nature portfolio

Peer Review Information

Journal: Nature Microbiology
Manuscript Title: Screening the Toxoplasma kinome with high-throughput tagging (HiT) identifies a regulator of invasion and egress
Corresponding author name(s): Sebastian Lourido

Reviewer Comments & Decisions:

Decision Letter, initial version:

Dear Dr Lourido,

Thank you for your patience while your manuscript "High-throughput functionalization of the Toxoplasma kinome uncovers a novel regulator of invasion and egress" was under peer-review at Nature Microbiology. It has now been seen by 4 referees, whose expertise and comments you will find at the end of this email. Although they find your work of some potential interest, they have raised a number of concerns that will need to be addressed before we can consider publication of the work in Nature Microbiology.

In particular, referee #1 states that "one key flaw of the mAID system employed in this study, is its inability to target exported proteins or proteins where the mAID tag is not accessible to the cytosol.". This referee also mentions that "Another issue highlighted by the authors but not discussed in great length is the out of frame integration of the tag leading to untagged transgenics." Referee #1 asks "Could they integrate a selected linked integration approach, similar to that used in Plasmodium falciparum, where a T2A self-cleaving peptide separates the tag and a selectable marker that would only be expressed upon correct integration of the repair template?" Editorially, we feel this will be important top address. Referee #2 would like more information on how false negatives impact the results. This referee also suggests to add additional characterization of SPARK, especially identifying candidate substrates. Editorially, we feel this would certainly strengthen the paper. Referees #2 and #3 also suggest to discuss in more detail how the current work represents an advance over the previous work by Sidik et al., 2016. Referee #4 suggests to provide better confocal microscopy images for Fig 5C.

Should further experimental data allow you to address these criticisms, we would be happy to look at a revised manuscript.

We are committed to providing a fair and constructive peer-review process. Please do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

We strongly support public availability of data. Please place the data used in your paper into a public data repository, if one exists, or alternatively, present the data as Source Data or Supplementary Information. If data can only be shared on request, please explain why in your Data Availability Statement, and also in the correspondence with your editor. For some data types, deposition in a public repository is mandatory - more information on our data deposition policies and available repositories can be found at https://www.nature.com/nature-research/editorial-policies/reporting-standards#availability-of-data.

Please include a data availability statement as a separate section after Methods but before references, under the heading "Data Availability". This section should inform readers about the availability of the data used to support the conclusions of your study. This information includes accession codes to public repositories (data banks for protein, DNA or RNA sequences, microarray, proteomics data etc…), references to source data published alongside the paper, unique identifiers such as URLs to data repository entries, or data set DOIs, and any other statement about data availability. At a minimum, you should include the following statement: "The data that support the findings of this study are available from the corresponding author upon request", mentioning any restrictions on availability. If DOIs are provided, we also strongly encourage including these in the Reference list (authors, title, publisher (repository name), identifier, year). For more guidance on how to write this section please see: http://www.nature.com/authors/policies/data/data-availability-statements-data-citations.pdf

When revising your manuscript:

* Include a "Response to referees" document detailing, point-by-point, how you addressed each referee comment. If no action was taken to address a point, you must provide a compelling argument. This response will be sent back to the referees along with the revised manuscript.

* If you have not done so already we suggest that you begin to revise your manuscript so that it conforms to our Article format instructions at http://www.nature.com/nmicrobiol/info/final-submission. Refer also to any guidelines provided in this letter.

* Include a revised version of any required reporting checklist. It will be available to referees (and, potentially, statisticians) to aid in their evaluation if the manuscript goes back for peer review. A revised checklist is essential for re-review of the paper.

When submitting the revised version of your manuscript, please pay close attention to our href="https://www.nature.com/nature-research/editorial-policies/image-integrity">Digital Image Integrity Guidelines. and to the following points below:

-- that unprocessed scans are clearly labelled and match the gels and western blots presented in figures.
-- that control panels for gels and western blots are appropriately described as loading on sample processing controls
-- all images in the paper are checked for duplication of panels and for splicing of gel lanes.

Finally, please ensure that you retain unprocessed data and metadata files after publication, ideally archiving data in perpetuity, as these may be requested during the peer review and production process or after publication if any issues arise.

Please use the link below to submit a revised paper:

{redacted}

Note: This url links to your confidential homepage and associated information about manuscripts you may have submitted or be reviewing for us. If you wish to forward this e-mail to co-authors, please delete this link to your homepage first.

Nature Microbiology is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. This applies to primary research papers only. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit please visit www.springernature.com/orcid.

If you wish to submit a suitably revised manuscript we would hope to receive it within 6 months. If you cannot send it within this time, please let us know. We will be happy to consider your revision, even if a similar study has been accepted for publication at Nature Microbiology or published elsewhere (up to a maximum of 6 months).

In the meantime we hope that you find our referees' comments helpful.

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Reviewer Expertise:

Referee #1: Toxoplasma genetic tools, Toxoplasma kinases
Referee #2: Toxoplasma biology
Referee #3: Toxoplasma actin biology
Referee #4: Toxoplasma biology

Reviewer Comments:

Reviewer #1 (Remarks to the Author):

Summary
Protein kinases play a fundamental role in regulating a plethora of cellular processes, however in Toxoplasma gondii, many kinases have yet to be characterised. To address this, Smith et al. employ an innovative high-throughput strategy combining CRISPR/Cas9-mediated gene editing coupled with a pool of vectors that would tag and allow for the conditional knockdown of the Toxoplasma kinome consisting of 145 proteins.

Using an arrayed approach, the authors generated singly tagged clones which represented 127/145 of the kinases. The localisation of 39 kinases was determined, and conditional depletion of 36 kinases was successfully achieved, allowing the authors to identify distinct morphological phenotypes associated with depletion of these kinases. The authors then went on to perform a pooled screen, which determined the

relative fitness cost associated with depletion of each kinase over the course of the lytic cycle, helping to identify kinases would lead to acute or delayed-death phenotypes upon their depletion. From this pooled screen, the authors characterised 4 kinases associated with delayed death, identifying two genes that appear to be involved in invasion. They further characterised one of these genes, which they name SPARK, showing that this kinase appears to be important in mediating Ca2+ release, identifying a novel protein involved in the egress pathway and demonstrating the strength of this screen.

