sonicated. Equal amounts of total protein from each lysate preparation were separated by SDS/PAGE, and radiolabeled proteins were visualized by autoradiography. Results are presented as means S.E. that were derived from three independent experiments. The Student's *t* test was used to determine the statistical significance.

Competitive parasite fitness assay using TaqMan probes

[00176] The comparative fitness assay was based on a protocol outlined in (Fohl and Roos, 2003). Following filter-purification, 5 X 10⁵ parental Δ Ku80 (referred to as wild-type, or WT) and TgIF2 α -S71A mutant parasites were mixed in 10 ml DMEM + 1% FBS and added to a T-25 cm² flask containing a monolayer of HFF host cells. A sample of the mixed parasites was collected every 72 hours for a total of 9 days. At each time point, 10⁵ parasites were used to

infect a fresh HFF monolayer. Genomic DNA was isolated from each parasite sample using the DNeasy kit (Qiagen). TaqMan-based allelic discrimination assay was performed using a forward and reverse oligonucleotides “TgIF2 α TaqMan for” and “TgIF2 α TaqMan rev” and a combination of probes used to identify the WT or mutant allele (“FAM-WT TgIF2 α ” and “VIC-TgIF2 α -S71A”, respectively). PCR reactions were performed in triplicate using the 7500 Real-time PCR system and analyzed with relative quantification software (SDS software Version 1.2.1, Applied Biosystems).

Parasite growth assays

[00177] *Toxoplasma* doubling assays were performed as previously described (Fichera *et al.*, 1995). Intracellular Δ Ku80 and TgIF2 α -S71A parasites were physically removed from host cells by syringe passage. 10^5 of each were immediately applied to a fresh monolayer of HFFs grown on coverslips (0 hr), or incubated in culture media at 37°C with 5% CO₂ in absence of host cells for 4 or 8 hours prior to infection. The number of parasites per vacuole was visualized every 8 hours by immunofluorescence assay (IFA) using the DNA intercalator 4',6-diamidino-2-phenylindole (DAPI). Experiments were carried out in triplicate using separate biological samples. *Toxoplasma* growth in culture was also evaluated by a standard plaque assay (Roos *et al.*, 1994). 500 Δ Ku80 or TgIF2 α -S71A mutant parasites were allowed to infect a HFF monolayer; the degree of host cell lysis was evaluated on day 5-7 using Coomassie Brilliant Blue staining. The area of clearing representing the degree of monolayer disruption was determined using Alpha Innotech Imaging system. Experiments were carried out in triplicate using separate biological replicates. Results from a single representative experiment are shown.

Toxoplasma adhesion and invasion assay

[00178] The red/green adhesion and invasion assay was carried out as previously described (Huynh *et al.*, 2003). Immediately following egress, 1×10^7 Δ Ku80 or TgIF2 α -S71A parasites were allowed to adhere/invade a confluent monolayer of HFF cells cultured on glass coverslips in 12-well plates. Prior to permeabilization, the infected cells were incubated with a mouse anti-Sag1 immune serum (Meridian Life Sciences) to visualize adhered parasites. The infected cells were then washed with PBS, permeabilized with 0.2% Triton X-100 in PBS and incubated with a second primary immune serum, rabbit anti-Sag1 (a generous gift from John Boothroyd, Stanford). A combination of anti-mouse Alexa fluor 488 and anti-rabbit Alexa fluor 594 was applied to the infected cells to distinguish between adhered versus invaded parasites. In the

merged images, the adhered parasites appear yellow and the invaded parasites are red. The numbers of adhered and invaded parasites were counted for a minimum of 6 different microscope fields. The standard error and Student's t test was applied as described above.

***Toxoplasma* motility assay**

[00179] 10^5 Δ Ku80 or TgIF2 α -S71A parasites were allowed to adhere and glide along a poly-L lysine coated coverslip for 30 min in DMEM containing 1% FBS. The adhered parasites and surface protein "trails" were detected with a mouse anti-Sag1 immune serum as previously described (Dobrowolski and Sibley, 1996). The number of Δ Ku80 or TgIF2 α -S71A parasites with trails was recorded from a minimum of 6 independent microscope fields.

***Toxoplasma* egress assay**

[00180] Parasite egress assays were performed as previously described (Black *et al.*, 2000). Briefly, 10^6 Δ Ku80 or TgIF2 α -S71A parasites were cultivated in HFFs at 37°C, 5% CO₂ overnight. To induce egress of the tachyzoites, infected monolayers were exposed to 2 μ M A23187 for 0, 0.5, 1, 2, 3, or 4 min. At each time interval, the parasites were fixed with cold methanol and the percentage of parasite egress was recorded from 10 random microscope fields.

Animal studies

[00181] Female BALB/c mice (18-20 grams) were injected intraperitoneally with 10 or 10^2 Δ Ku80 or TgIF2 α -S71A parasites (10 mice per group) harvested immediately after egress from HFF cells. Animals were monitored twice daily until significant illness was observed at which time the moribund animals were euthanized. Time to moribund state was recorded for each infected mouse.

Parasite culture

[00182] *Toxoplasma* tachyzoites were maintained in human foreskin fibroblasts (HFF) in Dulbecco Modified Eagle's Media (DMEM) containing 25 mM glucose and 4 mM glutamine (Invitrogen) supplemented with 1% heat inactivated fetal bovine serum (Gibco/Invitrogen) at 37°C and 5% CO₂.

Cloning of the *TgIF2K-D* cDNA

[00183] Tachyzoite mRNA was used to generate a cDNA library (Omniscript, Qiagen) for the amplification of the *TgIF2K-D* open reading frame (ORF). This PCR amplification employed primers specific to the *TgIF2K-D* gene that was annotated in the *Toxoplasma* database (www.toxodb.org, TgME49_119610). Tachyzoite mRNA was reverse transcribed using

SuperScript One-Step RT-PCR kit (Invitrogen) with random and oligo-dT primers according to the manufacturer's recommendations. All PCRs were carried out with Phusion DNA-polymerase (Finnzymes) using the provided GC buffer. The GeneRacer Kit (Invitrogen) was used for the 5'- and 3'-rapid amplification of cDNA ends (RACE) of the *TgIF2K-D* gene.

Generation of TgIF2K-D knockout parasites

[00184] In order to generate *TgIF2K-D* knockout parasites ($\Delta IF2K-D$), ~1.5 kb DNA fragments upstream and downstream of the start and stop codons encoded in the *TgIF2K-D* locus were amplified. Referring now to Table 2, oligonucleotide primers used to amplify the 5' flanking sequence were designated #1 and #2, and the primers used to amplify the 3' flanking sequence were #3 and #4. The amplified DNA was inserted into the pDHFR*-TSc3 vector, such that these fragments flanked opposing ends of a modified dihydrofolate reductase-thymidylate synthase (*DHFR*-TS*) minigene, which confers resistance to pyrimethamine. *Hinnebusch, A. G. 2005. The resulting knockout vector was designated $\Delta TgIF2K-D::DHFR^*$. Fifty μ g of the $\Delta TgIF2K-D::DHFR^*$ knockout vector was linearized with NotI and transfected into RH strain parasites lacking *Ku80*. Fox, B. A., J. G. Ristuccia, J. P. Gigley, and D. J. Bzik. 2009. Efficient gene replacements in *Toxoplasma gondii* strains deficient for nonhomologous end joining. Eukaryot Cell 8:520-529.* Transfected parasites were grown in HFF cells in the above defined DMEM supplemented with 1 μ M pyrimethamine and cloned by limiting dilutions. Individual parasite clones were screened by PCR to confirm the replacement of the *TgIF2K-D* genomic locus with the *DHFR*-TS* minigene.

[00185] To confirm that the correct insertion occurred at the *TgIF2K-D* locus, primers complementary to the 3'-UTR of the *DHFR** minigene (#10) and upstream of the insertion site (#9) were used in a PCR assay with genomic DNAs purified from the candidate knockout parasites. PCR assays using primers #5 and #6, which are complementary to exon III, and #7 and #8 that were used to generate the genetic tagging vector (see below) were carried out to verify the absence of the complete *TgIF2K-D* genomic locus. Loss of *TgIF2K-D* mRNA expression was verified by RT-PCR using primers complementary to sequences upstream (#11) and downstream (#12) of the encoded protein kinase domain. As control, a portion of *Toxoplasma* actin (TgME49_009030) mRNA was amplified by RT-PCR using primers #13 and #14.

Genetic tagging of *TgIF2K-D*

For the expression of TgIF2K-D tagged with hemagglutinin (HA) at its C-terminus, a 1.2-kb DNA fragment containing exon XVIII was amplified using *Toxoplasma* genomic DNA as the template and primers #7 and #8. The amplified DNA segment was then inserted into the vector 3xHA-LIC-DHFR-TS using the ligation-independent cloning method (40). LIC-HA3x-DHFR-Ts is a derivative of pYFP-LIC-DHFR in which the YFP coding fragment had been replaced with three contiguous HA tags. **Huynh, M. H., and V. B. Carruthers. 2009. Tagging of endogenous genes in a *Toxoplasma gondii* strain lacking *Ku80*. *Eukaryot Cell* 8:530-539.** Fifty µg of the TgIF2K-D-HA3x plasmid was linearized with the restriction enzyme *AscI* and then transfected into RHΔ*Ku80* parasites. Following limiting dilutions, positive clones were identified using a monoclonal antibody that specifically recognizes the HA tag (Roche).

[00186] The 1.2-kb DNA fragment containing exon XVIII (amplified with primers #7 and #8) was also ligated into a LIC-HA2X-DD-DHFR-TS vector to generate a *TgIF2K-D* fusion with 2xHA and a Shield-regulated destabilization domain (DD) at the C-terminus (2xDD). **Huynh, M. H., 2009.** Following transfection of this linearized plasmid, individual parasite clones were screened for the stabilization of TgIF2K-D_{2xDD} in the presence of Shield-1 (500 nM, Clontech) using the anti-HA monoclonal antibody.

Comparative fitness assay

[00187] The comparative fitness assay was carried out as described previously by Joyce et al. (16), with the exception that SYBR green-based quantitative real-time PCR (qPCR) was performed using primers that specifically delineated between parental Δ*Ku80*, referred to as wild-type (WT), and Δ*IF2K-D* parasites. In brief, equal numbers of filter-purified parental and Δ*IF2K-D* parasites (5×10^5) were co-cultured in the same flask of HFF host cells. At 72 hours post-infection, 10^5 parasites of the mixed population were isolated from the lysed culture and then transferred to a fresh HFF monolayer for an additional 72 hours. This resulted in a total of 6 days of HFF infection by using two serial passages. Genomic DNA (gDNA) from the parasite samples was isolated using the DNeasy Kit (Qiagen) and used in qPCR assays. Primers used to distinguish between WT from Δ*IF2K-D* parasites included #15 and #16, and #17 and #18, as indicated. qPCR measurements were normalized by amplifying the 5'-UTR of TgIF2K-D, which is present in both WT and Δ*IF2K-D* parasites (primers #19 and #20). 25 ng of gDNA was used in the qPCR assays, which were performed in triplicate using the 7500 Real-Time PCR System (Applied Biosystems). Relative quantification software (SDS software, version 1.2.1) was used

for the analysis. As a specificity control, SYBR green assays employing gDNA purified from either WT or $\Delta IF2K-D$ parasites were carried out to verify the specificity of primers in the qPCR assay (data not shown).

Parasite proliferation assays

[00188] *Toxoplasma* recovery from extracellular stress was analyzed using standard doubling and plaque assays. Parental $\Delta Ku80$ (WT), $\Delta IF2K-D$, and TgIF2 α -S71A parasites were physically released from host cells by syringe passage and then filter purified to remove host cell debris. 10^6 parasites were subjected to an extracellular stress assay for 0, 8, or 10 hours in culture medium at 37°C and 5% CO₂ without host cells prior to infecting HFF host cells. Parasites were quantitated using a standard counting assay with counts performed every 8 hours post-infection. Parasite counting assays were carried out in triplicate using separate biological samples and a representative experiment is shown. In the plaque assays, 500 WT, $\Delta IF2K-D$, TgIF2 α -S71A, or TgIF2K-D_{2xDD} parasites were used to infect HFF monolayers in 12-well plates following extracellular incubation for up to 10 hours, as indicated. The degree of host cell lysis at 7 days post-infection was determined by crystal violet staining of methanol fixed cells. Measurements of the lysed areas were determined using an Alpha Innotech Imaging system, and a representative experiment of three independent experiments presented.

Western blotting

[00189] Western blot analyses of TgIF2 α phosphorylation were carried out as previously described. To analyze the stabilization of the TgIF2K-D_{2xDD} protein, intracellular parasites were grown for up to 24 hours in medium supplemented with 500 nM Shield-1 prior to physical release from the host cells. HA-tagged proteins were detected by western blot analyses after resolving parasite lysate on a 3-8% Tris-acetate polyacrylamide gradient gel. Rat monoclonal antibody that specifically recognizes the HA tag (Roche) was used as a primary antibody and an anti-rat IgG antibody conjugated with horseradish peroxidase (GE Healthcare) was used as a secondary antibody. HA-tagged proteins were visualized using a chemiluminescence western blotting substrate (Pierce).

[00190] Measurements of protein synthesis. Intracellular parasites were mechanically released from host cells as described above and 2.5×10^7 /ml tachyzoites were transferred into *Toxoplasma* culture medium lacking methionine and cysteine. Labeling was initiated by adding [³⁵S]-Met/Cys-label (ICN) to a final concentration of 200 μ Ci/ml. After 1 hour incubation in

DMEM at 37°C and 5% CO₂, samples were immediately put on ice. Parasites were harvested by centrifugation at 4°C, cell pellets were washed twice with ice cold PBS and then lysed in 100 µl RIPA buffer. Uptake of the ³⁵S during the 1 hour pulse radiolabeling was similar between the WT and mutant parasites. For each sample, equal amounts of proteins were precipitated by adding trichloroacetic acid (TCA) to a final concentration of 10%. After incubating on ice for 30 min, samples were collected by centrifugation 10,000xg for 30 min at 4°C. The TCA precipitates were washed twice with acetone and resuspended in an equal volume of PBS. Incorporation of the radiolabeled amino acids was determined using a scintillation counter. All radiolabeling experiments were presented as an average of three independent samples, with *p*-values and standard errors determined using analysis of variance (ANOVA).

[00191] Immunofluorescence assays. HFF monolayers were grown on coverslips, infected for 24 hours, and then fixed in 3% paraformaldehyde. Immunofluorescence analyses using a rat monoclonal antibody that recognizes the HA tag (Roche) followed by goat anti-rat AlexaFluor488 as the secondary antibody (Invitrogen) was performed as previously described.

