Distinct signaling pathways control *Toxoplasma* egress and host-cell invasion

Sebastian Lourido, Keliang Tang and L. David Sibley

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1st Editorial Decision 19 July 2012

Thank you for submitting your manuscript for consideration by the EMBO Journal, and I apologize for the delay in getting back to you. The article has now been seen by three referees whose comments are shown below.

As you will see, while the three referees find the study of interest, they nevertheless raise some significant issues and suggest more experiments to strengthen your data.

Given these positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your
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revision.

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

This elegant study creates Toxoplasma strains that are differentially sensitive to a highly specific kinase inhibitor to demonstrate that a calcium-regulated kinase (TgCDPK3) contributes to induced parasite egress in vitro. The study will garner wide interest because of the plant-like nature of CDPK's, the signal transduction theme and the insight generated for egress, a poorly understood but crucial event in intracellular microbe biology. The authors use a chemical genetics approach to show that CDPK3 is required for rapid parasite egress, normal gliding motility and microneme secretion by one "pathway", but is not required for parasite cell invasion. They also provide data suggesting the existence of parallel pathways each governed by a different kinase, indicating the integration of signals. The study also lays the foundation for extending the approach to dissect the roles of additional kinases in parasite biology. While the study has considerable merit, addressing the following points will further strengthen the submission.

1. Making the time units the same between Fig 3C and D would make it easier for the reader to compare the findings.

2. At what time after adding an egress inducer was LHD release measured? This information was missing from the materials and methods and the figure legend. The data appear to be only from one time point. The results seem to be quite cut and dry with respect to CDPK3 being crucial for egress, but from the current data it is impossible to know if inhibition of CDPK3 results in an indefinite block in egress or rather a delay in egress. This distinction is important since the latter would suggest that additional supplementary pathways exist, a notion that is consistent with the finding that activation of PKG can compensate for the absence of CDPK3 activity. If only one time point (say 2 min) had been measured in e.g., the perforin-like protein knockout strain then the conclusion would have been that PLP1 is crucial for egress when in fact it is required for rapid egress of most vacuoles. The authors should perform a time course out to at least 20 min at an IC90 concentration of the inhibitor. If it is not feasible to use LDH as readout for a longer time course then microscopy could be used instead.

3. The calcium ionophore A23187 is used to stimulate and assess egress in the various strains, allowing the authors to establish the roles of CDPK1 and 3 in these processes. Egress requires microneme secretion. However, to assess the requirements of CDPK1 and 3 for microneme secretion the authors do not use A23187, but rather use ethanol or ethanol plus FBS. While the two treatments used reveal an interesting distinction for CDPK3, the absence of data from A23187 stimulation weakens the link between microneme secretion and the earlier egress and PV permeabilization data generated with the ionophore. This data should be collected and added along with stimulation with FBS alone to confirm that the FBS "pathway" is independent of CDPK3.

4. Pg 11 the statement that: ",..egress is under very tight control of three distinct kinases, each of which is essential for this process" is too bold in the absence of showing that the respective inhibitors indefinitely block egress.

Referee #2

This manuscript conducts a comparative analysis of two protein kinases in egress, gliding and invasion of T. gondii tachyzoites. In a previous paper the authors had converted the naturally small glycine gatekeeper of the TgCDPK1 kinase to a larger residue, rendering it resistant to a small molecule inhibitor, 3MB-PP1. The resulting mutant proved a powerful genetic tool to analyse
functions of this kinase. They now use the same 3MB-PP1-resistant line as a starting point to render another member of the same kinase family selectively susceptible to inhibition by introducing a very small glycine gatekeeper instead of the larger methionine. Using these sequential genetic modifications the authors produce a panel of mutants, which now enables them to use the same small molecule to dissect the functions of different members of the CDPK family in a comparative approach. This paper illustrates the power of chemical genetics for the functional analysis of protein kinases that each have multiple and subtly different roles in the biology of a cell.

It is this approach which I think makes this paper particularly attractive to a wide readership, beyond apicomplexan cell biology. Analog sensitive (AS) kinase alleles are not new. They have frequently been used to get at functions of protein kinases, and in yeast this has involved comparing multiple AS alleles in the same study (Benjamin et al., Genes & Development, 2003). However, I am not aware of a paper in which sequential genetic manipulations were used to switch an inhibitor from it's endogenous target to a structurally related target kinase in the same organism.