Overall, the authors establish this novel method as a high throughput strategy that can be used for temporal control of protein knock down and allows for visualisation of protein localisation - features that established screens lack. However, one key flaw of the mAID system employed in this study, is its inability to target exported proteins or proteins where the mAID tag is not accessible to the cytosol. Another issue highlighted by the authors but not discussed in great length is the out of frame integration of the tag leading to untagged transgenics. Despite these shortcomings, this work will be a vital tool that will be a major asset in the Toxoplasma field.

Major Issues

• Line 214: the authors state that the category "cell division I" and "cell division II" constitute smaller and larger vacuoles, respectively but the vacuoles don't look larger for cell division II. Have the authors quantified this? Considering the authors are making this statement, I assume they have, and would request that they add this information to the supplementary data, since this would be useful information to have for all the genes they characterised.

• Figure 3F: the authors spend a great deal of time explaining why they do not detect all the genes they expected to cluster in A but don't discuss the genes observed in cluster B - for example, PKG which is essential for lytic growth, comes up as non-essential. The authors should explain why they observed this and also discuss whether they observe any other genes, previously shown as being essential, that come up as non-essential.

• Line 295 - the authors state that a caveat in this work is that in the array they characterised clones where the tag was removed due to an error in homologous recombination - however in the array the authors looked at protein localisation - so it should have been evident which clones had an issue with removal of the tag or not. Can the authors clarify what they mean since this sentence is quite confusing and doesn't really explain the discrepancies they observe. The reverse could be true- where in the pool they see essential genes not dropping out since the tag is not being added so IAA addition doesn't lead to loss of that population.

• Line 343 - the authors say that they regenerated the lines. Why did they do this rather than characterise the clones they generated in the arrayed screen? Does this suggest there are issues with the clones from the arrayed screen, such that they are not characterisable?

• Line 402 - why was ionomycin used instead of A23187? The authors should clarify here.

• The authors do not state how many clones they got of each gene in the arrayed screen -did they see a massive enrichment of a small subset? Overall there doesn't seem to be much transparency on certain things often only discussing the positive aspects of the experiments rather than the downfalls, which as a methods paper is important information for anyone to know before using it as a tool to characterise their own subset of genes.

• The authors state that they get out of frame integration of the tag, rendering those transgenic lines useless, however the authors do not address how this seemingly major issue can be overcome. Could

they integrate a selected linked integration approach, similar to that used in Plasmodium falciparum, where a T2A self-cleaving peptide separates the tag and a selectable marker that would only be expressed upon correct integration of the repair template?

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• Sig Fig2: consider changing the images so that there is an overlay of the mNeon Green signal and the IMC1 signal (similar to in Fig. 2C) - in the current format it is difficult to tell where the protein is in relation to the parasite.
• Line 252: please state how many genes were tested and the % that 21 genes constitute as essential.
• Fig 4A: in this section in the text (starting line 332) the authors only talk about the delayed death genes, however the figure also includes acute death genes which are not referred to at all. The authors should either remove the acute death figure or mention it in the text if they want to draw comparisons between the two sets of genes.
• Line 614: for figure 5E-F the authors should add a sentence explaining what the shaded region is vs the thick line.

Reviewer #2 (Remarks to the Author):

Kinases are important regulators and attractive chemotherapeutic targets in Apicomplexan parasites, including Toxoplasma. Although several high throughput genetic screens have been conducted in Apicomplexan parasites, including a Toxoplasma knock-out screen, these do not include protein tagging or the use of degrons, which can facilitate functional analyses. In the current study, the Toxoplasma kinome (147 kinases) is targeted with tags incorporating a degron, a fluorophore and an epitope, followed by arrayed and pooled screening for localisation and degron-induced phenotypes. Development and improvement of the approach to library assembly and screening is documented in some detail. Several localisations and phenotypes confirm prior findings and validate the approach. The authors then focus on the identification and characterisation of a novel regulator of calcium signalling, invasion and egress - Store Potentiating/Activating Regulatory Kinase or SPARK.

1. Lines 456-459. False negatives arise due to failure to add the tag, failure to degrade the protein or disruption of protein function driven by the tag. I struggled a little to keep track of how many kinases were thought to be subject to these or other issues. Also, in terms of successful characterisation, I

believe 127 kinases were tagged, 109 defects were detected (15 morphological) and 39 were localised by microscopy. Could the authors add a simple Table to the main manuscript to clarify.

2. I understand that the degron approach provides greater temporal resolution, but could the authors briefly explain why the invasion and egress phenotypes revealed here by pooled screening were not or could not be revealed using the earlier knockout library (Sidik et al., 2016).

3. Line 545: "Jimenez-Ruiz and colleagues developed an alternative strategy for high-throughput phenotypic analysis of T. gondii". These authors also identify "essential genes required for independent steps during host cell egress and invasion" but there is no citation for this work.

4. Fig. 2C: Is it known why some clones display heterogeneity – 275610 for example?

5. Protein degradation may vary for different targets. It appears that the majority of degron fusions were effectively degraded but it is unclear whether many were degraded with similar efficiency. Can this be assessed?

6. Although the authors link SPARK to egress they found it "difficult to definitively place SPARK in the pathway" (lines 517-518). Could phosphoproteomic analysis be used to identify candidate SPARK-substrates?

7. Do any of the prior phenotypic screens in Plasmodium species suggest a similar role for the SPARK ortholog in these parasites?