REFERENCES

- [00192]** Balaji, S., Babu, M.M., Iyer, L.M., and Aravind, L. (2005). Discovery of the principal specific transcription factors of Apicomplexa and their implication for the evolution of the AP2-integrase DNA binding domains. *Nucleic Acids Res* 33, 3994-4006.
- [00193]** Black, M.W., Arrizabalaga, G., and Boothroyd, J.C. (2000). Ionophore-resistant mutants of *Toxoplasma gondii* reveal host cell permeabilization as an early event in egress. *Mol Cell Biol* 20, 9399-9408.
- [00194]** Black, M.W., and Boothroyd, J.C. (2000). Lytic cycle of *Toxoplasma gondii*. *Microbiol Mol Biol Rev* 64, 607-623.
- [00195]** Dever, T.E., Feng, L., Wek, R.C., Cigan, A.M., Donahue, T.F., and Hinnebusch, A.G. (1992). Phosphorylation of initiation factor 2 alpha by protein kinase GCN2 mediates gene-specific translational control of GCN4 in yeast. *Cell* 68, 585-596.
- [00196]** Dobrowolski, J.M., and Sibley, L.D. (1996). *Toxoplasma* invasion of mammalian cells is powered by the actin cytoskeleton of the parasite. *Cell* 84, 933-939.
- [00197]** Fennell, C., Babbitt, S., Russo, I., Wilkes, J., Ranford-Cartwright, L., Goldberg, D.E., and Doerig, C. (2009). PflK1, a eukaryotic initiation factor 2alpha kinase of the human malaria

parasite *Plasmodium falciparum*, regulates stress-response to amino-acid starvation. *Malar J* 8, 99.

[00198] Fichera, M.E., Bhopale, M.K., and Roos, D.S. (1995). In vitro assays elucidate peculiar kinetics of clindamycin action against *Toxoplasma gondii*. *Antimicrob Agents Chemother* 39, 1530-1537.

[00199] Finn, R. D., J. Mistry, J. Tate, P. Coghill, A. Heger, J. E. Pollington, O. L. Gavin, P. Gunasekaran, G. Ceric, K. Forslund, L. Holm, E. L. Sonnhammer, S. R. Eddy, and A. Bateman. 2010. The Pfam protein families database. *Nucleic acids research* 38:D211-222.

[00200] Fohl, L.M., and Roos, D.S. (2003). Fitness effects of DHFR-TS mutations associated with pyrimethamine resistance in apicomplexan parasites. *Mol Microbiol* 50, 1319-1327.

[00201] Fox, B.A., Ristuccia, J.G., Gigley, J.P., and Bzik, D.J. (2009). Efficient gene replacements in *Toxoplasma gondii* strains deficient for nonhomologous end joining. *Eukaryot Cell* 8, 520-529.

[00202] Francia, M. E., S. Wicher, D. A. Pace, J. Sullivan, S. N. Moreno, and G. Arrizabalaga. 2011. A *Toxoplasma gondii* protein with homology to intracellular type Na(+)/H(+) exchangers is important for osmoregulation and invasion. *Exp Cell Res*.

[00203] Hill, D.E., Chirukandoth, S., and Dubey, J.P. (2005). Biology and epidemiology of *Toxoplasma gondii* in man and animals. *Anim Health Res Rev* 6, 41-61.

[00204] Hinnebusch, A.G. (2005). Translational regulation of GCN4 and the general amino acid control of yeast. *Annu Rev Microbiol* 59, 407-450.

[00205] Huynh, M.H., and Carruthers, V.B. (2009). Tagging of endogenous genes in a *Toxoplasma gondii* strain lacking Ku80. *Eukaryot Cell* 8, 530-539.

[00206] Huynh, M.H., Rabenau, K.E., Harper, J.M., Beatty, W.L., Sibley, L.D., and Carruthers, V.B. (2003). Rapid invasion of host cells by *Toxoplasma* requires secretion of the MIC2-M2AP adhesive protein complex. *EMBO J* 22, 2082-2090.

[00207] Joyce, B. R., S. F. Queener, R. C. Wek, and W. J. Sullivan, Jr. 2010. Phosphorylation of eukaryotic initiation factor-2{alpha} promotes the extracellular survival of obligate intracellular parasite *Toxoplasma gondii*. *Proc Natl Acad Sci U S A* 107:17200-17205.

[00208] Khan, A., Behnke, M.S., Dunay, I.R., White, M.W., and Sibley, L.D. (2009). Phenotypic and gene expression changes among clonal type I strains of *Toxoplasma gondii*. *Eukaryot Cell* 8, 1828-1836.

- [00209] Lin, S. S., M. Blume, N. von Ahsen, U. Gross, and W. Böhne. 2011. Extracellular *Toxoplasma gondii* tachyzoites do not require carbon source uptake for ATP maintenance, gliding motility and invasion in the first hour of their extracellular life. *International journal for parasitology*.
- [00210] Mohrle, J.J., Zhao, Y., Wernli, B., Franklin, R.M., and Kappes, B. (1997). Molecular cloning, characterization and localization of PfPK4, an eIF-2 α kinase-related enzyme from the malarial parasite *Plasmodium falciparum*. *Biochem J* 328 (Pt 2), 677-687.
- [00211] Moraes, M.C., Jesus, T.C., Hashimoto, N.N., Dey, M., Schwartz, K.J., Alves, V.S., Avila, C.C., Bangs, J.D., Dever, T.E., Schenkman, S., *et al.* (2007). Novel Membrane-Bound eIF2{ α } Kinase in the Flagellar Pocket of *Trypanosoma brucei*. *Eukaryot Cell* 6, 1979-1991.
- [00212] Narasimhan, J., Joyce, B.R., Naguleswaran, A., Smith, A.T., Livingston, M.R., Dixon, S.E., Coppens, I., Wek, R.C., and Sullivan, W.J., Jr. (2008a). Translation regulation by eukaryotic initiation factor-2 kinases in the development of latent cysts in *Toxoplasma gondii*. *J Biol Chem* 283, 16591-16601.
- [00213] Narasimhan, J., Joyce, B.R., Naguleswaran, A., Smith, A.T., Livingston, M.R., Dixon, S.E., Coppens, I., Wek, R.C., and Sullivan, W.J., Jr. (2008b). Translation Regulation by Eukaryotic Initiation Factor-2 Kinases in the Development of Latent Cysts in *Toxoplasma gondii*. *J Biol Chem* 283, 16591-16601.
- [00214] Roos, D.S., Donald, R.G., Morrisette, N.S., and Moulton, A.L. (1994). Molecular tools for genetic dissection of the protozoan parasite *Toxoplasma gondii*. *Methods Cell Biol* 45, 27-63.
- [00215] Scheuner, D., Song, B., McEwen, E., Liu, C., Laybutt, R., Gillespie, P., Saunders, T., Bonner-Weir, S., and Kaufman, R.J. (2001). Translational control is required for the unfolded protein response and in vivo glucose homeostasis. *Molecular Cell* 7, 1165-1176.
- [00216] Schroder, M., and Kaufman, R.J. (2005a). ER stress and the unfolded protein response. *Mutat Res* 569, 29-63.
- [00217] Schroder, M., and Kaufman, R.J. (2005b). The mammalian unfolded protein response. *Annu Rev Biochem* 74, 739-789.
- [00218] Sonenberg, N., and Hinnebusch, A.G. (2009). Regulation of translation initiation in eukaryotes: mechanisms and biological targets. *Cell* 136, 731-745.

[00219] Sullivan Jr., W.J., Narasimhan, J., Bhatti, M.M., and Wek, R.C. (2004a). Parasite-specific eukaryotic initiation factor -2 (eIF2) kinase required for stress-induced translation control. *Biochem J* 380, 523-531.

[00220] Wek, R.C., and Cavener, D.R. (2007a). Translational control and the unfolded protein response. *Antioxid Redox Signal* 9, 2357-2371.

[00221] Wek, R.C., Jiang, H.Y., and Anthony, T.G. (2006). Coping with stress: eIF2 kinases and translational control. *Biochem Soc Trans* 34, 7-11.

[00222] While the novel technology has been illustrated and described in detail in the figures and foregoing description, the same is to be considered as illustrative and not restrictive in character, it being understood that only the preferred embodiments have been shown and described and that all changes and modifications that come within the spirit of the novel technology are desired to be protected. As well, while the novel technology was illustrated using specific examples, theoretical arguments, accounts, and illustrations, these illustrations and the accompanying discussion should by no means be interpreted as limiting the technology. All patents, patent applications, and references to texts, scientific treatises, publications, and the like referenced in this application are incorporated herein by reference in their entirety.

CLAIMS

We claim:

1. A method of identifying a compound, comprising the steps of:
providing at least one translation initiation factor identified in a single celled eukaryotic parasite; and
contacting the at least one translation initiation factor with a compound that binds to the translation initiation factor.
2. The method according to claim 1, wherein the single celled parasite is selected from the group of genera consisting of *Toxoplasma*, *Plasmodium* and *Cryptosporidium*.
3. The method according to claim 1, wherein the translation initiation factor is phosphorylated by any eIF2 α kinase.
4. The method according to claim 3, wherein the kinase is selected from the group consisting of: TgIF2K-A, TgIF2K-B, TgIF2K-C, TgIF2K-D.
5. The method according to claim 4, wherein the kinase has at least 90 percent homology to TgIF2K-D.
6. The method according to claim 4, wherein the kinase has at least 95 percent homology to TgIF2K-D.
7. The method according to claim 4, wherein the kinase has at least 80 percent identity to TgIF2K-D.
8. The method according to claim 4, wherein the kinase has at least 90 percent identity to TgIF2K-D.
9. The method according to claim 4, wherein the kinase has at least 95 percent identity to TgIF2K-D.
10. The method according to claim 3, wherein the translation initiation factor has about 90 percent homology to TgIF2 α .
11. The method according to claim 3, wherein the translation initiation factor has greater than or equal to about 95 percent homology to TgIF2 α .
12. The method according to claim 3, wherein the translation initiation factor has greater than or equal to about 75 percent identity to TgIF2 α .
13. The method according to claim 3, wherein the translation initiation factor has greater than or equal to about 85 percent identity to TgIF2 α .

14. The method according to claim 3, wherein the translation initiation factor has greater than or equal to about 95 percent identity to TgIF2 α .
15. The method according to claim 3, wherein the translation initiation factor is TgIF2 α .
16. The method according to claim 1, wherein the parasite is member of the genus *Toxoplasma*.
17. The method according to claim 1, wherein the parasite is *Toxoplasma gondii*.
18. The method according to claim 3, further including the step of:
measuring a signal generated by the binding of the compound with the eIF2 α kinase.
19. The method according to claim 3, further including the step of:
measuring a change in the activity of the eIF2 α kinase after contacting the eIF2 α kinase with said compound.
20. The method according to claim 3, further including the step of:
measuring a signal generated by the binding of the compound with translation factor TgIF2 α .
21. The method according to claim 3, further including the step of:
measuring a change in the activity of the translation factor after contacting the eIF2 α kinase with said compound.
22. A method of reducing the infectivity of a single celled eukaryotic parasite, comprising the steps of:
providing a single celled eukaryotic parasite, wherein the parasite includes at least one eIF2 α kinase and at least one translation initiation factor wherein the at least one kinase phosphorylates the at least one translation factor; and
contacting the parasite with at least one compound that interferes with or eliminates the phosphorylation of the translational initiation factor.
23. The method according to claim 22, wherein the parasite is selected from the group consisting of; *Toxoplasma*, *Plasmodium* and *Cryptosporidium*.
24. The method according to claim 22, wherein the kinase is selected from the group consisting of: TgIF2K-A, TgIF2K-B, TgIF2K-C, TgIF2K-D.
25. The method according to claim 24, wherein the kinase has at least 90 percent homology to TgIF2K-D

26. The method according to claim 24, wherein the kinase has at least 95 percent homology to TgIF2K-D.
27. The method according to claim 24, wherein the kinase has at least 80 percent identity to TgIF2K-D.
28. The method according to claim 24, wherein the kinase has at least 90 percent identity to TgIF2K-D.
29. The method according to claim 24, wherein the kinase has at least 95 percent identity to TgIF2K-D.
30. The method according to claim 22, wherein the translation initiation factor has about 90 percent homology to TgIF2 α .
31. The method according to claim 22, wherein the translation initiation factor has greater than or equal to about 95 percent homology to TgIF2 α .
32. The method according to claim 22, wherein the translation initiation factor has greater than or equal to about 75 percent identity to TgIF2 α .
33. The method according to claim 22, wherein the translation initiation factor has greater than or equal to about 85 percent identity to TgIF2 α .
34. The method according to claim 22, wherein the translation initiation factor has greater than or equal to about 95 percent identity to TgIF2 α .
35. The method according to claim 22, wherein the translation initiation factor is TgIF2 α .
36. A method for vaccinating a patient, comprising the steps of:
 providing a mutant of a single celled organism selected from the genera of parasites consisting of: *Toxoplasma*, *Plasmodium* and *Cryptosporidium*, wherein said mutant is deficient in eIF2 α kinase activity; and/or a translation initiation factor that can be phosphorylated by said eIF2 α kinase activity; and
 contacting a mammal with said mutant form of the parasite.
37. The method according to claim 36, wherein said mutant parasite is from the genus *Toxoplasma*.
38. The method according to claim 36, wherein said mutant parasite is a mutant of *Toxoplasma gondii*.
39. The method according to claim 36, wherein the eIF2 α kinase is selected from the group consisting of TgIF2K-A, TgIF2K-B, TgIF2K-C, TgIF2K-D.

40. The method according to claim 38, wherein the eIF2 α kinase is has at least 90 percent homology to TgIF2K-D.
41. The method according to claim 38, wherein the mutant has no TgIF2K-D activity.
42. The method according to claim 38, wherein the translation factor has at least 90 percent homology to TgIF2 α -S71A and cannot be phosphorylated by TgIF2K-D .
43. The method according to claim 36, wherein the translation factor has at least 80 percent identity to TgIF2 α -S71A and cannot be phosphorylated by TgIF2K-D .
44. The method according to claim 36, wherein the patient is an animal is selected from the group of animals consisting of humans, ovines, felines, and bovines.

