Systematic functional analysis of *Leishmania* protein kinases identifies regulators of differentiation and survival

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Summary

Differentiation between distinct stages is fundamental for the life cycle of intracellular protozoan parasites and for transmission between hosts, requiring stringent spatial and temporal regulation. Here we applied kinome-wide gene deletion and gene tagging in *Leishmania mexicana* promastigotes to define protein kinases with life cycle transition roles. Whilst 162 were dispensable, 44 protein kinase genes were refractory to deletion and are likely core genes required for parasite replication. Phenotyping of pooled gene deletion mutants using bar-seq and projection pursuit clustering revealed functional phenotypic groups of protein kinases involved in differentiation from metacyclic promastigote to amastigote, growth and survival in macrophages and mice, colonisation of the sand fly and motility. This unbiased interrogation of protein kinase function in *Leishmania* allows targeted investigation of organelle-associated intrinsic and extrinsic signalling pathways required for successful intracellular parasitism.

Keywords
Leishmania, Protein kinase, CRISPR-Cas9, Differentiation

Introduction

The life cycles of many eukaryotic microbial pathogens are intricate due the requirement to transition between insect and mammalian hosts and cell type differentiation is central to their ability to adapt to different environments [1]. Some parasitic protozoa undergo cell cycle arrest in response to autocrine signals in their host. These arrested cells can undergo differentiation in response to environmental cues to produce a distinct cell type that can proliferate. Little is known about the molecular mechanisms that underpin these events, although recent advances have been made in the malaria parasite *Plasmodium* [2–4] and the African trypanosome *Trypanosoma brucei* implicating protein kinase signalling pathways [5,6]. As models to investigate life cycle progression *Plasmodium* is relevant for its mosquito transmission and intra-erythrocytic replication, whilst *Trypanosoma brucei* is pertinent for its transmission via the tsetse fly and extracellular replication. *Leishmania,* a kinetoplastid parasitic protozoa that is the causative agent of leishmaniasis, also has a complex life cycle with a requirement to replicate and differentiate in both mammalian and sand fly (Diptera: Psychodidae) hosts. The parasite is transmitted during the vector blood meal on an infected vertebrate with the ingestion of infected cells or amastigote forms that have reached the extracellular milieu in the mammalian host. In the vector midgut, the protozoa are enclosed in the peritrophic matrix, in contact with the blood to be digested, and differentiate into procyclic promastigotes. After the blood digestion, the parasites attach to midgut epithelium, replicate, differentiate and migrate anteriorly to reach the cardia of the sand fly to be transmitted to the mammalian host via a new blood meal. The infective stage is the metacyclic promastigote, adapted to migration, transmission and invasion [7,8]. In the mammalian host *Leishmania* has adapted to survive and proliferate in the acidic environment of the parasitophorous vacuole within phagocytes. *In vitro* systems are available for most life cycle stages of *Leishmania*, although these models do not completely reflect the natural environment and may not activate some of the intrinsic pathways required for normal differentiation.

Phosphorylation mediated signal transduction in eukaryotes is a key regulator of protein function and likely plays a pivotal role in *Leishmania* differentiation considering the changes reported for protein phosphorylation in different life cycle stages [9]. *Leishmania* has 195 eukaryotic protein kinases (ePKs) divided in six groups, classified according to catalytic domain conservation (CMGC, AGC, CAMK, STE, NEK, CK1), and an additional group here classified as 'Others', which contains diverse important eukaryotic cell signal transduction functions such as Aurora, Polo and AMP kinases. Atypical protein kinases (aPKs) lacking a conserved ePK structure but otherwise

catalytically active are present in *Leishmania* that harbors, for example, 11 phosphatidylinositol 3' kinase-related kinases (PIKK) resembling lipid kinases [10]. *Leishmania*'s kinome is greatly reduced in comparison to the human equivalent, lacking tyrosine kinases (TK) and tyrosine kinase-like (TKL) groups while CMGC, STE and NEK groups are significantly expanded.

A number of studies have explored the role of individual *Leishmania* protein kinases in promastigote survival, metacyclic differentiation and amastigote replication, as well as their role in infectivity. Less than 10% of the kinome, mostly from the CMGC group, have been investigated using genetic and chemical approaches [11–28]. There are 17 mitogen activated protein kinases (MAPK) in *Leishmania* [29] and they were the focus of most of those studies, which highlighted the roles of MPK9 and MPK3 on flagellum maintenance, the latter being phosphorylated by MKK1 [12,13,21]. Another example is MPK2, previously demonstrated to modulate aquaglyceroporin 1 (AQP1) and amino acid transporter (APP3), proteins involved in drug resistance and the arginine depletion response, respectively, and therefore intra-macrophage amastigote survival [18,19]. Amastigote growth suppression and persistence was observed in TOR3 and MAPK4 null mutants [15,22], MPK10 has been implicated in amastigote differentiation, and CRK1 and CRK3 in cell cycle control and amastigote survival [11,24,30]. Only DYRK1 was shown to be essential for differentiation to metacyclic promastigotes [28], and there is a gap of knowledge on the role of protein kinases in differentiation from amastigotes to promastigotes and survival in the sand fly.

Here, we systematically tagged protein kinases with mNeonGreen fluorescent protein for localisation studies, and attempted to create individual null mutants for *Leishmania mexicana* ePKs and PIKK using CRISPR-Cas9. We performed pooled infections and assessed the relative proportion of barcodes representing 159 *Leishmania* protein kinase mutants to establish their importance in survival, differentiation and ultimately infection success *in vitro* and *in vivo* in the invertebrate and vertebrate hosts.