Reviewer #3 (Remarks to the Author):

In this work, Smith et al. report a multiplex, high-throughput screen of the Toxoplasma kinome. The platform combines phenotypic characterization, localization analysis, and sensitive pooled screening for uncovering a wide-range of fitness defects. The screening platform is well designed, scalable, and has broad applications for identifying genes critical for the lytic cycle of the parasite. The targeted screen of the parasite kinome led to the discovery of a number of new kinases that are critical for parasite replication or invasion, including SPARK, a novel member of the AGC kinase family. The characterization of SPARK reveals that it is likely to be one of the major missing links in the calcium signaling pathway. The manuscript is extremely well written and the results are clearly presented. Both the technological advance and biological discoveries are exciting and open up numerous new directions for the field. I am particularly impressed with the rigorous analysis and comparison of the arrayed and pooled screens, and thoughtful discussion on potential caveats. I am however puzzled by Figure 3G, which showed that there are some significant differences in relative fitness measurements between the current screen and the previous large-scale CRISPR-based screen (Sidik et al., 2016). In the Auxin-AID based assay, some of the genes with moderate phenotype scores (e.g. Tg202160, Tg205550, Tg231070) from the CRISPR screen displayed much more severe phenotype than many genes with more significant phenotype scores. It would be helpful if the authors could provide some discussion or data to explain this. Is it possible that the Auxin-AID system exacerbates the phenotype for certain genes? Have the authors tried to make knockout (instead of knockdown) mutants for Tg202160, Tg205550 or Tg231070? The Sidik

CRISPR screen essentially measured the persistence of a deleted locus in a population. A gene assigned a moderate phenotype score in that assay (e.g. the KO mutant persists for a decent amount of time in the population) seems to be unlikely to display a severe phenotype when knocked out.

Reviewer #4 (Remarks to the Author):

This manuscript describes the development of a High-throughput (HiT) CRISPR-mediated screening in Toxoplasma that can be used in both arrayed and pooled formats with spatiotemporal resolution. It allows obtaining protein localization and phenotypic description simultaneously at scale. Here, the authors specifically profiled the T. gondii kinome. The authors have been able to assign several kinases that had not previously been studied to cell cycle progression. Pooled screen profiles allowed to differentiate between acute and delayed-death phenotypes. In the latter category, the authors discovered SPARK, a novel regulator of parasite invasion and egress, through the stimulation of parasite intracellular Ca2+ stores. The kinome profiling will provide a great resource to the field of Toxoplasma cell biology, but also for other Apicomplexa because many kinases are conserved in related parasites such as Plasmodium. The HiT vector system is scalable, flexible for others tags, and adapted to address a wide variety of biological questions.
The manuscript is well-written, and mostly approachable for the general reader. The experiments are carefully done and data adequately discussed. I have few minor comments.

Specific Comments:

1. Fig. 1A: the selection cassette (grey) and gRNA (blue) are in the opposite direction on the linearized fragment

2. It would be great to add the knockout phenotype score from Sidik et al, in the table corresponding to the pooled screen.

3. Fig. 2: Panels C and D are difficult to read with respect to the text. I suggest: 1) to show an example of each of the 9 locations presented in the scheme of figure 2C and to add the accession numbers of the proteins of each group, 2) to group the images by location in figure S2, 3) to add an IFA illustrating the location of proteins in figure 2D, in order to easily associate the location of the protein with the phenotype; and finally 4) when known to add the name of the kinase to the panel.

5. Fig. 4C: TGGT1_239885 does not cause severe reduction in plaque size, as stated in the text line 326

6. Lines 184-186: The discontinuity in the IMC marker appears also in ‘singlets I’. There are parasite morphological differences between Singlets I and II that suggest a block in the G1 phase for singlet I and growth arrest in the S/M of the cell cycle for singlet II.

7. Line-106: what is the accession number of CRK3 which is not in the text, nor in the table and is also not annotated in ToxoDB

8. It is not always clear in figure 3 and text, whether the data derive from both the HiT 3'CDPK3 and HiT 3'SAG1 screens or only from HiT 3'CDPK3 screen. Please clarify.

9. The location of the SPARK is difficult to appreciate in Figure 5C. Better images by confocal microscopy are required here.

10. 'SPARK exhibits a phenotypic profile distinct from PKG, for which knockdown or chemical inhibition cannot be rescued by Ca2+ ionophore (refs 23,47,85)'. I could not find this information in reference 47 (Jia et al) and ref 85 (Sidik et al), only in ref 23 (Brown et al)

11. To better understand the rational of using ionomycin, add in the text that it specifically mobilizes neutral calcium stores.

12. 3'UTR and 5'UTR: often the prime ' is missing

Author Rebuttal to Initial comments

Reviewer #1 (Remarks to the Author):

Summary
Protein kinases play a fundamental role in regulating a plethora of cellular processes, however in Toxoplasma gondii, many kinases have yet to be characterised. To address this, Smith et al. employ an innovative high-throughput strategy combining CRISPR/Cas9-mediated gene editing coupled with a pool of vectors that would tag and allow for the conditional knockdown of the Toxoplasma kinome consisting of 145 proteins.

Using an arrayed approach, the authors generated singly tagged clones which represented 127/145 of the kinases. The localisation of 39 kinases was determined, and conditional depletion of 36 kinases was successfully achieved, allowing the authors to identify distinct morphological phenotypes associated with depletion of these kinases. The authors then went on to perform a pooled screen, which determined the relative fitness cost associated with depletion of each kinase over the course of the lytic cycle, helping to identify kinases would lead to acute or delayed-death phenotypes upon their depletion. From this pooled screen, the authors characterised 4 kinases associated with delayed death, identifying two genes that appear to be involved in invasion. They further characterised one of these genes, which they name SPARK, showing that this kinase appears to be important in mediating Ca2+ release, identifying a novel protein involved in the egress pathway and demonstrating the strength of this screen.

Overall, the authors establish this novel method as a high throughput strategy that can be used for temporal control of protein knock down and allows for visualisation of protein localisation - features that established screens lack. However, one key flaw of the mAID system employed in this study, is its inability to target exported proteins or proteins where the mAID tag is not accessible to the cytosol. Another issue highlighted by the authors but not discussed in great length is the out of frame integration of the tag leading to untagged transgenics. Despite these shortcomings, this work will be a vital tool that will be a major asset in the Toxoplasma field.