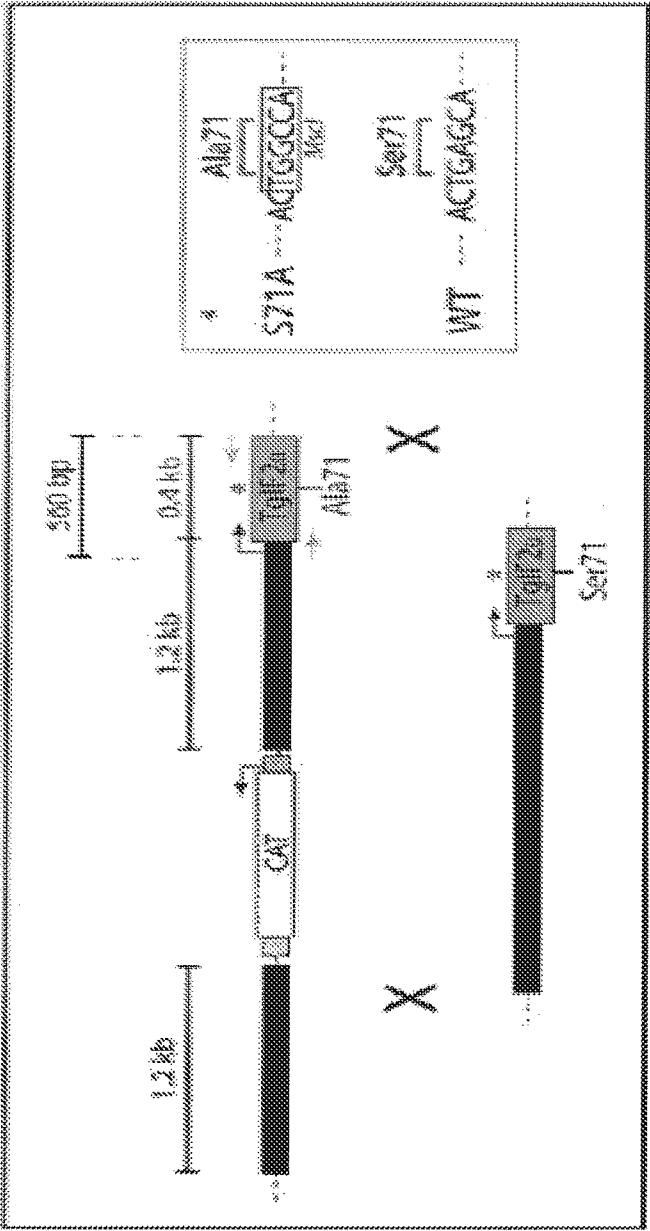


FIG. 1A

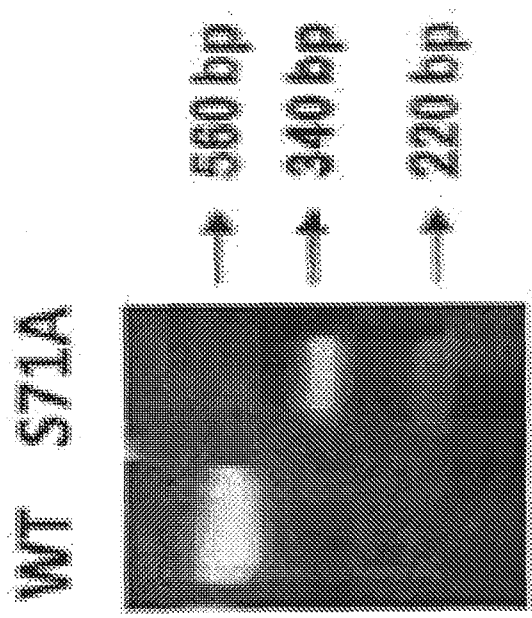


FIG. 1B

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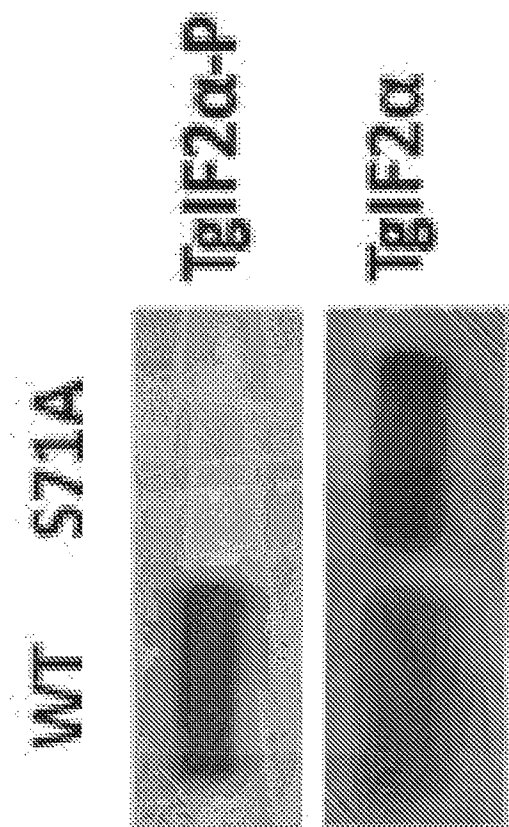


FIG. 1C

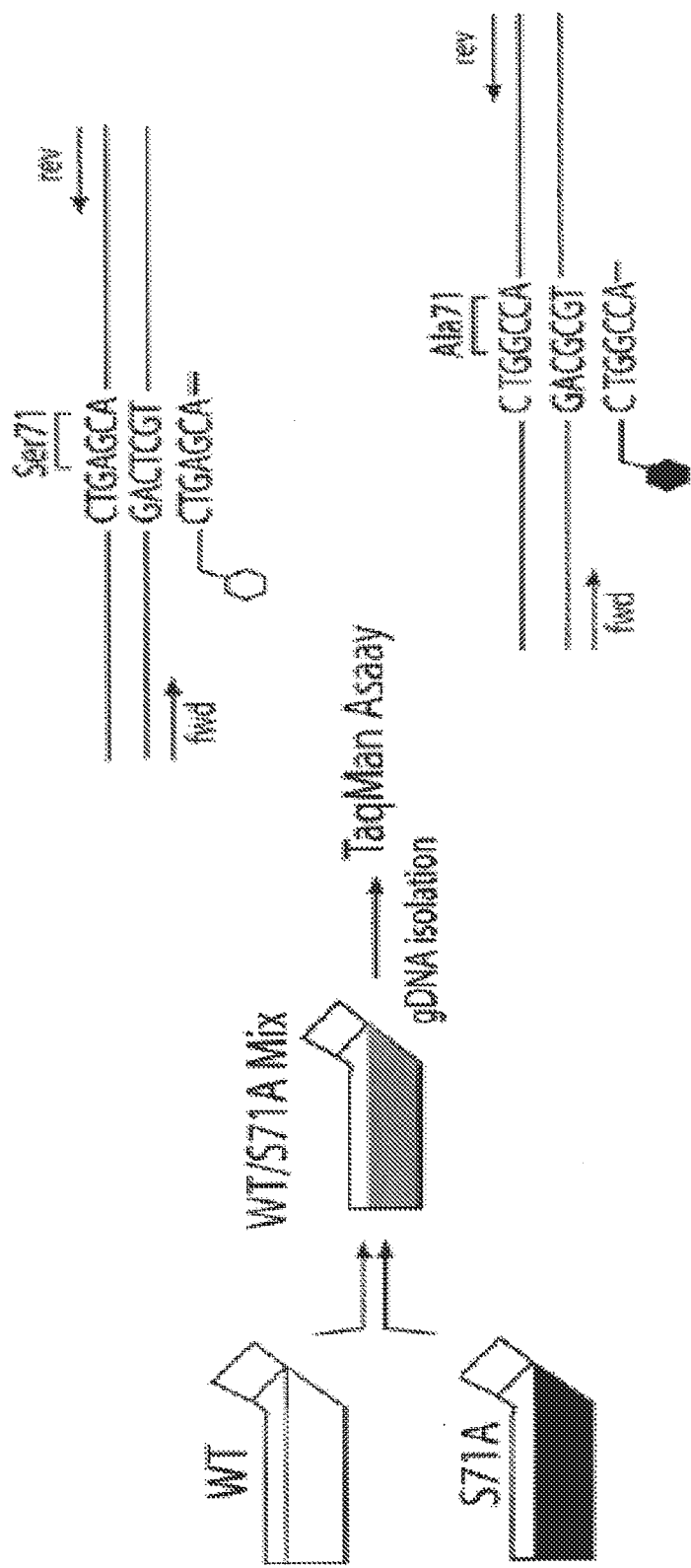


FIG. 2A

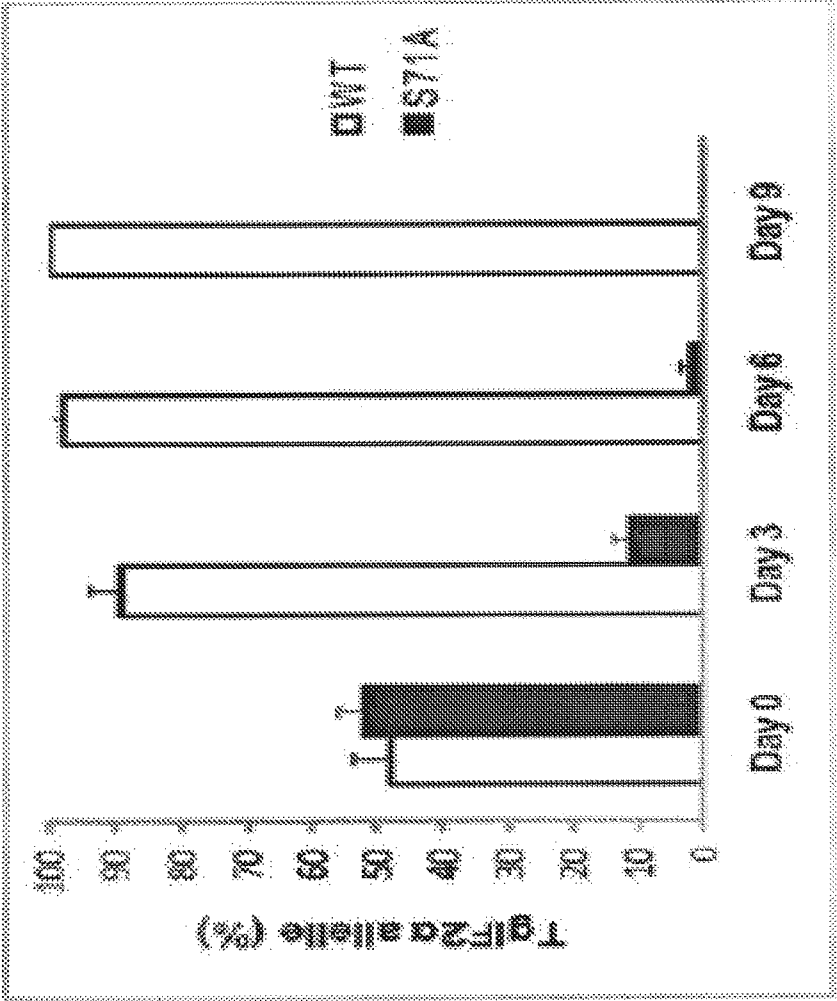


FIG. 2B

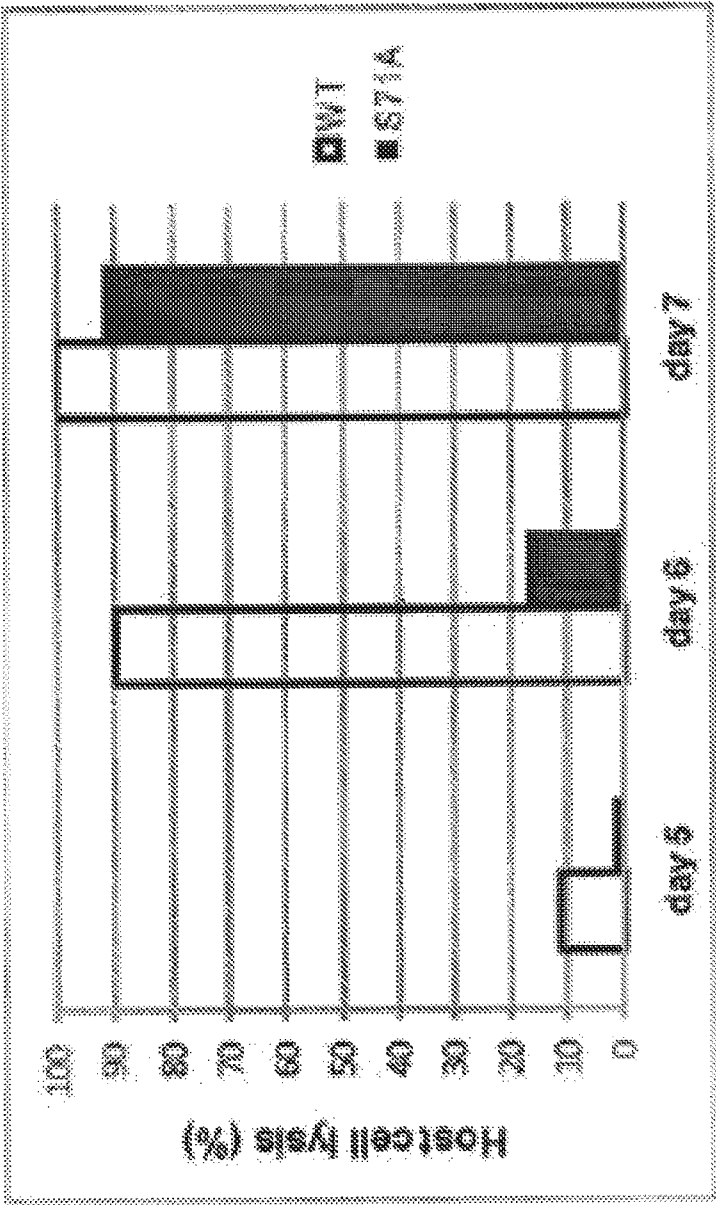


FIG. 2C

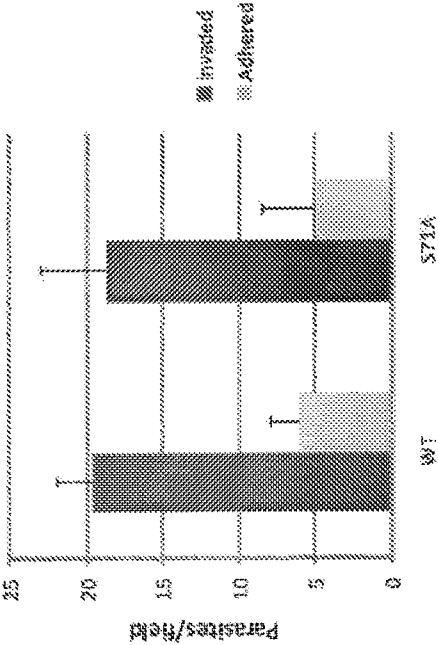
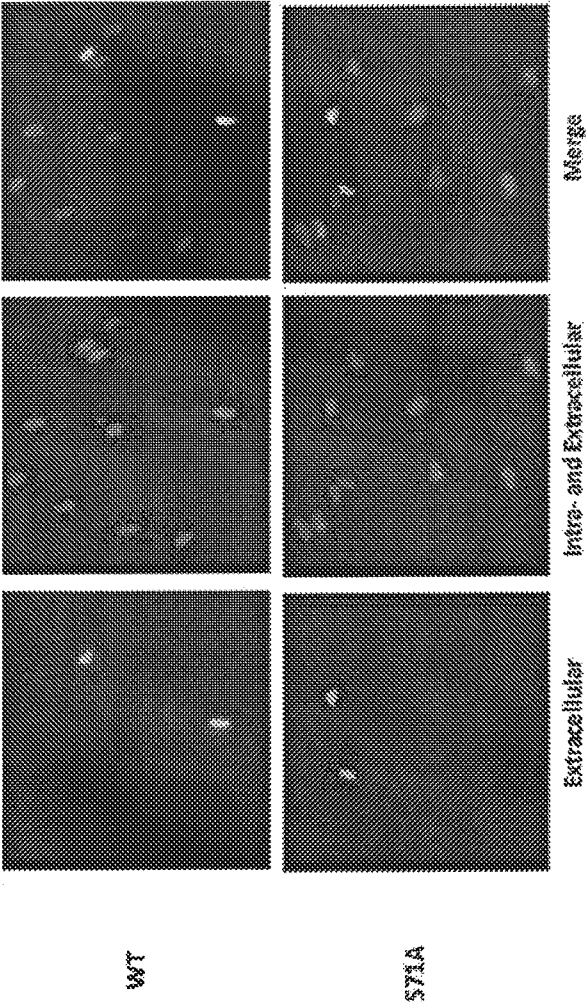