As far as the partly overlapping, yet distinct functions of the CDPK1 and 3 protein kinases in egress and invasion are concerned, the paper's conclusions are generally well supported by the data. Regarding the role of the parasite's cyclic GMP dependent protein kinase in egress, however, Fig. 5 presents intriguing data, but all conclusions remain based on inhibitors. Neither the selective elevation of CGMP levels by zaprinast, nor the selectivity of the PKG inhibitor compound 1 are demonstrated. These data therefore remain preliminary and the paper falls short of demonstrating conclusively a role for protein kinase G in egress. Statements in abstract, results section and discussion to this effect should be toned down significantly.

The role for TgCDPK3 in regulating directly the molecular motor that powers gliding is an important and potentially controversial question that this manuscript tries to deal with. The only motility assay that is used looks at antigen trails left by a small fraction of gliding parasites on a slide (Figure 2A). That only 12-25% of parasites ever seem to glide in this assay casts some doubt on its relevance. The authors conclude that CDPK3 is required for the onset of motility, but not to sustain gliding, but this is not convincingly supported by Fig. 2A. It would be important to use quantitative video microscopy to measure the impact of 3MB-PP1 on average gliding speed, distance travelled and number and frequency of gliding episodes to support this important point.

Figure 4B - I do not understand how with CDPK3M, 3MB-PP1 can enhance EtOH induced MIC2 secretion (i.e. give negative inhibition). Is there a systematic error in this figure? How reproducible is it? We are only shown relative data. Is baseline secretion in all mutants the same in the absence of inhibitor and in response to both combinations of stimuli? The gatekeeper mutation could easily affect baseline secretion and inducibility, for instance by modifying activity of the kinase to some degree, without causing a marked phenotype in other assays. The enhancement of secretion by the inhibitor in 3M makes the apparent absence of inhibition in 3G impossible to interpret. Does the inhibitor have no effect at all on EtOH induced secretion by CDPK3G or does it reverse the enhancement observed in CDPK3M, resulting in no net effect? I am not convinced if and how EtOH and FBS/EtOH behave differently as triggers in these mutants.

Other points:

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This is a well constructed manuscript reporting elegant studies on the highly significant subject of Apicomplexan invasion. It describes the functional analysis of the TgCDPK3 gene, comparing it with TgCDPK1. The authors build on their previous chemical genetic analysis of the TgCDPK1 gene, and engineer inhibitor sensitive forms of TgCDPK3 for in vivo analysis following inhibition. The key finding is that in contrast to TgCDPK1 which is required for both parasite egress and
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defect appears to derive from microneme secretion for the initiation of motility, with intriguing
differences between the response of microneme secretion to different triggers being regulated by
TgCDPK3. Finally, some evidence is presented that suggests that PKG interacts with TgCDPK3.
Overall, this paper demonstrates the efficacy of chemical genetic approaches for the analysis of
parasite kinases and introduces the function of the TgCDPK3 gene. It also lays the foundation for
looking at combinatorial signaling by PKG, TgCDPK in the processes. A few further experiments
will strengthen the evidence for their conclusions.

Comments:
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could easily look at phosphorylation of some of the actinomyosin motor components.

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or knockdown approach? Eg. specificity of inhibitors and potential of hitting multiple targets?

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This elegant study creates Toxoplasma strains that are differentially sensitive to a highly specific kinase inhibitor to demonstrate that a calcium-regulated kinase (TgCDPK3) contributes to induced parasite egress in vitro. The study will garner wide interest because of the plant-like nature of CDPK's, the signal transduction theme and the insight generated for egress, a poorly understood but crucial event in intracellular microbe biology. The authors use a chemical genetics approach to show that CDPK3 is required for rapid parasite egress, normal gliding motility and microneme secretion by one "pathway", but is not required for parasite cell invasion. They also provide data suggesting the existence of parallel pathways each governed by a different kinase, indicating the integration of signals. The study also lays the foundation for extending the approach to dissect the roles of additional kinases in parasite biology. While the study has considerable merit, addressing the following points will further strengthen the submission.

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Response: We have revised the axes in Figure 3 as suggested.