We appreciate the reviewer's evaluation of our work and agree with the limitations of the mAID approach. We have attempted to highlight those limitations in the Results by stating that *"the AID system is restricted to downregulation of proteins with cytosolically exposed termini…"* and in the Discussion mentioning that, *"alternative tags or conditional expression systems, such as split fluorophores or the U1 system, could also extend HiT screening to additional biological questions and make it compatible with secreted or compartmentalized proteins inaccessible to the AID approach."* We also detail our inability to downregulate two inaccessible proteins that were present among our targets in **Figs. S2E & S3A,** mentioning in the Results that *"lack of degradation may reflect inaccessibility to TIR1."* Despite these drawbacks, the mAID system remains the best method to rapidly degrade proteins of interest in *Toxoplasma* and ideally suited to cytosolic signaling proteins such as the kinases targeted by our study. The strategy will have to be modified to study other classes of proteins, like exported or compartmentalized proteins, and our new data (**Fig. 1D-J**) shows examples of how the HiT vectors can be modified to enable transcriptional repression or co-translational expression of reporters.

The reviewer brings up an important point regarding out-of-frame integration of the tags, which motivated the sequencing of clones from the CDPK1 tagging experiments in **Figure 1C**. As stated in the text, the rate of out-of-frame integrants is <10% in the case of CDPK1, which likely reflects the expected error rate of homologous recombination in *Toxoplasma* (Sidik et al. 2014. PLoS One). Since the fitness of such clones will depend on the targeted protein, it is possible they will outcompete correctly tagged clones, but in such cases the problem would be detected by a decrease in relative abundance for guides against the gene of interest compared to the library, absence of an IAA effect, and lack of signal in the arrayed screen. As stated above, some proteins will certainly be incompatible with HiT screening, but can be rationally excluded from the analysis. Nevertheless, we demonstrate that even in its present form the screens can yield critical insight on a large number of proteins.

Major Issues

• Line 214: the authors state that the category "cell division I" and "cell division II" constitute smaller and larger vacuoles, respectively but the vacuoles don't look larger for cell division II. Have the authors quantified this? Considering the authors are making this statement, I assume they have, and would request that they add this information to the supplementary data, since this would be useful information to have for all the genes they characterised.

We appreciate the reviewer highlighting this issue. The categorization had been qualitative and was not validated by quantification. However, it was clear from the reanalysis of all the mutants that what distinguishes the two groups—and perhaps resulted in the false perception of different vacuole size—is the distribution of the IMC. We have therefore retained the two categories but describe them as follows in the Results:

*"We cataloged phenotypes involving compromised cell division that resulted in vacuoles of intermediate sizes. Although the precise characteristics of the cell division phenotypes differ between mutants, they could be broadly categorized by exhibiting either continuous (cell division I) or fragmented (cell division II) distribution of IMC (**Fig. 2C**)."*

• Figure 3F: the authors spend a great deal of time explaining why they do not detect all the genes they expected to cluster in A but don't discuss the genes observed in cluster B - for example, PKG which is essential for lytic growth, comes up as non-essential. The authors should explain why they observed this and also discuss whether they observe any other genes, previously shown as being essential, that come up as non-essential.

Indeed the goal of mapping the previous screening results on Figure 3F was to highlight discrepancies between the two approaches to the reader. We have also highlighted issues like that of PKG in the Results stating that *"Out-of-frame integration of the HiT construct may be frequent for proteins rendered hypomorphic by the tag and drive known essential genes like PKG into cluster B."* We also discuss these genes in the Discussion stating that *"... 29 genes previously reported to be fitness-conferring were missed by our pooled HiT screens..."* and detailing possible sources of the discrepancy. We hope that by providing the complete picture, investigators will be able to design experiments to directly test the source of screening discrepancies, which is outside the scope of the present study.

• Line 295 - the authors state that a caveat in this work is that in the array they characterised clones where the tag was removed due to an error in homologous recombination - however in the array the authors looked at protein localisation - so it should have been evident which clones had an issue with removal of the tag or not. Can the authors clarify what they mean since this sentence is quite confusing

and doesn't really explain the discrepancies they observe. The reverse could be true- where in the pool they see essential genes not dropping out since the tag is not being added so IAA addition doesn't lead to loss of that population.

Due to variable levels of expression, some genes are not observable by live-cell microscopy. In the absence of a measurable phenotype, it is therefore not apparent whether a clone that lacks a detectable signal has integrated the tag in-frame or out-of-frame. We have revised the text to clarify that some genes are not observable by live cell microscopy, precluding the use of localization as a general proxy for integration:
"Discrepancies between the two screens may also be explained by array clones that resulted from errors in homologous recombination that removed the tag. Although out-of-frame clones could theoretically be excluded from the analysis on the basis of lacking expression of the tag, the low abundance of many protein kinases precludes the implementation of such a strategy."

• Line 343 - the authors say that they regenerated the lines. Why did they do this rather than characterise the clones they generated in the arrayed screen? Does this suggest there are issues with the clones from the arrayed screen, such that they are not characterisable?

We have no reason to suspect anything is wrong with the clones from the arrayed screen. The delayed and acute death analysis of the pooled screens was completed after the arrayed screen had been completed and the entire 1160 clone array was not preserved. In addition, not all of the delayed death candidates were present in the arrayed library. Finally, regenerating the mutants was an opportunity to validate the results of the screen. For these reasons we derived new clonal lines of each candidate. We have added a sentence clarifying this point:
"Although three of the candidates were present in the array, we rederived conditional mutants to validate the results with independently derived clones."

• Line 402 - why was ionomycin used instead of A23187? The authors should clarify here.

We are accustomed to use ionomycin for bulk measurements due to spectral interference between A23187 and Fura-2; however, we agree that this is not an issue with the GCaMP measurements and for consistency have repeated the relevant assays with A23187 (**new Fig. 5I**), observing similar results. Stimulation with either ionophore rescues the effects of SPARK depletion on extracellular parasites.