FIG. 3

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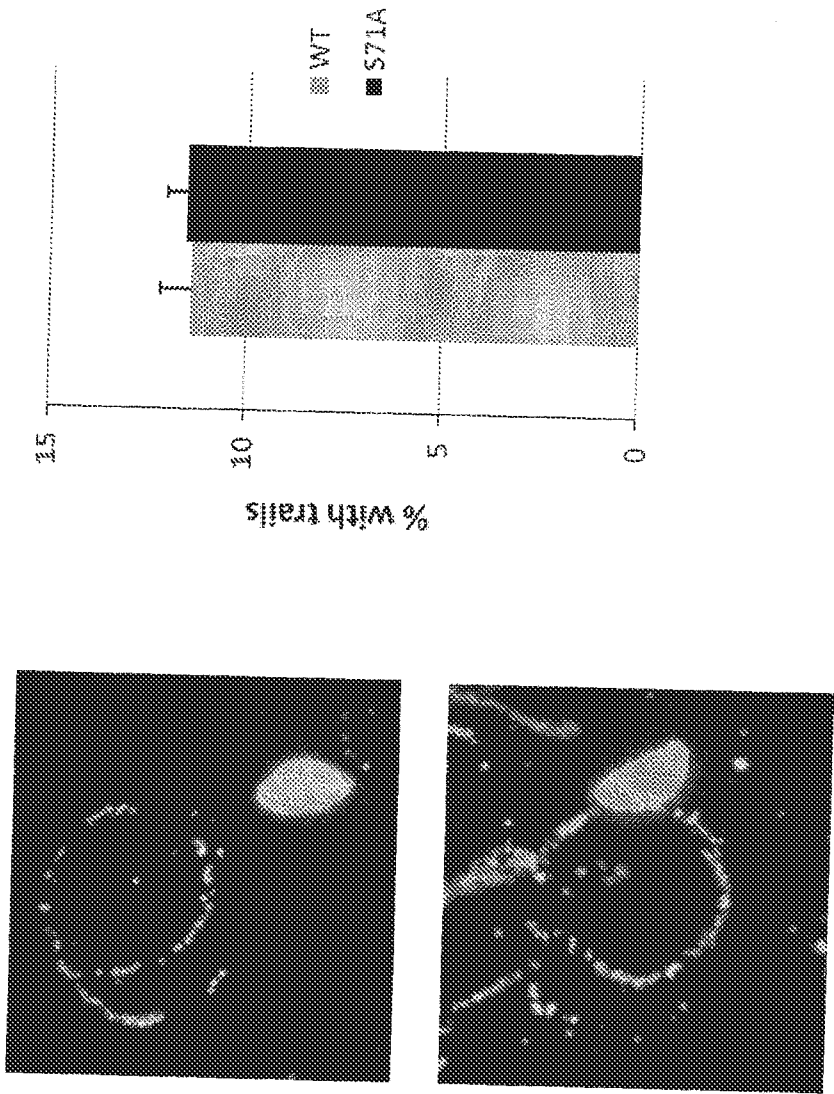


FIG. 4A

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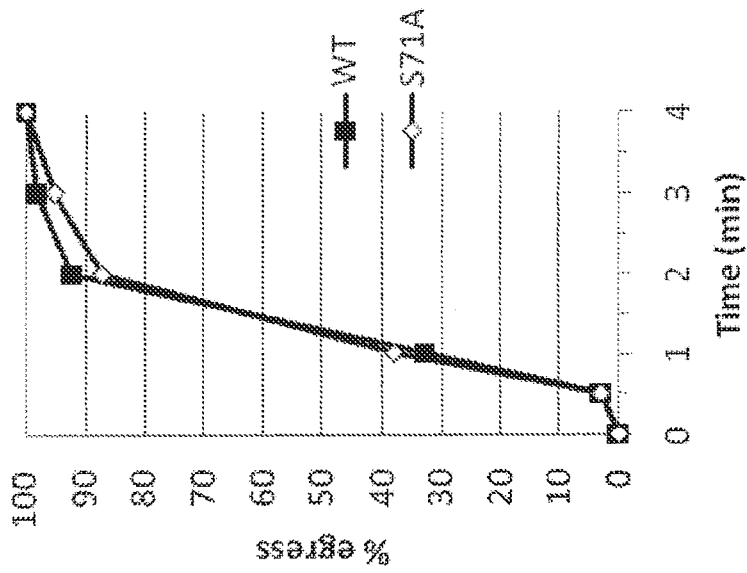


FIG. 4B

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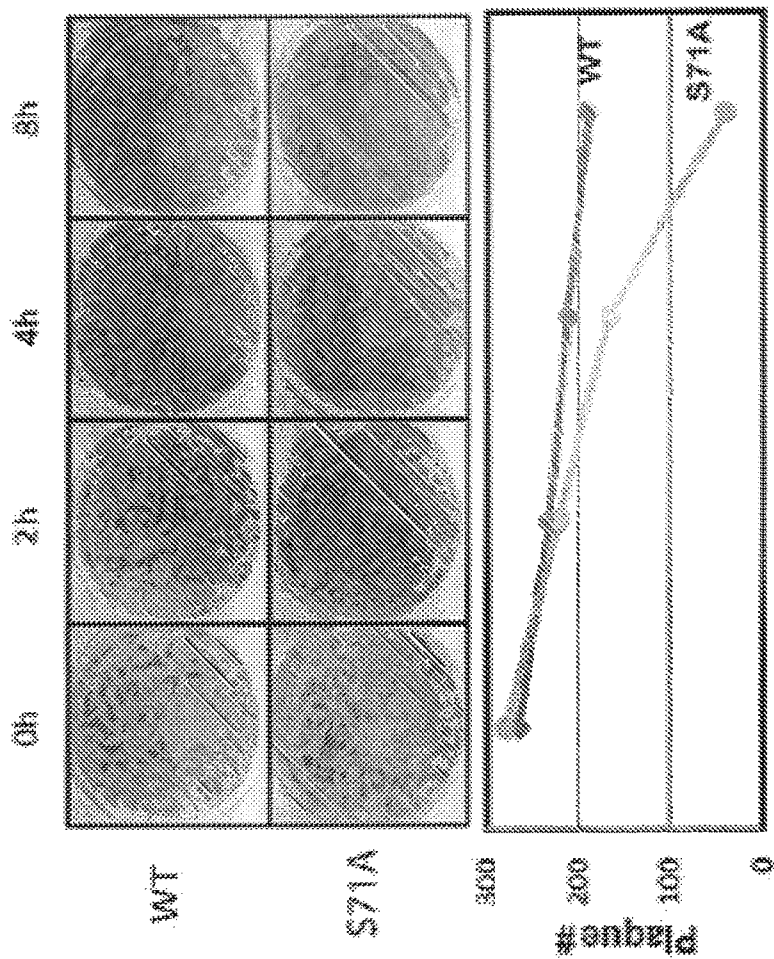