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Response: In terms monitoring PLP1 function we have examined vacuolar permeability for 10 min by video microscopy, as plotted in Figure 3D and shown in the supplemental videos. The assay shown in Figure 3B was conducted at 5 min as stated in the legend. We have added new data to the supplement (Supplementary Figure 3) showing the result for 30 min. The inhibition of egress by treatment of CDPK1 or CDPK3 sensitive strains (expressing G alleles) is observed at both time points. Hence, the block in egress appears to be complete rather than transient.

3. The calcium ionophore A23187 is used to stimulate and assess egress in the various strains, allowing the authors to establish the roles of CDPK1 and 3 in these processes. Egress requires microneme secretion. However, to assess the requirements of CDPK1 and 3 for microneme secretion the authors do not use A23187, but rather use ethanol or ethanol plus FBS. While the two treatments used reveal an interesting distinction for CDPK3, the absence of data from A23187 stimulation weakens the link between microneme secretion and the earlier egress and PV permeabilization data generated with the ionophore. This data should be collected and added along with stimulation with FBS alone to confirm that the FBS "pathway" is independent of CDPK3.
Response: We agree that this combination of agonists was not optimal to test the requirement of CDPK1 vs. CDPK3 in secretion. To provide data on secretion that better parallels the studies on egress, we have removed the assays using FBS and replaced them with the calcium ionophore A23187 (Figure 4C). Our rationale for removing FBS as a trigger for secretion is that the precise component of serum that triggers secretion is not known nor is the signaling pathway that it evokes (i.e. it has not been shown to depend on intracellular calcium). Hence, we feel it is best to leave further analysis of the FBS-triggered pathway to futures studies. Instead, we have used A23187, which acts to equilibrate calcium across membranes, thus resulting in a prolonged increase in intracellular calcium. Consistent with the response seen in egress, both kinases are required for secretion triggered by A23187 (i.e. secretion by parasites expressing CDPK1G or CDPK3G are both strongly inhibited by 3-MB-PP1). In contrast, the requirement for CDPK3 in secretion triggered by ethanol is far less apparent than for CDPK1. Ethanol has been shown to work by elevating IP3, leading to increases in intracellular calcium (J Biol Chem. 2002 Jul 19;277(29):25870-6.). Although the precise reason why CDPK3 is less crucial for ethanol vs. A23187-induced secretion is not known, it may relate to spatial, temporal or quantitative differences in the calcium signals that are...
evoked by this different triggers. Alternatively, it may relate to additional signaling pathways stimulated by ethanol but not A23187. Collectively, these data support our original conclusion that both CDPK1 and CDPK3 are required for egress, while they show differential requirements for secretion depending on the agonist employed.

4. Pg 11 the statement that: "...egress is under very tight control of three distinct kinases, each of which is essential for this process" is too bold in the absence of showing that the respective inhibitors indefinitely block egress.

Response: It is difficult to assess whether the block is indefinite, since prolonged ionophore treatment is likely to affect other aspects of the parasite and integrity of the host cell monolayer. However, as shown in the new Supplementary Figure 3, the block in egress is sustained for at least 30 min. As suggested, we have revised the text to indicate that all three kinases contribute to the control of microneme secretion and hence impact egress, motility and invasion to various degrees.

Referee #2

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1) As far as the partly overlapping, yet distinct functions of the CDPK1 and 3 protein kinases in egress and invasion are concerned, the paper's conclusions are generally well supported by the data. Regarding the role of the parasite's cyclic GMP dependent protein kinase in egress, however, Fig. 5 presents intriguing data, but all conclusions remain based on inhibitors. Neither the selective elevation of cGMP levels by zaprinast, nor the selectivity of the PKG inhibitor compound 1 are demonstrated. These data therefore remain preliminary and the paper falls short of demonstrating conclusively a role for protein kinase G in egress. Statements in abstract, results section and discussion to this effect should be toned down significantly.