• The authors do not state how many clones they got of each gene in the arrayed screen -did they see a massive enrichment of a small subset? Overall there doesn't seem to be much transparency on certain things often only discussing the positive aspects of the experiments rather than the downfalls, which as a methods paper is

important information for anyone to know before using it as a tool to characterise their own subset of genes.

This information was available in the original submission and remains present throughout the manuscript, even if it is not possible to list clone numbers for all 155 targeted genes in the main text. We provided a detailed list of analyzed clones in **Table S2** and plots for the number of clones per guide and per gene in the array (**Fig. S2B**). Since we now summarize patterns of expression by subcellular localization, we also included the number of clones for each gene in parenthesis (**Figure 2B–C**).

• The authors state that they get out of frame integration of the tag, rendering those transgenic lines useless, however the authors do not address how this seemingly major issue can be overcome. Could they integrate a selected linked integration approach, similar to that used in Plasmodium falciparum, where a T2A self-cleaving peptide separates the tag and a selectable marker that would only be expressed upon correct integration of the repair template?

We have generated additional HiT vectors utilizing T2A linked to an mKate2 fluorophore to show that the reviewer's suggestion is in principle feasible (**new Figure 1D–G**). However, we opted not to utilize the T2A system in our screens to avoid selection bias against lowly expressed proteins. As we demonstrated for the tagging of CDPK1, the rates of correct homologous recombination are >90% for the 3′$_{CDPK3}$ HiT vector. As expected the strategy is not compatible with every gene, as discussed above and referenced in the manuscript.

Minor Issues

• Fig 2: the current format is confusing since the reader has to look for the number of the gene in two separate panels to see the localisation data (C) and the knockdown phenotype (D). The authors should consider merging the two figure panels, making a panel for each gene showing the dual colour localisation image on top, with the IMC1 localisation -IAA in the middle and the IMC1 localisation +IAA is on the bottom. This would make it much easier for the reader to navigate through.

We have made several improvements to **Figure 2**, with the hopes of improving accessibility. Unfortunately, the set of genes for which we obtain subcellular information is not the same as the set that displays morphological changes upon conditional degradation. Based on another reviewer's suggestion, we have simplified the display of the localizations (**Fig. 2B**). Detailed information for each gene is still available in the supplement to allow readers to examine the localization of individual genes (**Fig. S2–S4**).

• Sig Fig2: consider changing the images so that there is an overlay of the mNeon Green signal and the IMC1 signal (similar to in Fig. 2C) - in the current format it is difficult to tell where the protein is in relation to the parasite.

Due to the varying expression levels of each gene, merged images are likewise difficult to interpret as the localizations of lowly expressed proteins are obscured by the IMC1 signal. We believe the current display provides the most transparent version of the results, allowing motivated readers to generate merged images from the provided grayscale images.

• Line 252: please state how many genes were tested and the % that 21 genes constitute as essential.

Thank you for the suggestion. We have added this information to the text:
"Based on this metric, the 109 clones represented 21 genes (17% of genes tested) that caused severe defects in fibroblast clearance when conditionally knocked down"

• Fig 4A: in this section in the text (starting line 332) the authors only talk about the delayed death genes, however the figure also includes acute death genes which are not referred to at all. The authors should either remove the acute death figure or mention it in the text if they want to draw comparisons between the two sets of genes.

We have revised the text to more clearly state the comparison between representatives of the two sets of genes:

*"We characterized four delayed-death candidates that displayed a pronounced delay in gRNA loss that contrasted sharply with the rapid IAA-induced depletion of representative acute-death genes (**Fig. 4A**)"*

• Line 614: for figure 5E-F the authors should add a sentence explaining what the shaded region is vs the thick line.

We have revised the figure legend to clarify that the shaded regions represent +/- S.D.

Reviewer #2 (Remarks to the Author):

Kinases are important regulators and attractive chemotherapeutic targets in Apicomplexan parasites, including Toxoplasma. Although several high throughput genetic screens have been conducted in Apicomplexan parasites, including a Toxoplasma knock-out screen, these do not include protein tagging or the use of degrons, which can facilitate functional analyses. In the current study, the Toxoplasma kinome (147 kinases) is targeted with tags incorporating a degron, a fluorophore and an epitope, followed by arrayed and pooled screening for localisation and degron-induced phenotypes. Development and improvement of the approach to library assembly and screening is documented in some detail. Several localisations and phenotypes confirm prior findings and validate the approach. The authors then focus on the identification and characterisation of a novel regulator of calcium signalling, invasion and egress - Store Potentiating/Activating Regulatory Kinase or SPARK.

1. Lines 456-459. False negatives arise due to failure to add the tag, failure to degrade the protein or disruption of protein function driven by the tag. I struggled a little to keep track of how many kinases were thought to be subject to these or other issues. Also, in terms of successful characterisation, I believe 127 kinases were tagged, 109 defects were detected (15 morphological) and 39 were localised by microscopy. Could the authors add a simple Table to the main manuscript to clarify.

We have attempted to clarify these results in a variety of ways, while providing detailed results to the reader. First, we have changed the display of the localization information by summarizing the number of genes observed in each subcellular compartment (**Fig. 2B**). All of the genes that displayed morphological phenotypes in the array are included in **Fig. 2C**. We have specified in the text and figure that the clones

that displayed lytic assay phenotypes (**Fig. 2D**) represent 21 genes. We provide complete results for each gene in the pooled and arrayed screens in **Table S2**.

2. I understand that the degron approach provides greater temporal resolution, but could the authors briefly explain why the invasion and egress phenotypes revealed here by pooled screening were not or could not be revealed using the earlier knockout library (Sidik et al., 2016).