FIG. 5A

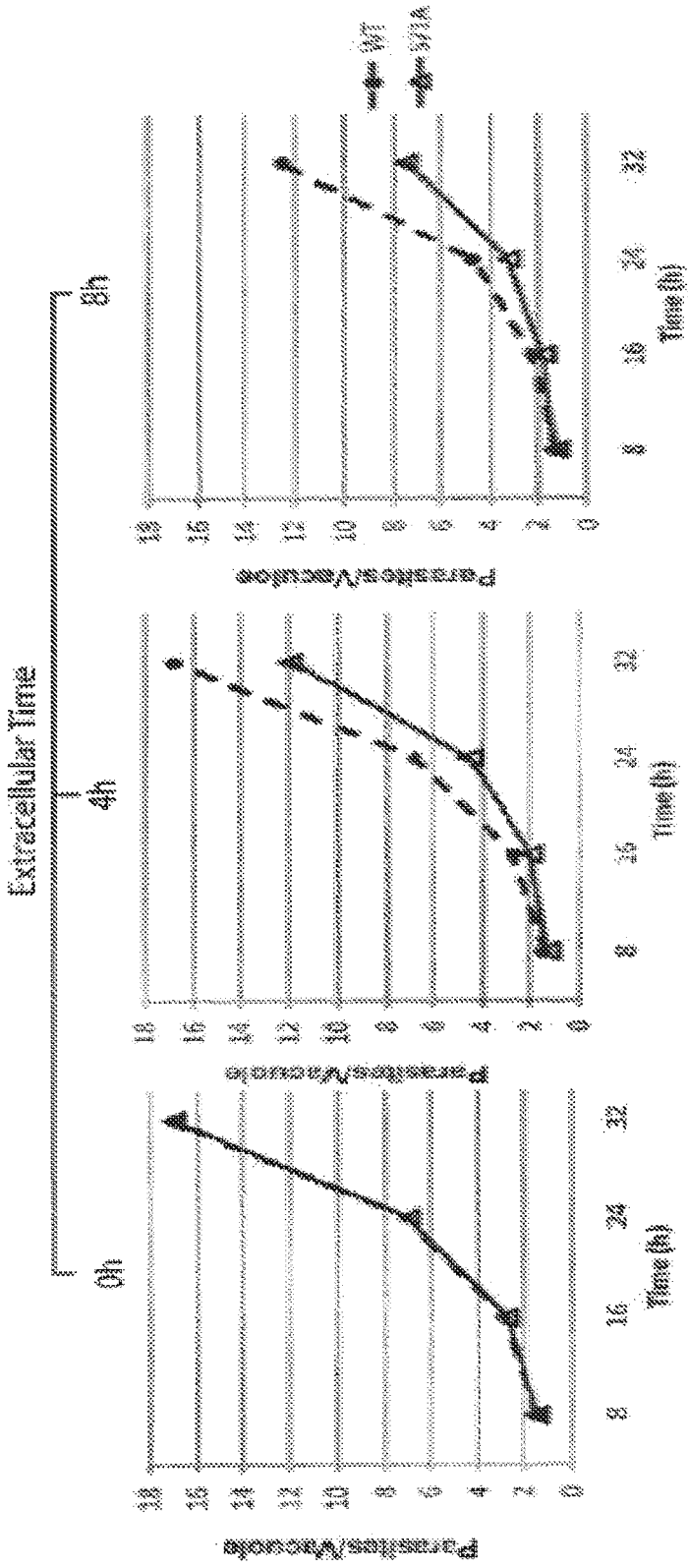


FIG. 5B

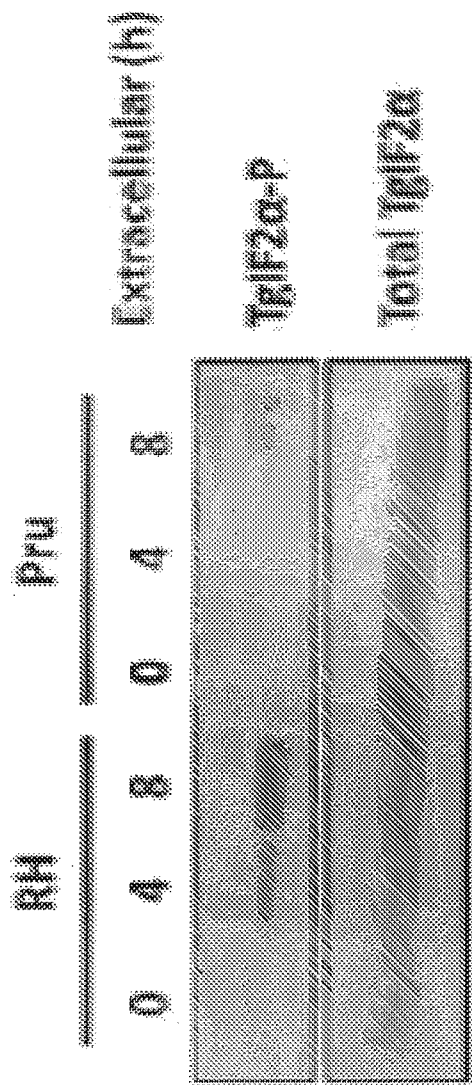


FIG. 5C

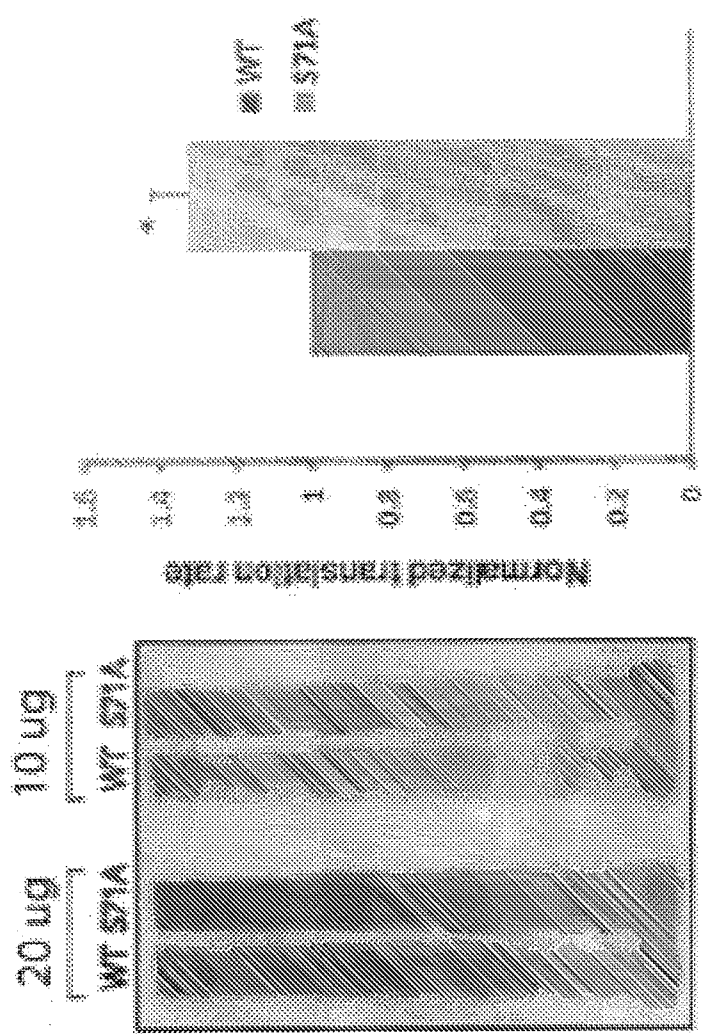


FIG. 5D

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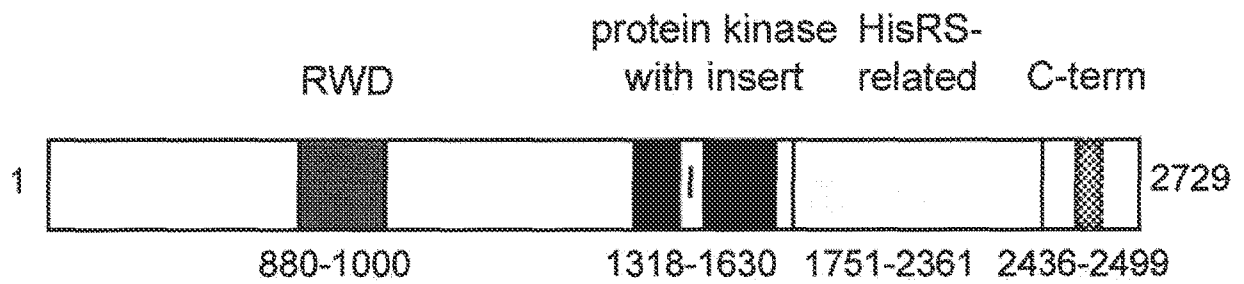


FIG. 6

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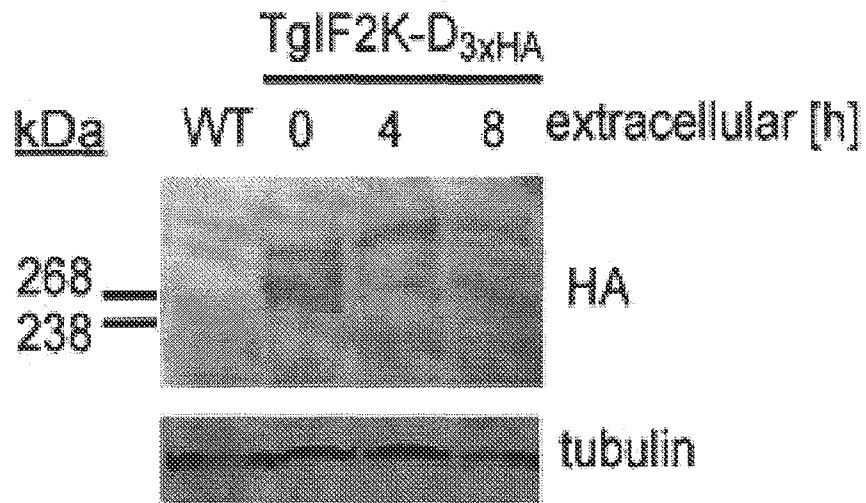


FIG. 7A

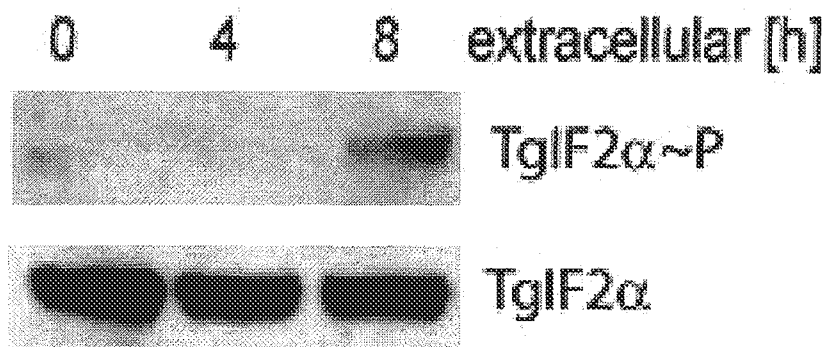


FIG. 7B

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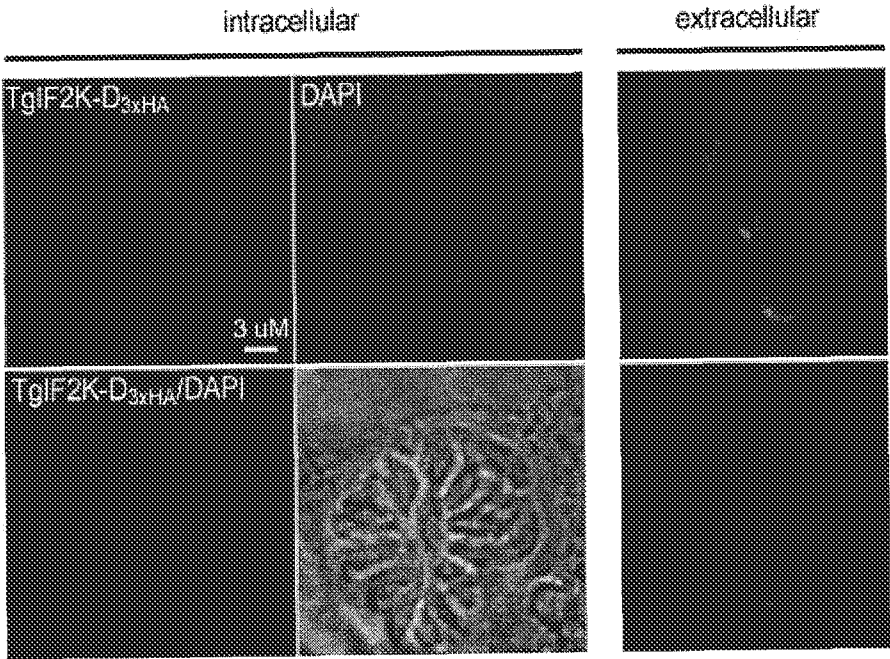


FIG. 7C

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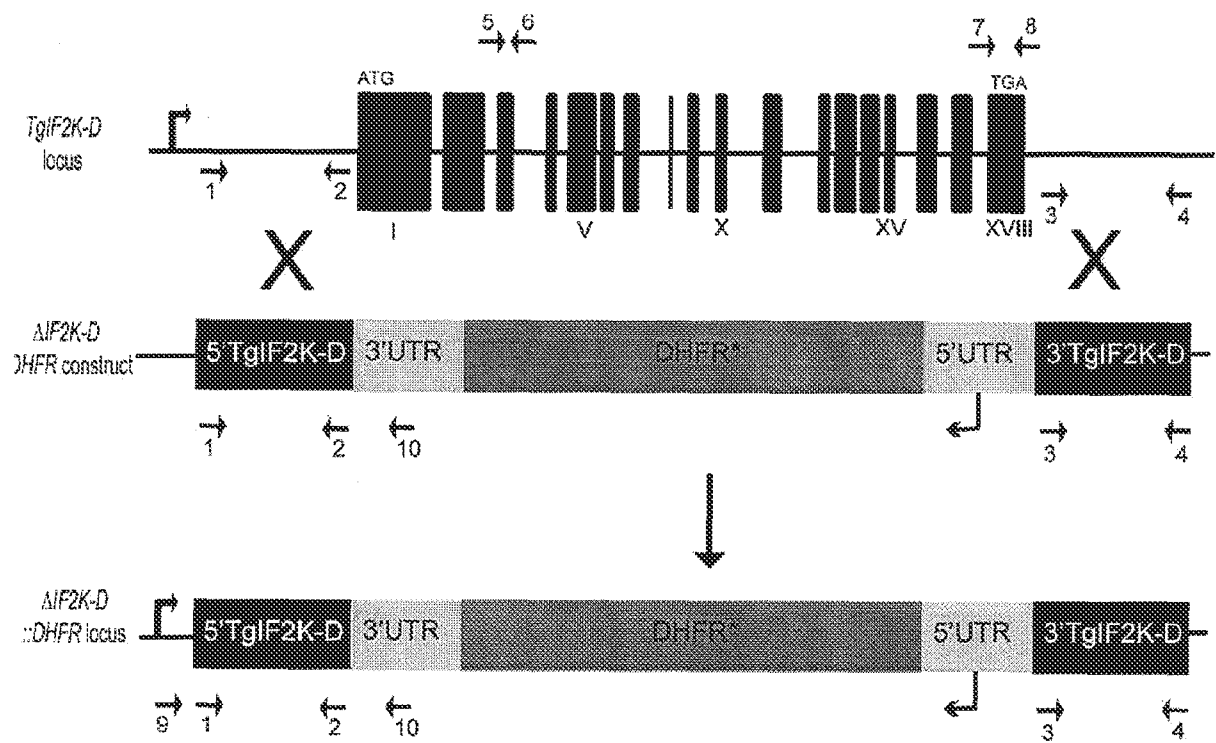


FIG. 8A

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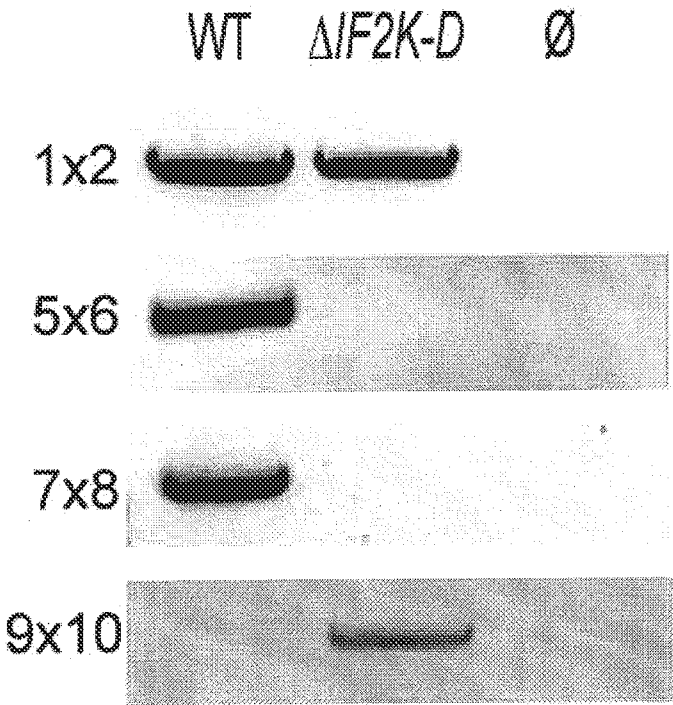


FIG. 8B

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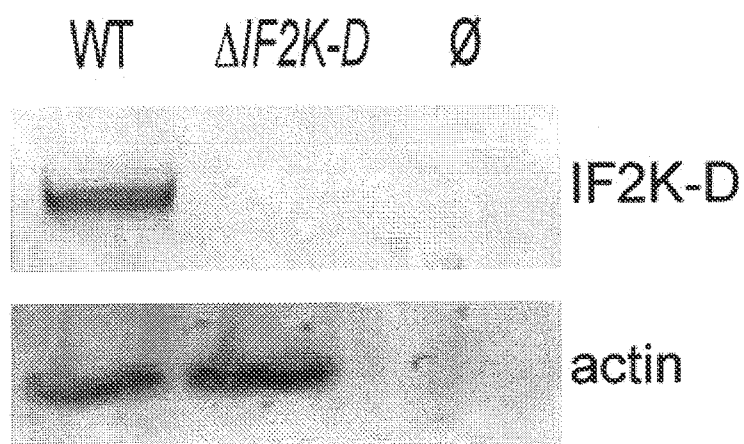


FIG. 8C

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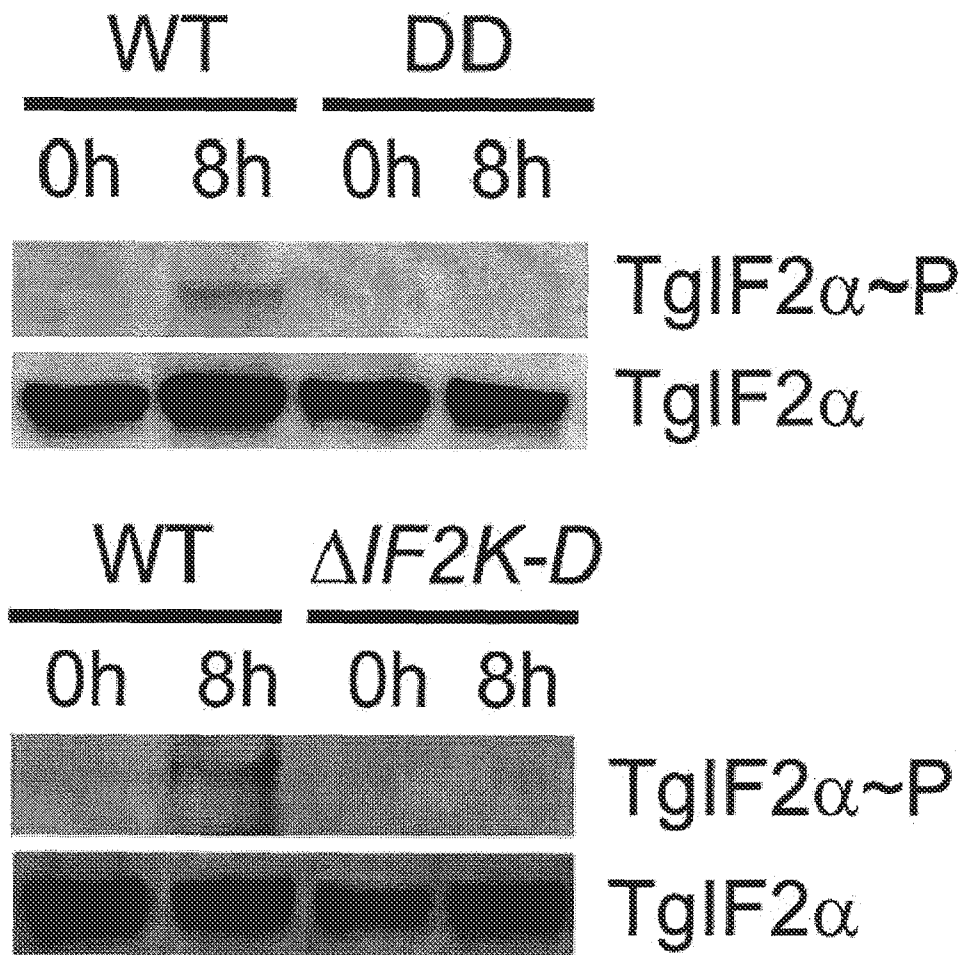