Response: The selectivity of compound 1 for PKG in T. gondii was previously shown using similar chemical genetic strategies described here (Eukaryot Cell. 2002 Jun;1(3):317-28). Although this previous study did not examine egress, we feel the specificity of this compound has been well established in T. gondii. Prior studies have concluded that although CDPK1 shows some sensitivity to compound 1 in vitro, the primary target of its action in vivo is PKG (Mol Biochem Parasitol. 2006 Sep;149(1):86-98). These citations are now found in the results and discussion, and we feel they adequately support our conclusions about the role of PKG. We have not monitored levels of cGMP in response to Zaprinast and we acknowledge in the discussion that our conclusions are based on the assumption that it elevates cGMP and activates PKG. We agree that future studies should be designed to address this point. However, it is worth noting that the effects of Zaprinast are fully reverse by compound 1, consistent with the model we have proposed. We have modified the text to indicate that our results are consistent with the role of PKG in rescuing the function of CDPK3, although further work would be necessary to definitively show this.
2) The role for TgCDPK3 in regulating directly the molecular motor that powers gliding is an important and potentially controversial question that this manuscript tries to deal with. The only motility assay that is used looks at antigen trails left by a small fraction of gliding parasites on a slide (Fig. 2A). That only 12-25% of parasites ever seem to glide in this assay casts some doubt on its relevance. The authors conclude that CDPK3 is required for the onset of motility, but not to sustain gliding, but this is not convincingly supported by Fig. 2A. It would be important to use quantitative video microscopy to measure the impact of 3MB-PP1 on average gliding speed, distance travelled and number and frequency of gliding episodes to support this important point.

Response: We examined motility by video microscopy not by staining of trails (even though a stained trail assay is shown in Figure 1). The results of these assays are shown in Figure 4A and gliding speed is now quantified in Figure 4B. The apparent frequency of gliding (12-25%) is entirely normal for these in vitro assays, based on prior work from our laboratory and many others (PLoS Pathog. 2011 Oct;7(10):e1002280, PLoS Pathog. 2006 Aug;2(8):e84., Mol Biol Cell. 1999 Nov;10(11):3539-47). The reason for this low response is not known but might relate to metabolic status of the cells. Nevertheless, the reduced frequency of gliding in CDPK1G and CDPK3G strains treated with 3-MB-PP1 is quite apparent from the data in Figure 4. However, the lack of differences in gliding speed is consistent with normal activity of the motor complex. Combined with the clear defect in microneme secretion, we feel that these data indicate a role for CDPK3 in release of microneme adhesins, which are needed for gliding, rather than an activity that impacts the motor complex directly.

3) Figure 4B - I do not understand how with CDPK3M, 3MB-PP1 can enhance EtOH induced MIC2 secretion (i.e. give negative inhibition). Is there a systematic error in this figure? How reproducible is it? We are only shown relative data. Is baseline secretion in all mutants the same in the absence of inhibitor and in response to both combinations of stimuli? The gatekeeper mutation could easily affect baseline secretion and inducibility, for instance by modifying activity of the kinase to some degree, without causing a marked phenotype in other assays. The enhancement of secretion by the inhibitor in 3M makes the apparent absence of inhibition in 3G impossible to interpret. Does the inhibitor have no effect at all on EtOH induced secretion by CDPK3G or does it reverse the enhancement observed in CDPK3M, resulting in no net effect? I am not convinced if and how EtOH and FBS/EtOH behave differently as triggers in these mutants.

Response: Because the secretion assay is inherently variable, we have chosen to present the data as normalized internally for each strain. That is, the % enhancement or inhibition for each strain is compared based on the response with 3-MB-PP1 vs. without. We are confident the differences seen are not due to differences in the various strains used, as baseline secretion is similar in all strains in the absence of 3-MB-PP1 (Supplemental Figure 4). The reviewer is correct that secretion by parasites containing a bulky gatekeeper mutation is consistently elevated when treated with 3-MB-PP1, although this effect is modest compared to the inhibition caused in sensitive strains (Figure 4C). The molecular basis for this is not known, but might represent an off-target activity of the inhibitor. If so, it would be expected to occur equally in all strains, and yet it would only be apparent when secretion is not inhibited (i.e. it would be masked by any inhibition). In this case, the degree of inhibition seen in Figure 4C might be higher by 10-15% for CDPK1G and CDPK3G strains. However, such an effect does not negate the obvious differences in inhibition of secretion in the 3-MB-PP1 sensitive strains.