We had observed that gRNAs were lost from the populations over the course of several lytic cycles in the published gene disruption screens. This likely results from a lag between gene disruption and loss of gene expression based on protein and RNA turnover. Additionally, the transfected DNA generates a background signal that makes it difficult to exclusively detect integrated constructs in the first few lytic cycles. To emphasize the improved temporal resolution of our present screen, we compare our new results with the effects observed for the first and second lytic cycles in the 2016 data (**Fig. S4F**). The results show no distinction between delayed- and acute-death phenotypes in our previous screens. We have modified the Results to indicate that *"..., this temporal resolution was not captured by conventional CRISPR-based screens (Fig. S4F)."*

3. Line 545: "Jimenez-Ruiz and colleagues developed an alternative strategy for high-throughput phenotypic analysis of T. gondii". These authors also identify "essential genes required for independent steps during host cell egress and invasion" but there is no citation for this work.

The work had been co-submitted to the journal, but is now available on *BioRxiv*. We have added the appropriate citation.

4. Fig. 2C: Is it known why some clones display heterogeneity – 275610 for example?

These instances are likely due to cell cycle–dependent expression. For example, available transcriptomic data (ToxoDB) shows 275610 peaks in expression during the S/M phase of the cell cycle, consistent with its localization to daughter cell nuclei. We have provided this explanation in the text: *"Some proteins, such as TGGT1_275610, consistently displayed heterogeneous expression, likely indicating cell cycle regulation."*

5. Protein degradation may vary for different targets. It appears that the majority of degron fusions were effectively degraded but it is unclear whether many were degraded with similar efficiency. Can this be assessed?

Due to the low level of expression of many of the targeted genes, this is challenging to address quantitatively. However, for those fusions that could be analyzed by microscopy we observe that *"36 of the 40 proteins…showed complete depletion after 24 hours of IAA treatment…TGGT1_226040, TGGT1_322000, TGGT1_320630, and TGGT1_234950 showed no or only partial signal reduction under IAA treatment."* Microscopy images illustrating these results are available in **Figs. S2–S4**.

6. Although the authors link SPARK to egress they found it "difficult to definitively place SPARK in the pathway" (lines 517-518). Could phosphoproteomic analysis be used to identify candidate SPARK-

substrates?

The parasite phosphoproteome is extensively rewired in response to increased cytosolic calcium (Treeck et al. 2014. *PLoS Pathog*). Since we know that the release of calcium stores is altered in SPARK-depleted parasites, it will be challenging to distinguish direct from indirect effects of kinase depletion. The present study focuses on our ability to use HiT screening to identify new regulators of the parasite lytic cycle, and the phenotypic characterization of the SPARK mutant makes that point. The proposed analysis would come at great expense (~$7,000 for a quantitative phosphoproteome) and achieve only a speculative list of candidates requiring extensive validation. Further molecular characterization of SPARK and its targets therefore falls outside the scope of the present study—although we agree that future research will need to address this important question.

7. Do any of the prior phenotypic screens in Plasmodium species suggest a similar role for the SPARK ortholog in these parasites?

Indeed, available data from *Plasmodium falciparum* corroborates the importance of SPARK in these parasites. Although the otholog appears to have been absent from the PlasmoGEM screen, we have included a discussion of the results from the PiggyBAC screen, which describes the *P. falciparum* ortholog as fitness-conferring. We also discuss the recent manuscript from the Voss lab, which also describes the essentiality of the SPARK ortholog:
"The Plasmodium falciparum *ortholog of TGGT1_268210 was classified as fitness-conferring in previous screens and has recently been linked to regulation of protein kinase A (PKA)."*

Reviewer #3 (Remarks to the Author):

In this work, Smith et al. report a multiplex, high-throughput screen of the Toxoplasma kinome. The platform combines phenotypic characterization, localization analysis, and sensitive pooled screening for uncovering a wide-range of fitness defects. The screening platform is well designed, scalable, and has broad applications for identifying genes critical for the lytic cycle of the parasite. The targeted screen of the parasite kinome led to the discovery of a number of new kinases that are critical for parasite replication or invasion, including SPARK, a novel member of the AGC kinase family. The characterization of SPARK reveals that it is likely to be one of the major missing links in the calcium signaling pathway. The manuscript is extremely well written and the results are clearly presented. Both the technological advance and biological discoveries are exciting and open up numerous new directions for the field. I am particularly impressed with the rigorous analysis and comparison of the arrayed and pooled screens, and thoughtful discussion on potential caveats.

We appreciate the reviewer's positive comments about our work and hope to be able to share our peer-reviewed results soon with the broader community.

I am however puzzled by Figure 3G, which showed that there are some significant differences in relative fitness measurements between the current screen and the previous large-scale CRISPR-based screen (Sidik et al., 2016). In the Auxin-AID based assay, some of the genes with moderate phenotype scores (e.g. Tg202160, Tg205550, Tg231070) from the CRISPR screen displayed much more severe phenotype than many genes with more significant phenotype scores. It would be helpful if the authors could provide some discussion or data to explain this. Is it possible that the Auxin-AID system exacerbates the phenotype for certain genes? Have the authors tried to make knockout (instead of knockdown) mutants for Tg202160, Tg205550 or Tg231070? The Sidik CRISPR screen essentially measured the persistence of a deleted locus in a population. A gene assigned a moderate phenotype score in that assay

(e.g. the KO mutant persists for a decent amount of time in the population) seems to be unlikely to display a severe phenotype when knocked out.

In order to be consistent with the analysis of the pooled HiT screens, we followed the output of the classifier in **Fig. 3F** when selecting which genes to plot in **Fig. 3G**. However, the reviewer keenly identifies three genes that are clearly discordant between the HiT screen and the previous gene- disruption screen. As discussed in response to Reviewer #1, the goal of mapping the previous screening results on **Fig. 3F** was to highlight discrepancies between the two approaches to the reader. We have also highlighted examples of false negatives in the Results stating that *"Out-of-frame integration of the HiT construct may be frequent for proteins rendered hypomorphic by the tag and drive known essential genes like PKG into cluster B."* We also discuss these genes in the Discussion stating that *"... 29 genes previously reported to be fitness-conferring were missed by our pooled HiT screens..."* and detailing possible sources of the discrepancy.