FIG. 9A

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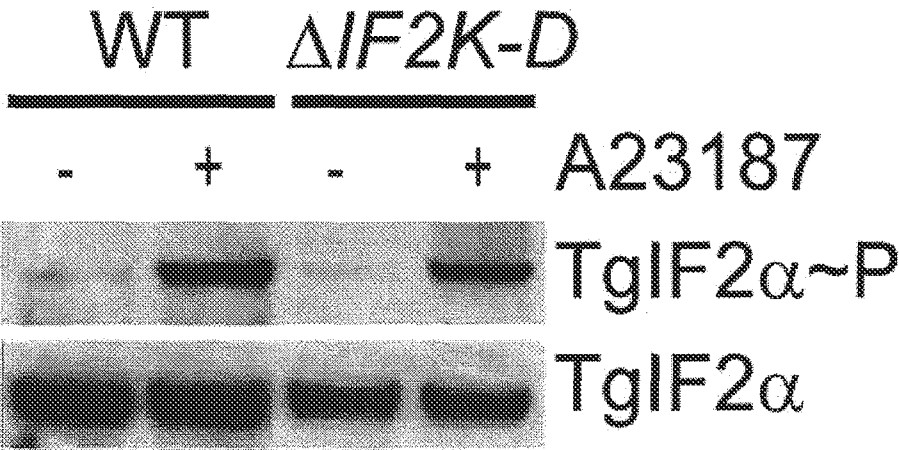


FIG. 9B

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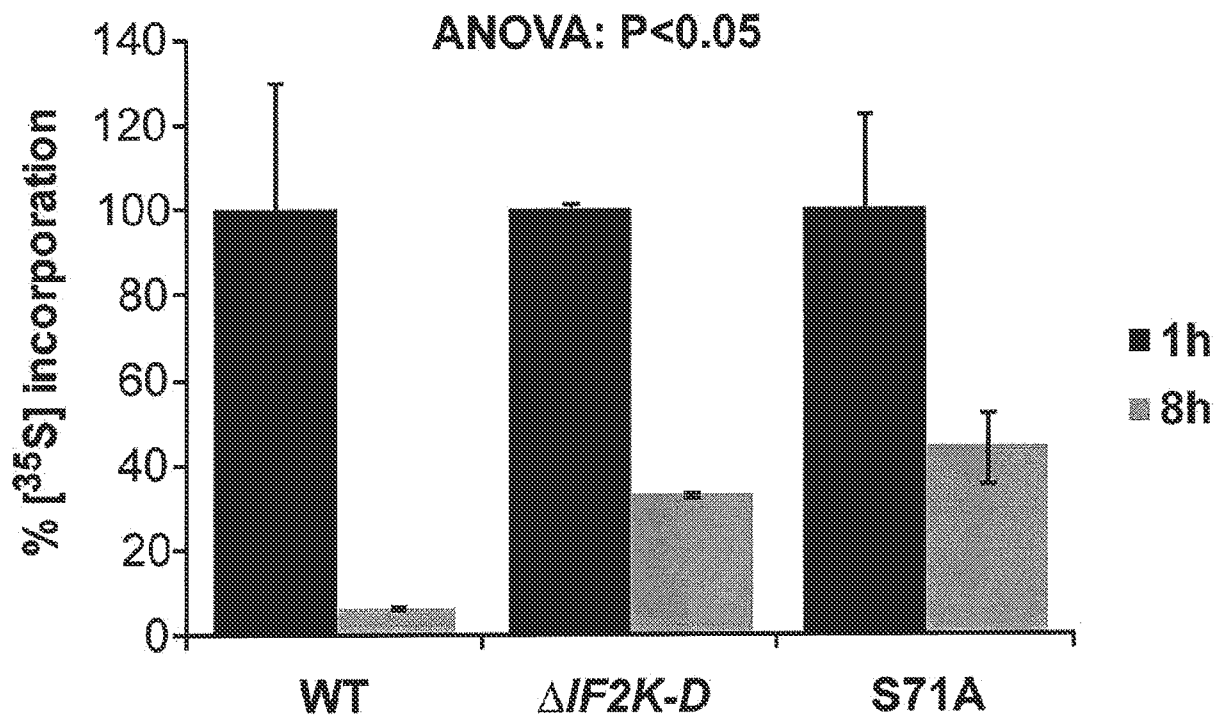


FIG. 9C

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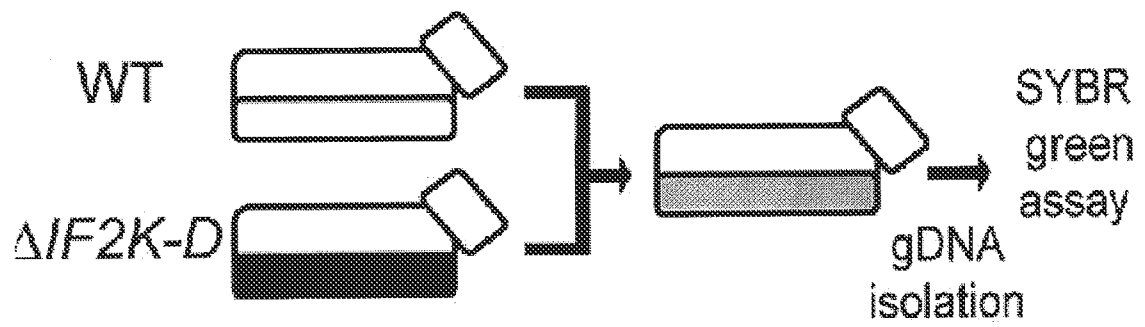


FIG. 10A

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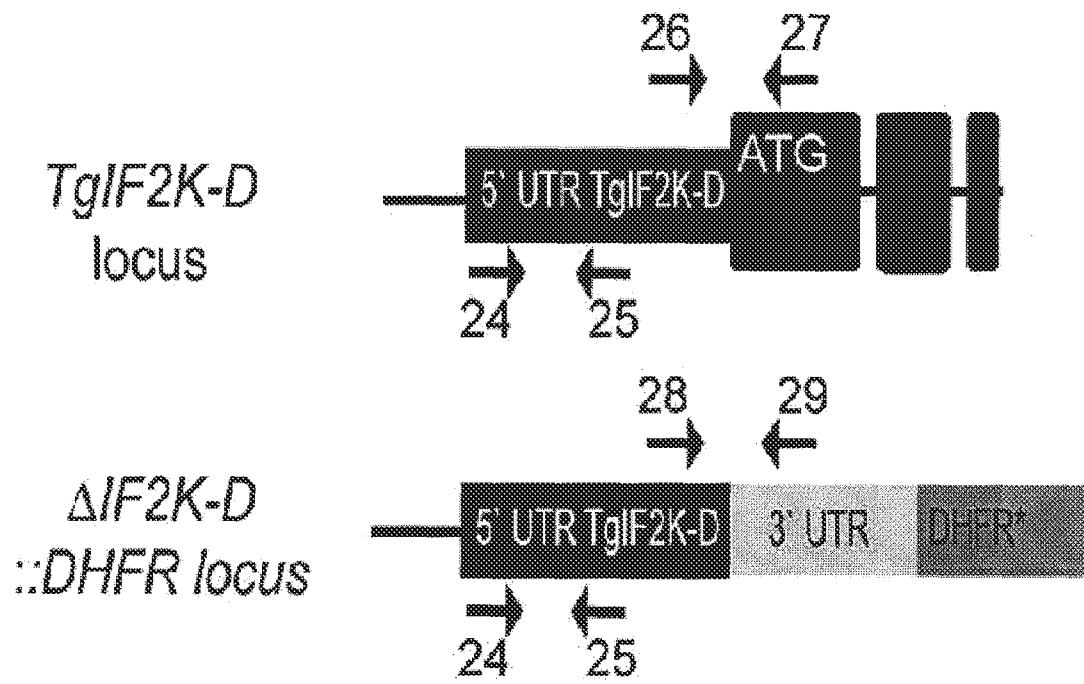


FIG. 10B

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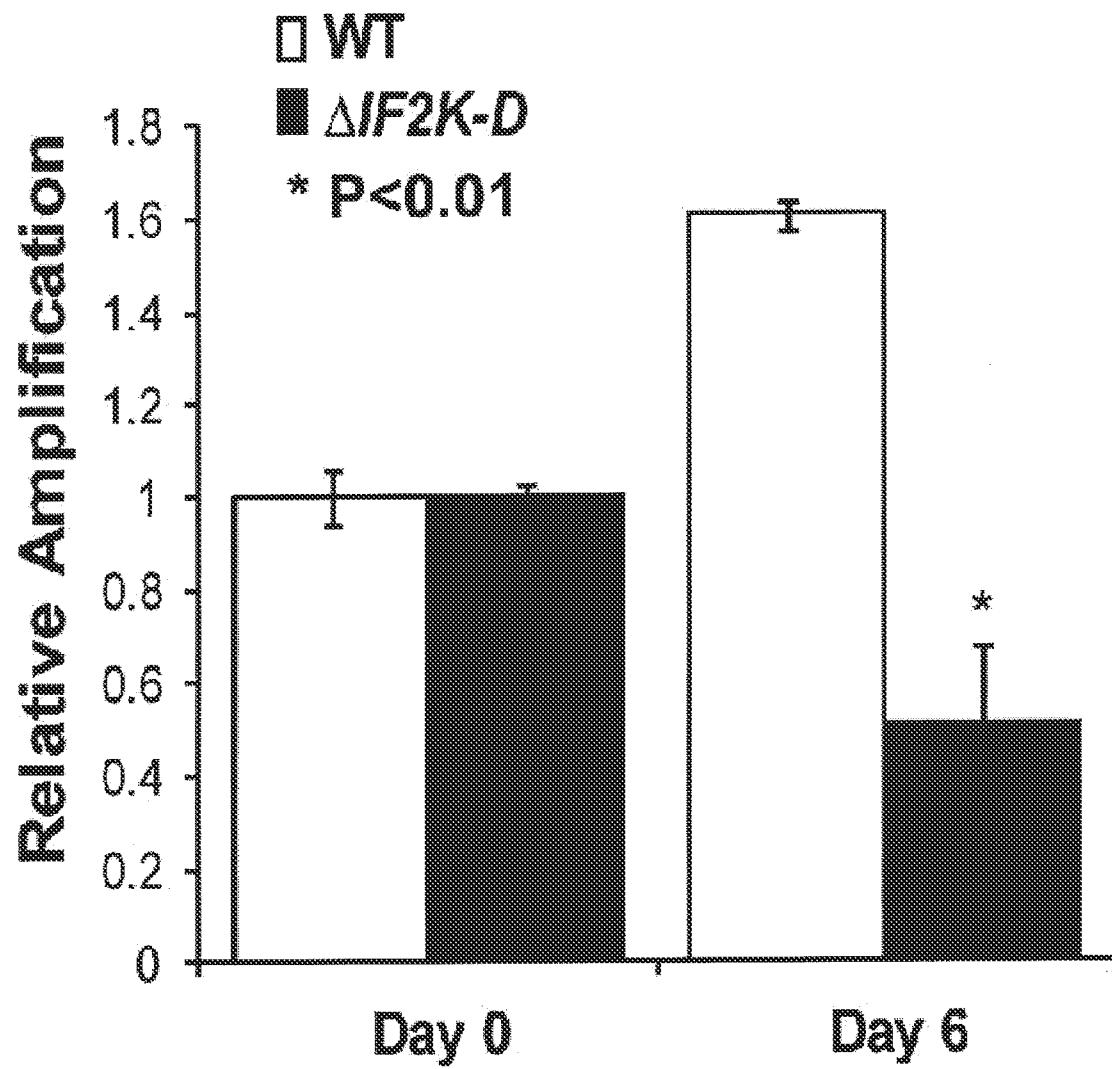


FIG. 10C

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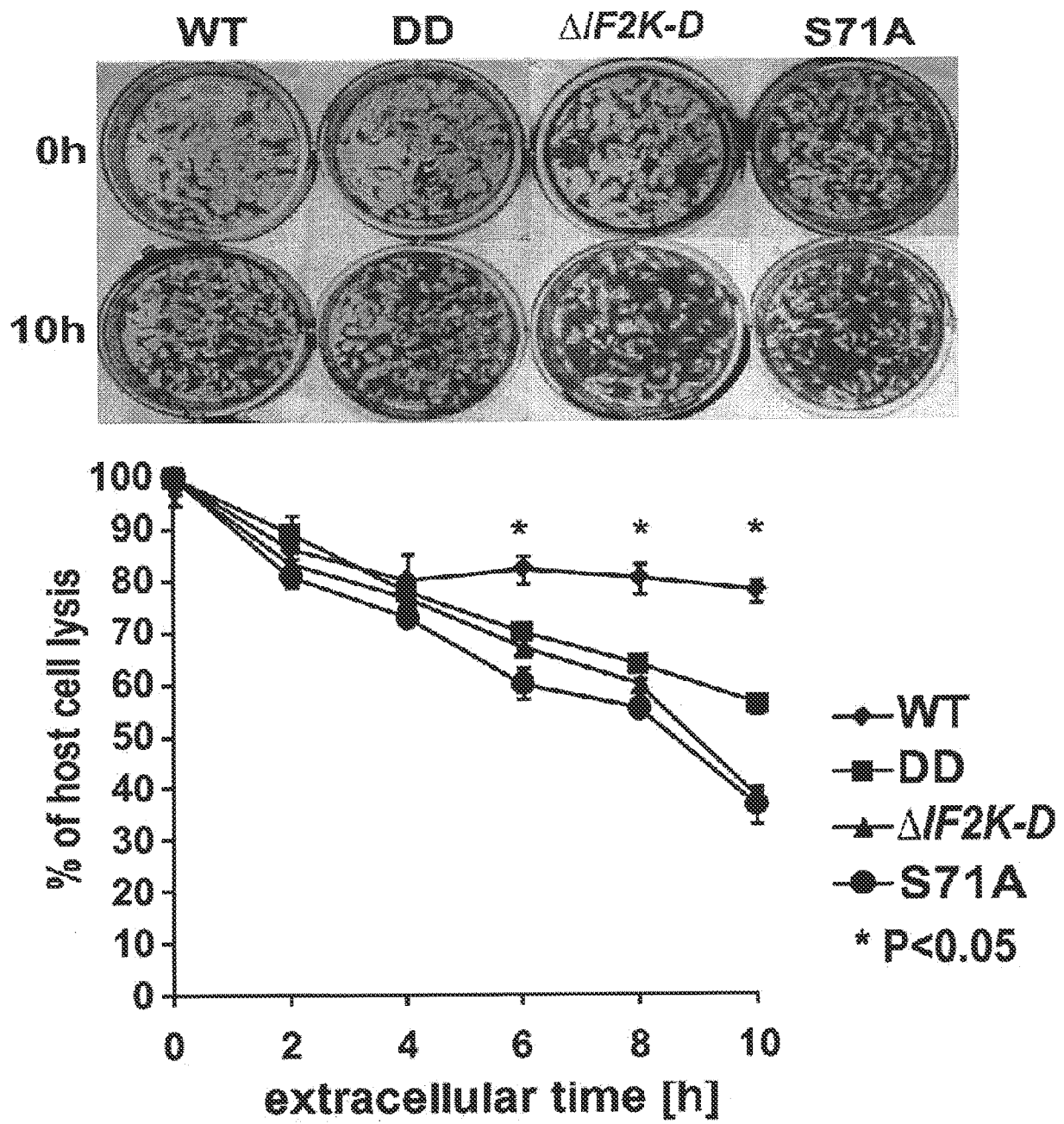