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with TgCDPK1. The authors build on their previous chemical genetic analysis of the TgCDPK1 gene, and engineer inhibitor sensitive forms of TgCDPK3 for in vivo analysis following inhibition. The key finding is that in contrast to TgCDPK1 which is required for both parasite egress and invasion from host cells, TgCDPK3 is only required for egress. Further, it is found that this appears defect appears to derive from microneme secretion for the initiation of motility, with intriguing differences between the response of microneme secretion to different triggers being regulated by TgCDPK3. Finally, some evidence is presented that suggests that PKG interacts with TgCDPK3. Overall, this paper demonstrates the efficacy of chemical genetic approaches for the analysis of parasite kinases and introduces the function of the TgCDPK3 gene. It also lays the foundation for looking at combinatorial signaling by PKG, TgCDPK in the processes. A few further experiments will strengthen the evidence for their conclusions.

Comments:
1. The CDPKs are postulated to initiate motility, but then a significant effort is made in the Discussion to critique previous data on the in vitro phosphorylation of the actinomyosin motor by PfCDPK1. However, it is possible that the phosphorylation is required for initiation of the actinomyosin motor but not continued operation? Further, with the mutants now in hand the authors could easily look at phosphorylation of some of the actinomyosin motor components.

Response: We do not disagree with prior studies showing that CDPK3 can phosphorylate the motor complex in vitro; however, such a process has not been shown to play a role in vivo. To account for the possibility that CDPK3 might be required for activating or assembling the motor, but not for its sustained activity, we treated intracellular parasites expressing CDPK3 with 3-MB-PP1, mechanically harvested, and tested them for invasion. Such pretreatment also failed to block invasion, suggesting that CDPK3 is not required for initiation of motor function (see Supplementary Figure 2). We have also added data to Figure 4B showing that once motility is initiated, the speed of gliding is the same for all strains treated with 3-MB-PP1. Although we could examine phosphorylation of putative motor complex members, we do not think this would be productive since none of the putative phosphorylation sites in the motor complex that are presumed to be targets of CDPK3 (or CDPK1 in Plasmodium) have been shown to be functionally important. Moreover, the phenotypes we have revealed here indicate that CDPK3 controls microneme secretion, rather than motility per se. Nonetheless, we acknowledge in the discussion that future work might reveal that CDPK3 also phosphorylates components of the motor complex.

2. The rationale that TgCDPK1 is the ‘closest homolog’ for PfCDPK1 and therefore should be orthologous is weak. A straightforward functional complementation experiment might establish this orthology. What other criteria justify using TgCDPK1 to study PfCDPK1? (note the reviewer presumably means TgCDPK3 vs PfCDPK1)

Response: We realize the numbering of CDPKs has lead to some confusion: TgCDPK3 is the closest homologue of PfCDPK1 based on alignment of the kinase domains, phylogeny, conservation of post-translational modifications, and cellular localization. We have revised the text to make this clearer and also to acknowledge that differences may exist between the two systems.

3. A dose-response curve of the inhibition of invasion by 3-MB-PP1 against CDPK1-G and CDPK3-G, would clearly establish the relative sensitivity of the two kinases.

Response: We have added a range of doses for the inhibition of CDPK1 and CDPK3 by 3-MB PP1 (Supplementary Figure 1). These findings indicate that CDPK1 is required for invasion, while CDPK3 is not, even at high doses of inhibitor.

4. CDPK1-G inhibition of egress is not complete at even high concentrations of inhibitor, compared to CDPK3-G. Why is this?

Response: Inhibition of CDPK1 leads to partial vacuolar lysis, and hence egress is not completely blocked, as seen here and shown previously (Lourido Nature. 2010 May 20;465(7296):359-62.). In contrast, inhibition of CDPK3 leads to complete lack of vacuole permeability. This suggests that these two kinases have different targets and hence non-equivalent roles in the process. This is also implied by the fact that they are non-redundant. However, precisely defining their roles awaits identification of their downstream targets. At present, we feel our report is important in calling
attention to the different roles for each kinase in the processes of egress and invasion.

5. Could differences in dose-sensitivities for egress of CDPK3-G and CDPK1-G be due to differences in sensitivity of the two target enzymes to intracellularly-produced metabolites of the drug? How can this possibility be excluded?

Response: This prospect seems unlikely since the binding of the inhibitor to Src and CDPK1 has been studied in detail at the structural level. The specificity of 3-MB-PP1 appears to be due to the parent molecule rather than a metabolite. It is more likely that this different sensitivity is the result of each kinase controlling a different step(s) in the process.