There are multiple possible explanations for discordant results between the two screens. For example, gRNAs used in the gene disruption screens are clustered around the start of the CDS and inaccurate gene models may not actually disrupt the gene leading to an artificially high phenotype score. As the reviewer suggests, it is also formally possible that tagging might render a protein toxic to the cell; indeed the effect that drove the three genes identified into cluster A appear to originate from a modest overall loss in guide abundance, rather than an auxin-dependent effect. Our goal with the classifier was to agnostically select genes for followup studies; however, all of the data are provided in **Table S2** for readers to directly examine genes of interest.

Reviewer #4 (Remarks to the Author):

This manuscript describes the development of a High-throughput (HiT) CRISPR-mediated screening in Toxoplasma that can be used in both arrayed and pooled formats with spatiotemporal resolution. It allows obtaining protein localization and phenotypic description simultaneously at scale. Here, the authors specifically profiled the T. gondii kinome. The authors have been able to assign several kinases that had not previously been studied to cell cycle progression. Pooled screen profiles allowed to differentiate between acute and delayed-death phenotypes. In the latter category, the authors discovered SPARK, a novel regulator of parasite invasion and egress, through the stimulation of parasite intracellular Ca2+ stores. The kinome profiling will provide a great resource to the field of Toxoplasma cell biology, but also for other Apicomplexa because many kinases are conserved in related parasites such as Plasmodium. The HiT vector system is scalable, flexible for others tags, and adapted to address

a wide variety of biological questions. The manuscript is well-written, and mostly approachable for the general reader. The experiments are carefully done and data adequately discussed. I have few minor comments.

We sincerely appreciate the reviewer's positive evaluation of our work.

Specific Comments:

1. Fig. 1A: the selection cassette (grey) and gRNA (blue) are in the opposite direction on the linearized fragment

We have adjusted the figure to maintain accurate and consistent orientation of the selection cassette.

2. It would be great to add the knockout phenotype score from Sidik et al, in the table corresponding to the pooled screen.

We have added a column containing this information to the "Pooled_Genes" tab of **Table S2**.

3. Fig. 2: Panels C and D are difficult to read with respect to the text. I suggest: 1) to show an example of each of the 9 locations presented in the scheme of figure 2C and to add the accession numbers of the proteins of each group, 2) to group the images by location in figure S2, 3) to add an IFA illustrating the location of proteins in figure 2D, in order to easily associate the location of the protein with the phenotype; and finally 4) when known to add the name of the kinase to the panel.

We have revised the figures and supplement to incorporate the reviewer's helpful suggestions. Localizations are now summarized using a representative image grouped with the gene IDs with similar subcellular distribution (**new Fig. 2B**). We have also grouped the supplementary figures by subcellular localization (**new Figs. S2–S4**). Because there isn't a perfect concordance between the genes for which we obtained localizations and those that displayed morphological phenotypes, we have not attempted to merge the results from the two assays.

5. Fig. 4C: TGGT1_239885 does not cause severe reduction in plaque size, as stated in the text line 326

To more empirically measure changes in plaque size, we quantified plaque area and calculated *p* values. By this metric, loss of TGGT1_239885 does appear to cause a reduction in plaque size, whereas TGGT1_204280 does not (**new Fig. 4D**).

6. Lines 184-186: The discontinuity in the IMC marker appears also in 'singlets I'. There are parasite morphological differences between Singlets I and II that suggest a block in the G1 phase for singlet I and growth arrest in the S/M of the cell cycle for singlet II.

We appreciate the reviewer's suggestion and have revised the Discussion to state:
"Knockdowns in each of four nuclear kinases caused arrests at the single-parasite stage, reminiscent of either a block in G1 (singlets I) or S/M (singlets II)."

7. Line-106: what is the accession number of CRK3 which is not in the text, nor in the table and is also not annotated in ToxoDB

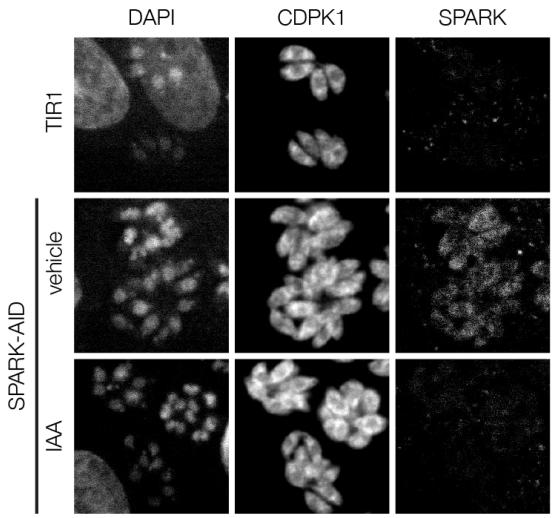
We have added the accession number of TgCRK3. The designation of TgCRK3 was given in the reference (Alvarez and Suvorova, 2017). The text now reads:
"TGGT1_249260 (TgCrk3) also displayed no defects by microscopy or lytic assay, consistent with the observation that knockdown resulted in reduced plaque size but normal morphology."

8. It is not always clear in figure 3 and text, whether the data derive from both the HiT 3'CDPK3 and HiT 3'SAG1 screens or only from HiT 3'CDPK3 screen. Please clarify.

We added clarifying language to more explicitly state when data was being derived from both screens.

9. The location of the SPARK is difficult to appreciate in Figure 5C. Better images by confocal microscopy are required here.

SPARK was difficult to detect in the tagged strains, likely due to its low expression levels—in other studies, it is hardly detectable in global proteomics. We attempted confocal imaging, but it did not improve the localization (see below), we have therefore opted to retain the original images in the manuscript.



10. 'SPARK exhibits a phenotypic profile distinct from PKG, for which knockdown or chemical inhibition cannot be rescued by Ca2+ ionophore (refs 23,47,85)'. I could not find this information in reference 47 (Jia et al) and ref 85 (Sidik et al), only in ref 23 (Brown et al)

We have revised the references accordingly.