FIG. 11A

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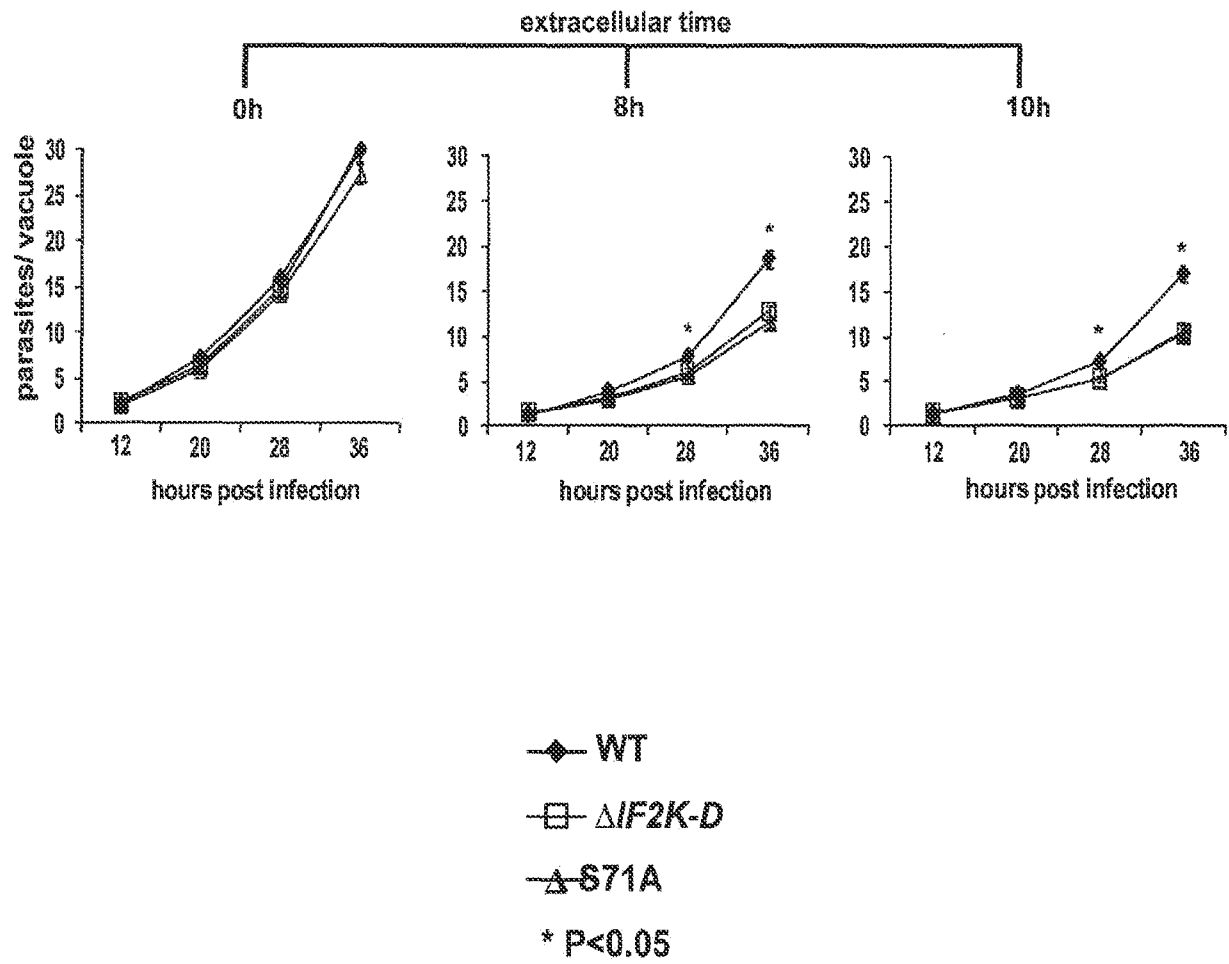


FIG. 11B

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aacgattcctctgtgccccctctgtctcttctcattgatttgactttccccagctctgcagaatgtccacattttacca
ccgtgtcagctgcctccaaggtcgaccgtgtttgtgtcgccgaaagtgaacagtcacactctcggtgattctagccg
tcagttctcttttctccgggggaaacgttcacctttctctctgactttctgtctgtctcctcgctgactgggggtcttt
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gcagaactccttttcaaacgaaagtccctgtgtagagccgtaggatcgccgttttccgctccagagtggtccgtaacg
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ggacaaaggcgatttcgggggagtgaaacggggccctcgaggctccagagaagagaaacagagagaggagaagatcc
agatttccggtggcttttccgttcatccagaggaagagaaacggacggagctggagcctgacgaggaagagagaagaa
agacaagtgtactcgatgctggaggaacaagtgtgagtgatgagcactccgcccgtgtcaacccgggtgctctgacgcg
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gttccgacttctcgacgggcaacggaggagactctctgttctcctcattttctgttacgcccgtgagcgagaactgtcgc
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tccactcttccccctccttccccactggcgccacgggtcacagacagcgttctgttaagccagagggccgggaagcgactgc
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agaaogttgggtctgcgcctctgtatctgtcttaacgggtacggcgttactcactcttgtcccgctcctctgtagggt
ctgggttcccttccgaatctcctccttctgttcttctgtcctttatcttcttccatttagctgcctcgttctgtctccaat
tatgcatacgtggatttaacccctcataaggacagggccacg**ATGCGGATTGGCACAGAGACGCCCGGGGGGSAACGC**
GAGCTGCGAGCGAAGACGACCGAGACGATGGATCTTGGACACTGGTGGCGTCCGAAACGACGCGAGCCCTCCTCCTCGC
ACCCCTCCGGGCTTGGCTCAGCTTCGACGCTCGCGTCCGCGGGGACGCGCCCGCGGCTCTCTCTCAACCCCGCC
GTAGGAGTCTTTTCGACGCTCGCGTCCGCTCGGCGAGGAGCGCGTCCGACCGGTTTCAACCCCGTGGCCTTGAGTG
CTTCGGCGGTGCGGGATCCGACAGACAGTTGGAGCGAGTGGGGAGGACGAGGACGCGAGGCCCTTCGAGGGGGCGCGC
AGGAGCAGGGGACAGCGCGAGACCTGCGAGAGCAGTGGCCGAGTTGGCCGCTGCTGCCAAGCAGGCTGGGGCGAGAG
CGCCCCCGCGCGGACCTCCAGTTGGGCGACAGTCCGCGCAGGAAGCTGCGGGTCCGCGCGGGCGCCTCTGGGGC
AGGCTCTGCGACCTGTCTCTCGACGCCCACGGCCGCGAGGCCCGCCCGAGCGAACGTCGCCGGGCGAGGCCCTGCGG
TGTGGCGGAGCCCGGAGGCGGGGGCGAAGGCCAGTGCGCCGTGAGGAGTTCCGCGCGCCAGAGATGCCACGGGA
GGTCCAACGCGACCGCAAACCGCGCGAGGGCCTGCAGAGGCCGCGACGGCCAGTGGTTTGGTCTCTGGGGCGGAGTCC
GAAAGGTCCGCGCGGAGGCGAAACAGAGGGGAGACGAGGAGCTTGGGCGAGAGAGGAGAGAGTCCGATGGGAAGGCT
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GCGAGAGAAGCAGGGGGCTCTGTGAGACGAACGGCGAAGCTGCAGAAGCAAAGCGAGGCCCCCTCGAAGAAGGAGCAC
CCTGACAAGGTTGCGGAAGAGGCCAGGATGCAGGAAGAAAGCAAAGGGAACACAAGGCGGAAGCAAGACAGACACCTG
GAAGAAAACCTCTCTGGCGAGAAGAAACGAGTGCAGACCTCCACACAGAACCAGCGGGGGCTGCGGGCCTCCCGTCG
ACGGCTGGGATACGCCAAAAGCTCCTTCCGGCACCGGACATCCCTGACGAGGCCCTCAAACCCCGCCCGCTGTCTT
CATCTTCGGCGCTGGCGTCTCCTTTGAGTCCATGTCCCTTACAGTCCGGCAGCACAGTCTCCTTTGCGGGCTTCT
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AAGGCCTGGAAAGTGCCGAGACCAAGGAGAGGAAGGCGAGCGGGGACTCTGAGCGGAGGCCGAGAAGAAGAAGG
AGAAGCACTTGCACTTTTACAGAAGAGGAAAAAAGAATCAGTCTCAAGACCTTCCGCGCGGGGCTGCGCTTCTGT
TGCCAGTGCGAACAGGGCGACAGCGCTACCGAGGAGCAAGAGGAAGATGAAGTTTCGAGCCATGGATGCCATGTACAC
CCCGGTGAAGATTCTGCAACCTGGTTTCTCCCGGACCTTCTCGCTGAGCTCCACGACCTCTGCTGGACTTCTGT
CCGCGCTGCGCTCACGCGCCGGCGCTCCCGGACCGGACAGGGGAGCGCGGAGCCAGCGGGGTGACGGACGACCT
ACTGCTTGCCCTGCCAGCGGACTTCTCTCTGGAAGAACCAGTCTGTGTGCGCTGTGTTGACGCCGGCTCTCACGC

FIG. 12

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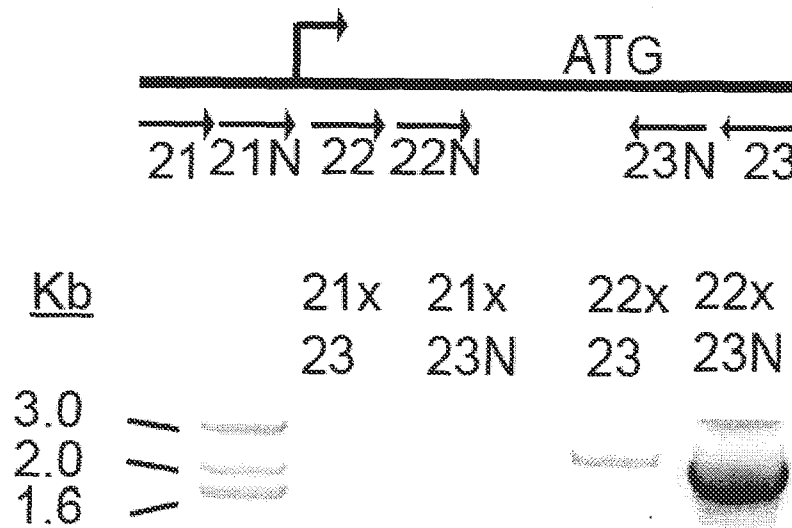


FIG. 13

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	I	II	III
(SEQ ID NO:14) TgIF2K-D	RYORDEIIOLEVLGSRERYRIALVYHIIDKH----	KYIVKQVFP	PCGP-
25-- VESALQOVTTCALQHPV	1398		
(SEQ ID NO:15) TgIF2K-C	RYHTEIIEIQGLGSGGFGSVTKVQEQGG----	IFAVKCHP	PAR-
105- AVGYMEIATMFAIDDKH	1057		
(SEQ ID NO:16) NcIF2K-D	RYORDEIIOLEVLGVDRDHYRIALVYHIIDKH----	KYIVKQVFP	PCGP-
26-- VESALQOVTTCALQHPV	1576		
(SEQ ID NO:17) PfeIK1	RYRDEFEDEKILGCGGFGYVVKVONKKFNI----	TAIVKILRI	SNSK-
15-- NSYIMEIATMFAIDDKH	499		
(SEQ ID NO:18) Pf14 0264	SYELTEFFNFMVFCNRDFNIFCNILNDKKG-15-	EVANKCVI	TQNK-
31-- IKKILCEHAKITKTHKY	648		
(SEQ ID NO:19) PbeIK1	RYRDEFESESVLGC GGFGYVVKVONKKFNI----	AVAVKCHP	SINK-
32-- NSLIMEIATMFAIDDKH	501		
(SEQ ID NO:20) TbeIF2K1	RYRSEIEIQGLGSGGFGYVVKVONKKFNI----	LYAVKCHP	TAIR
KNEAEK-----ALNMQSIAAISHKH	330		
(SEQ ID NO:21) AtGCN2	RYLNDFEELKPLGSGGFGYVVKVONKKFNI----	QYAVKCHP	IRL
KDKEIPV--NSRIMVATSRIOH	483		
(SEQ ID NO:22) DmGCN2	RLRTDEEVIMYLCKCAFCVLCVRIADNR----	EYAVKCHP	PLP
ARSRQL--YKKMTMVEISRMEN	581		
(SEQ ID NO:23) ScGCN2	RYASDTEELAVLGCAGFGYVVKVONKKFNI----	YAVKCHP	IRH
TEEKLST-----IISAMMFAISNHQY	654		
(SEQ ID NO:24) MmGCN2	RYFIEFEELQHLCKGARGAVIKVONKLDGC----	CYAVKCHP	TPASRH
---FRRKCGVTHSRPHEN	646		
(SEQ ID NO:25) HsGCN2	RYFIEFEELQHLCKGARGAVIKVONKLDGC----	CYAVKCHP	TPIN
PASRQ---FRRKCGVTHSRPHEN	647		
	IV	V	VIA
TgIF2K-D	RYRYDQAWTQ-39--VYVOMEFCER--SLEDDEIKRTVL-----		HWDSQQ
RYRYDQAWTQ	1483		
TgIF2K-C	RYRYDQAWTQ-24--EFILMEYCP-GRILREAITD SGLQLPPASFRQTLRRR-98-		MGLRAIK
RYRYDQAWTQ	1238		
NcIF2K-D	RYRYDQAWTQ-43--VYVOMEFCER--SLEDDEIKRT-----		HWDSQQ
RYRYDQAWTQ	1665		
PfeIK1	RYRYDQAWTQ-99--RYILMEYCP-GKILREAITDCGFI-----		CRNEKI
RYRYDQAWTQ	644		
Pf14 0264	RYRYDQAWTQ-282-LYTCCEHCK-GQSEKEHENNFF-----		QKNKYL
RYRYDQAWTQ	976		
PbeIK1	RYRYDQAWTQ-57--RYILMEYCP-GKILREAITDCGFI-----		YKNEKI
RYRYDQAWTQ	604		