6. What are the minimal concentrations of drug needed for defects in gliding motility and microneme secretion- again a dose-response curve would help?

Response: We are not sure that a full dose response curve would be informative here. Rather, we have established the effective concentration based on egress (Figure 3B) and invasion (Supplementary Figure 1), assays that are much easier to quantify and hence obtain accurate dose response curves. Granted differences might occur in other assays, but we feel the relative roles of CDPK1 and CDPK3 in microneme secretion, motility and invasion are well illustrated using the doses chosen here. Furthermore, all of the assays are internally controlled using the resistant strains.

7. Need to expand (perhaps quantitate) the important finding that once movement was initiated, pattern and parasite movement appeared normal.

Response: We have added data to Figure 4B showing that once motility is initiated, the speed of gliding is the same for both mutants treated with 3-MB-PP1.

8. The differential response of microneme secretion to the two triggers is interesting. It would be important to use just FBS in these experiments to support the conclusions. Further explanation and maybe even experimentation to follow-up this fascinating observation is warranted.

Response: We have revised this figure to now include responses to A23187 alone, since this is the condition used to test egress. The results demonstrate that similar to egress, both CDPK1 and CDPK3 are required for A23187-stimulated secretion, while CDPK1 plays a more prominent role during ethanol-induced secretion. We have removed the data for FBS as explained in response to reviewer 1, comment 3.

9. It is said that high concentrations of zaprinast was used- is this the 0.5 mM as stated in the Material & Methods. Why do the authors state that this is high- is this high concentration a concern?

Response: This concentration is similar to what was shown to affect Plasmodium (McRobert PLoS Biol. 2008 Jun 3;6(6):e139). Hence, we do not think it is a concern and have removed the wording about the dose being “high”.

10. It would be informative to see dose-response curves for the effect of zaprinast on triggering egress, together with CDPK3. What is the evidence that they are working together? Could synergy or additive effects be demonstrated using isobolograms?

Response: This is a really interesting idea, but not one that we have included here. At present we do not have two completely independent agonist or inhibitors with which to simultaneously interrogate these pathways. Instead we have alluded to the fact that future studies along these lines might aid in determining if the pathways controlled by CDPK1, CDPK3, and PKG are the same, inter-dependent.

11. The suggestion that the three kinases are somehow working together might benefit from the development of a model and inclusion of a figure.

Response: We can certainly appreciate this suggestion but at this stage there are too many possibilities to allow us to draw a very robust model. At a later date when specific targets have been defined, and when their interdependence is better understood, such a model will be more information.
12. What are the disadvantages of the chemical genetics approach, if any, vis a vis a gene knockout or knockdown approach? Eg. specificity of inhibitors and potential of hitting multiple targets?

Response: The current inhibitors have been shown to have a very high degree of specificity for small gatekeeper kinases. As shown in Figure 2, small gatekeepers are exceedingly rare in *T. gondii* (and in mammalian systems). However, there is some potential for the inhibitors used here to affect A, S, or T gatekeeper kinases, which have intermediate sensitivity to 3-MB-PP1. All of the assays performed here utilize compound treatments that are quite short, thus hopefully minimizing such off target activity. Additionally, all of the assays are internally controlled using the bulky gatekeeper mutants that are resistant (any off target effect would still be apparent in these controls). However, we have added a note that at higher concentrations or prolonged incubation, off target effect may confound the interpretation of chemical genetics experiments.

13. Figure 3A would benefit from a dose-response curve.
Response: Results from different doses are now included in Supplemental Figure 1.

Acceptance letter  
16 October 2012

Thank you for submitting your revised manuscript to the EMBO Journal. Your study has now been re-reviewed by referees # 1 and 2. As you can see below, both referees appreciate the introduced changes and support publication in the EMBO Journal. I am therefore very pleased to accept the paper for publication in the EMBO Journal.

Thank you for submitting your interesting manuscript to the EMBO Journal.

Yours sincerely

Editor  
The EMBO Journal

REFeree REPORTS

Referee #1

The authors have adequately addressed my comments. Accordingly, my view is that the submission is now ready for publication.

Referee #2

I am now happy for this ms to be accepted for publication. The authors have responded well to all comments and have provided additional information and data, for instance concerning the modality and speed of gliding in the mutants.