11. To better understand the rational of using ionomycin, add in the text that it specifically mobilizes neutral calcium stores.

In response to a comment from Reviewer #1, we repeated the assays with A23187 (**new Fig. 5l**) with similar results—addition of either ionophore rescues the effects of SPARK knockdown in extracellular parasites.

12. 3'UTR and 5'UTR: often the prime ' is missing

We have scanned the text to correct any such instances.

Decision Letter, first revision:

Dear Sebastian,

Thank you for submitting your revised manuscript "High-throughput functionalization of the Toxoplasma kinome uncovers a novel regulator of invasion and egress" (NMICROBIOL-21092365A). It has now been seen by the original referees and their comments are below. The reviewers find that the paper has improved in revision, and therefore we'll be happy in principle to publish it in Nature Microbiology, pending minor revisions to satisfy the referees' final requests and to comply with our editorial and formatting guidelines.

If the current version of your manuscript is in a PDF format, please email us a copy of the file in an editable format (Microsoft Word or LaTex)-- we can not proceed with PDFs at this stage.

We are now performing detailed checks on your paper and will send you a checklist detailing our editorial and formatting requirements in about a week. Please do not upload the final materials and make any revisions until you receive this additional information from us.

Thank you again for your interest in Nature Microbiology Please do not hesitate to contact me if you have any questions.

Sincerely,
{redacted}

Reviewer #1 (Remarks to the Author):

The authors have clarified all questions. Congratulations on a nice manuscript!

Reviewer #2 (Remarks to the Author):

The authors have satisfactorily addressed my previous comments. I only spotted a couple of issues with Figure citations - Fig 2D is cited before Fig. 2C and the reference to Fig. 4D on line 319 should be to Fig. 4E.
David Horn

Reviewer #3 (Remarks to the Author):

Overall the authors have provided a comprehensive response. However, I still have some remaining questions about the pooled HiT screens. Just as the authors pointed out, the three genes I asked about in the last review, Tg202160, Tg205550, Tg231070, displayed a loss of guide abundance in the two pooled screens when mAID tagged. The relative guide abundance in fact is consistently higher in the IAA- vs vehicle-treated samples over passages. Thus the assignment of these three genes as fitness-conferring by the HiT screens is rather questionable. This suggests that in the UMAP analysis, the loss of guide abundance over passages is heavily weighted even when it is not related to IAA-treatment. This is an important caveat of the analysis, and should be pointed out and discussed.

My understanding is that for the UMAP analysis, each gene is represented in a multidimensional space with the fold changes for vehicle- and IAA-treated samples. Given the discrepancies seen for Tg202160, Tg205550, and Tg231070, wouldn't the RELATIVE fold changes of IAA- over vehicle-treatment be a better representation for the position of each gene in the fitness space?

Related, for figure 3G, the labels "acute death" and "delayed death" seem a bit misleading, as the pooled screens only assess relative fitness in a population. How about using "early loss" and "delayed loss" instead?

Reviewer #4 (Remarks to the Author):

By changing the organization of the figures, the authors have greatly improved the reading of the results, facilitating the reader to navigate in all the information obtained. The authors have answered all my concerns, only a few minor points remain.

1. Add the rational to use the SAG1 UTR as in the previous version of the manuscript: « Since the screens were performed in parallel to the analysis of UTR function, we generated libraries using the two different 3' UTRs. »

2. Lane 165 : Fig. 2C not 2D

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3. Ref 79 is now published.

4. Lane 303, add the accession number for the Plasmodium falciparum ortholog (Pf3D7_1121900) and its name (PfDK1).

5. Statistic : Fig. 4B, the numbers of biological replicates is missing.

6. Page 415 : remove rhoptry from the sentence

Decision Letter, final checks:

Dear Dr. Lourido,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Microbiology manuscript, "High-throughput functionalization of the Toxoplasma kinome uncovers a novel regulator of invasion and egress" (NMICROBIOL-21092365A). Please carefully follow the step-by-step instructions provided in the attached file, and add a response in each row of the table to indicate the changes that you have made. Please also check and comment on any additional marked-up edits we have proposed within the text. Ensuring that each point is addressed will help to ensure that your revised manuscript can be swiftly handed over to our production team.

We would like to start working on your revised paper, with all of the requested files and forms, as soon as possible (preferably within two weeks). Please get in contact with us if you anticipate delays.

When you upload your final materials, please include a point-by-point response to any remaining reviewer comments.

If you have not done so already, please alert us to any related manuscripts from your group that are under consideration or in press at other journals, or are being written up for submission to other journals (see: https://www.nature.com/nature-research/editorial-policies/plagiarism#policy-on-duplicate-publication for details).

In recognition of the time and expertise our reviewers provide to Nature Microbiology's editorial process, we would like to formally acknowledge their contribution to the external peer review of your manuscript entitled "High-throughput functionalization of the Toxoplasma kinome uncovers a novel regulator of invasion and egress". For those reviewers who give their assent, we will be publishing their names alongside the published article.

Nature Microbiology offers a Transparent Peer Review option for new original research manuscripts submitted after December 1st, 2019. As part of this initiative, we encourage our authors to support increased transparency into the peer review process by agreeing to have the reviewer comments,

author rebuttal letters, and editorial decision letters published as a Supplementary item. When you submit your final files please clearly state in your cover letter whether or not you would like to participate in this initiative. Please note that failure to state your preference will result in delays in accepting your manuscript for publication.

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Final Decision Letter:

Dear Dr Lourido,

I am pleased to accept your Article "Screening the Toxoplasma kinome with high-throughput tagging (HiT) identifies a regulator of invasion and egress" for publication in Nature Microbiology. Thank you for having chosen to submit your work to us and many congratulations.

Over the next few weeks, your paper will be copyedited to ensure that it conforms to Nature Microbiology style. We look particularly carefully at the titles of all papers to ensure that they are relatively brief and understandable.

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