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TbeIF2K1 **IVRYAYDAWIE**-100-**LYAIOMELCE**-HS**IRHL****ED**CDKEEGSLLTA-----GNGDKVAT
KIRROL 484
 AtGCN2 **IVRYIQAWE**-43--**LYAIOMELCE**-**IR**QV**FESY**-N-----HF**DKDFA**
WHIRROL 570
 DmGCN2 **IVRYFNSWIE**-102-**LYAIOMELCE**-C**IR**TA**ED**DN-**I**-----FN**TDRL**
WRIRROL 728
 ScGCN2 **IVRYIAAWIE**-119-**LYAIOMELCE**-R**IR**Y**DL**HSENI-----NQORDEY
WRIRROL 819
 MmGCN2 **IVRYYNAWIE**-139-**LYAIOMELCE**S-**IR**DT**ED**QG-**I**-----FR**DT SRL**
WRIRROL 830
 HsGCN2 **IVRYYNAWIE**-146-**LYAIOMELCE**S-**IR**DT**ED**QG-**I**-----YR**DT SRL**
WRIRROL 832

	VIA	VIB	VII
TgIF2K-D	LEALVYIRNRVCHLS IR PS NI EP AYGYN----		VKIGDFG IT SLFSIV-----
---- 1530			
TgIF2K-C	VGGVAY IR ES FG VI HR DI IR PS NI EP KV QD RLC----		VKIGDFG IT TVLK KG PGGSQAAGR-58-
PLSS 1356			
NcIF2K-D	LEALVYIRNRVCHVS IR PS NI EP AYGYN----		VKIGDFG VT SLFDIV-----
---- 1712			
PfeIK1	IKGIS IR DM KI MD IR DI IR PS NI EP QITDNILI----		AKIGDFG IT IRIG-----
---- 689			
Pf14_0264	IES ES IR ML KOK IR Y IK IR NS ON IR Y ED NDKYGAH----		IKHINYS IC NMIDYFYFYHYSYKNN-38-
KESK 1075			
PbeIK1	IKGIVY IR DM KI MD IR DI IR PS NI EP QINDDILS----		AKIGDFG IT IKID-----
---- 649			
TbeIF2K1	ETVVS IR FR OG VI HR DI IR PS NI EP QSSVSSDDVGT IR VA DF GL ART LRSMKHSPSNVELN---		
---- 546			
AtGCN2	VEGLA IR IR OG VI HR DI IR PS NI EP QSSVSSDDVGT IR VA DF GL ART LRSMKHSPSNVELN---		IKIGDFG LA AKFLK-----
---- 612			
DmGCN2	AEGLA IR IR OG VI HR DI IR PS NI EP QSSVSSDDVGT IR VA DF GL ART LRSMKHSPSNVELN---		IKIGDFG LA TTSTFLA-----
---- 772			
ScGCN2	LEALSY IR IR OG VI HR DI IR PS NI EP QSSVSSDDVGT IR VA DF GL ART LRSMKHSPSNVELN---		VKIGDFG LA KNVHRS LD ILK LD SON----
---- 873			
MmGCN2	EDGLA IR IR OG VI HR DI IR PS NI EP QSSVSSDDVGT IR VA DF GL ART LRSMKHSPSNVELN---		VKIGDFG LA TDHLA FT AE GK QDDQA----
---- 884			
HsGCN2	EDGLA IR IR OG VI HR DI IR PS NI EP QSSVSSDDVGT IR VA DF GL ART LRSMKHSPSNVELN---		VKIGDFG LA TDHLA FT AE GK QDDQT----
---- 886			

	VIII	IX
TgIF2K-D	-----HNVHSPISRL Y TA ED LI-	
CA ADR KF ASLTMRDAD CA DM Y SL CV IR AE WGRHEM GRS 1587		
TgIF2K-C	FPGSHPSDTPSLRL SAG VI IR Y Y MA ED QAT GR SR-----	YDOKAD IR PS LG V IR EMWAP--
PR ITA 1414		
NcIF2K-D	-----HNVHSPISRL Y TA ED LI-STADGKSSSLTMRDAD KAD MY S IG V LE AE W G	
RHEM GRS 1769		
PfeIK1	-----DTQ IN PSACT IR Y Y SP EO IN GE P-----	FNEKAD IR PS LG V IR EMWAP--
PR ITA 737		
Pf14_0264	EEDIFVKKCNSFEIKNDSSCKE IN DE K INKEKNVP-56-KKYDY E EL DL PS LG V IR EMWAP--	
PR ITA 1195		
PbeIK1	-----NTQ IN PSACT IR Y Y SP EO IN GE H-----	FNEKAD IR PS LG V IR EMWAP--
PR ITA 697		
TbeIF2K1	DVRPLDELEVGPSP IR GN LG SV Y CA ED Q ER GES-----	YDOKAD IR PS LG V IR EMWAP--
AGQGF 606		
AtGCN2	-----V AG SGVDS IR QACT IR Y Y TA ED LI ED WPK-----	YDOKAD IR PS LG V IR EMWAP--

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DmGCN2 677 -----ITSAEDGTGTEKVGHTLVAPETGNASKSV-----YNOKVIMYTHGILFEMCOP--
ScGCN2 841 -----LPGSSDNLTSAICTAMVATVETDGTGH-----YNEKIDMYSLGTTHFMIMY---
MmGCN2 925 -GDGVIKSDPSGHLITCMVCHALVVSPEV-QGSTKSA-----YNOKVDLPSLGTTHFFEMSYH--
PmVHA 943 -GD-LIKSDPSGHLITCMVCHALVVSPEV-QGSTKSA-----YNOKVDLPSLGTTHFFEMSYH--
HsGCN2 944

```

```

X
TgIF2K-D CSRIEFITTSIVKERNLO-----QLNVPSAAVKTIKHISPNPGDRPSMTTHQSSI
PpPAVEED 1646
TgIF2K-C MERASVIGQITLDHEQQMRRLLPQ-94-QYVPSEVAPILAMIQODPASRPAADAILVDD
VtLPLELSQ 1574
NcIF2K-D CSRIEFITTSIVKERNLO-----QLNVPSAAVKTIKHISPNPADRPSSMTTHPPAV
EEELFKAF 1127
PfeIK1 MERSITISNLIKGIYPYIM-----KADNKKFQFISSIAINPQERCCAYNTHES
VtFTFEKDF 796
Pf14_0264 EEKLINITTIKEKTFSESFIKN-----IDNDSLKVLKFIISTINYDTNEKKITKLEY
VdILNLNI 1257
PbeIK1 MERSIVISNLIKCIYPSI-----RSDNKIFQFILSIEIDPQNRLSAYS
ImHENFFFSYEKNF 756
TbeIF2K1 RSRFNIMTDISRGKPIQWF-----YAWNPRMAEVIASIERDPGQRTSEETIN
KADFGDPADV 667
AtGCN2 MERHVIITNKLKGEPLK-----WVNEFPQASILRRIMSPSPSDRPSATEIKHAFP
PrMESEL 738
DmGCN2 MERAQTHMAIRNV SINIPD-----AMKDPKYEKTVMKLQWLNHDPACRPTAEE
HtISDLVPAQLEA 905
ScGCN2 MERVNIKKIRS-VSIEFPP-----DFDDNKMKVKKIIRLIDHDIPNKRPGARTAL
NSGWIPVKHQDE 988
MmGCN2 SERIFVINQIRDPSTPKFP-----DDFDDGEHTKOKSVISWLNHDEAKRPTAME
HtKSELPPQMEE 1007
HsGCN2 SERIFVINQIRDPSTPKFPE-----DFDDGEHAKOKSVISWLNHDPKRPTATE
HtKSELPPQMEE 1008

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FIG. 14

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(SEQ ID NO:26) TgIF2K-D -----ADIFPLDAQSSGRLSPSSTLTSPSGPVSAPSRSPVSSPSPDGT-----
- ATQSTNRAGATTEAALSQRCLCSG 912

(SEQ ID NO:27) NcIF2K-D -----ASSASRSPVSSPAPDGPPA
SCAAQETR-- GSDPPIPRCQLCLG 970

(SEQ ID NO:28) Pf14_0264 MDNDNNKRRKTKKKKVRNVNKNSENNKRRKGIQENQDEQNY
TEQYDQIEHLAENIF PPMYVDNFGHVKKSDLPKDLM 80

(SEQ ID NO:29) MmGCN2 -----MAGGRGASGRGRAEPOESRSGRQDHET
GAEFAHYGSDIQRIDIRGRVRE----- 51

(SEQ ID NO:30) DmGCN2 -----MAD-----EKAKESPRROAQHHEVHKS
REGCQVEPRPOANPSLWN----- 42

(SEQ ID NO:31) ScGCN2 -----MSLSHLTLDQTLICN
EKEAARSTYMDDET DITKRRKSSWDKO----- 43

TgIF2K-D -----ECQVECYR-----MRR-VSETORAFFAVDMLPRNTEAGPEYVAVGL
SHAMPRTVEVORELR 970

NcIF2K-D -----DCQVECYR-----MRR-VSETQQAFFAIDMLPRNTEAGPEYVAVGLSAAMP
REEDPQIRN 1028

Pf14_0264 KRNEYIKNSKDLSENIIVDINDEINKNTCLTSMPIINIDNIMEKYATTFMYELIPEYSYENNVIIHNN
TKLNEEQKNDVTL 160

MmGCN2 -----PPEINLMD-----MPQG-LAGEEVYVQVEPOYKCEPTEDDVPEEELK
NAGGLSNESVNLKES 108

DmGCN2 -----PTPRRQO-----TPLEDSSNGLETYVCTNHVTCESKTEKLDLQSLK
ESKGSMDOLIFAPRN 101

ScGCN2 -----PCQIFET-----TURS-YDKEPVESSTIMFAMTPMTEYTAPETEFA
NVQNMATISQPMKES 99

TgIF2K-D LRRDEFASGCEVVLVRRGCIQLOSPMAELSSTSCLSNLWEEMAFKRRORHLLRRRQ---HMTHOSLKA
RCGMSHPF-- 1045

NcIF2K-D SLREYEASRGEVVLVRRGCIQLOSPMAELSSTLCSNLWEEMAFKRRORHLLRRRQ---HMTHRNKA
RCGMSHPFGP 1105

Pf14_0264 NIKKICARNYCRITPESICIFINEYENKIFNN-DQNLWEEMAFKRRORHLLRRRQ---HMTHRNKA
HNEIEDGRKIDNYKNVQ 239

MmGCN2 HSEELAKKQCCEVMIPERAAHVQSITSEHNKPPPKS-FHEEMPRQAEKQORLEA---RRREGE-QRE-
RHHIQRH 182

DmGCN2 QPQAQSQELRGEVVLVRRGCIQLOSPMAELSSTLCSNLWEEMAFKRRORHLLRRRQ---HMTHRNKA
QRCITIDEVERR 175

ScGCN2 EFKRIHNTSRGCEITPESITSTCEKDEQNVVNTQSHEDDRQSIKETKEQEKSE---REKQQTIKR---
EQROR 173

FIG. 15

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(SEQ ID NO:32)      TgIF2K-D      --LDYSLQAVVIAQSAKQPLGESHVCRILLRRCIRAELRVTPV
VEISYFQRLRR--SRRILYHVOIQHHRASVAAHATLT- 2513
(SEQ ID NO:33)      NcIF2K-D      --DEAAIQAVVIAQSAKQPLGESHVCKILLRRCVRAELRVSP
VVEISYFQRLRR--SRRILYHVOIQHHRASVSSHAAT-- 2739
(SEQ ID NO:34)      Cmu 027700    --LQSCYKQAVVIAQSAKQPLGESHVCRILLRRCIRAELRVTPV
YLSIFVDKIKKGTLEVLLVVMKTKPTNSLYNASNI 1300
(SEQ ID NO:35)      PF14 0264     LLIDFHSPSVVIAKAYKCHYAFSLYNSLIONGKCECRIPDM
ETSKEEKSIAK--YKNINIHVOINOKISSSTSLNIEDM 2300
(SEQ ID NO:36)      Pb 101620     -
FIDFHSPSVVIAKAYKCHYAFSLYNSLIONGKCECRIPDM--YNHINIHVOINOKISSSTSLNIEDM
PTV-CSDM 1892
(SEQ ID NO:37)      PkH 113740
MHIDFHSPSVVIAKAYKCHYAFSLYNSLIONGKCECRIPDM--YSDINIHVOINOKISSSTSLNIEDM
FLNIEDM 2053
(SEQ ID NO:38)      Pvx 085120    --
IDFHSPSVVIAKAYKCHYAFSLYNSLIONGKCECRIPDM--YGDINIHVOINOKISSSTSLNIEDM
NIEDM 2219

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FIG. 16

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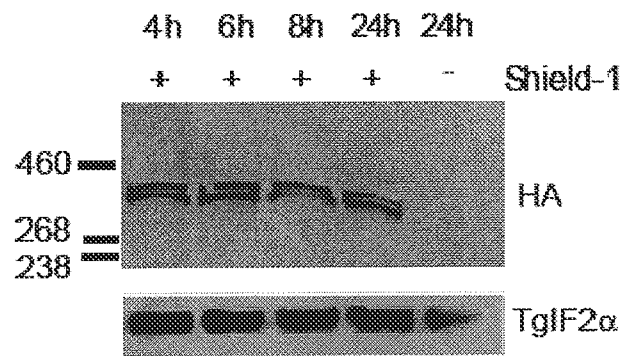


FIG. 17