Results

Generation of gene deletion mutants identify non-essential protein kinases in promastigotes

Leishmania mexicana eukaryotic protein kinases (ePK) and PIKK family atypical protein kinases (aPKs) gene sequences were retrieved from TriTrypDB, using *L. major*, *Trypanosoma brucei* and *Trypanosoma cruzi* gene ID's reported previously [10]. We investigated 195 *L. mexicana* ePKs distributed into 8 groups and 11 aPK PIKKs (the kinome; Fig. 1a; Supplementary Table 1). We also

identified protein kinases with orthologs and paralogs across a selection of trypanosomatids, including 27 found only in the combined *Leishmaniinae* family [31] and *Blechomonas ayalai* and 17 *Leishmaniinae* unique kinases (LUKs), represented across several kinase groups (1 AGC, 2 CK1, 3 CMGC, 3 STE, 1 NEK and 7 grouped as non-related 'Others'; Fig. 1b, 1c).

The requirement for these protein kinases in promastigote survival was assessed by the attempted generation of gene deletion lines using CRISPR-Cas9. Briefly, two repair templates containing drug resistance markers (*BSD* and *PUR*) were amplified using long primers with 30bp homology arms containing a unique 12bp barcode and two guides [32]. The repair templates and guides were transfected into *L. mexicana* procyclic promastigotes expressing CAS9 and T7 RNA polymerase (Fig. 2a). PCR amplification was used to determine the presence of the coding sequence (CDS) and integration of the drug resistance cassette (Fig. 2b, Supplementary Data 1). PCR confirmation of an exemplar gene deletion mutant, Δ*25.0853*, showed a PCR product for integration of the drug resistance cassette in the specific locus, and an absence of the CDS (Fig 2bi). In contrast, when a gene deletion mutant could not be generated, for example with *PKAc1,* either no populations were recovered after transfection, or cell lines had both a CDS and an integration PCR product (Fig. 2bii). This may be due to gene or chromosome duplication, which is a common mechanism by which *Leishmania* retains an essential gene following attempts at generating gene deletion mutants [33,34]. Whole genome sequencing of 18 mutant cell lines (15 gene deletion mutants and three that retained an additional CDS) confirmed loss of reads over the CDS of interest and presence of resistance markers in this location (Fig. 2c). If a gene deletion mutant was unable to be identified after three transfection attempts, it was classed as 'essential' with a 1 star quality classification [35]. Forty four protein kinases fell into this category (Fig. 1a, 1c).

Gene deletion mutants were successfully produced for 162 protein kinases, equating to 79% of the kinome. Only 2/22 (9%) NEKs were found to be refractory to gene deletion compared to 13/35 (27%) CMCGs and 4/11 AGCs (36%) (Fig.1c). Gene deletion mutants were obtained for all 11 pseudokinases, which lack all three of the key conserved residues thought to be important for catalytic activity (Supplementary Fig. 1) [10], demonstrating no essential requirement in promastigotes. A further 40 protein kinases were found to lack either one or two of these residues and gene deletion mutants could be generated for 33/40 (82.5%). As previous work showed that it is possible to mutate the lysine residue and retain some ATP binding activity [36], we have classified these protein kinases as putatively active.

The essentiality of protein kinases was considered in relation to orthology across 10 trypanosomatid species. Of the 174 kinases represented across all of these species, 21% were deemed essential in this study. This percentage increases to 30% of the protein kinases found in the *Leishmaniinae* plus *Blechomonas* group and 41% when considering the 17 LUKs alone (Fig. 1b). This suggests that *Leishmania* promastigotes require these unique kinases, specifically for their life cycle adaptations. Of note, two of the eleven non-essential pseudokinases are LUKs and one pseudokinase is found in the *Leishmaniinae* plus *Blechomonas* grouping.

Our gene deletion attempts indicate that 44 of the protein kinases are essential in promastigotes. For additional validation, we generated gene deletions for both endogenous alleles of *LmxM.04.0650* whilst providing an extrachromosomal copy of the gene. Δ*04.0650* [*6xmyc-04.0650*] (Fig. 2d) retained the episome for at least seven generations without drug pressure. In some instances genes were refractory to the anticipated genetic manipulation and in the case of *CLK1* (*LmxM.09.0400*) and *CLK2* (*LmxM.09.0410*) this was found to be due to incorrect assembly of the reference genome. A recent nanopore genome allowed design of new primers and the successful generation of Δ*clk1* and Δ*clk2* gene deletion mutants, suggesting that disruption to one of the two *CLK* genes can be compensated for by the other (Supplementary Fig. 2).

Localisation of protein kinases

We generated 199 N- or C-terminal mNeonGreen tagged protein kinases for localisation studies in procyclic promastigotes (Fig. 3a). We used the atlas of *Leishmania* cellular landmarks [37] to localise proteins to the cytoplasm (48.5%), basal body (11.6%), nucleus (10.1%), endomembrane (7.1%), flagellum (6.5%), lysosome (6.5%), flagellar pocket (3%), pellicular membrane (2.5%), cytoplasmic organelles (2%) and mitochondrion (1%) (Fig. 3b,c, Supplementary Data 2 and Supplementary Table 1). The fluorescence signal for some protein kinases varied during the cell cycle, for example PKAC1 accumulates in the nucleus in early G1/S and at late G2/M in the cytoplasm and flagellar cytoplasm. This also holds true for MPK6 (LmxM.31.3250) which is localised within the cytoplasm and flagellar in G1 but is located on the pellicular membrane during S phase. In other cases protein kinases were found in more than one localisation, for example LmxM.14.1070 was found in the basal body, flagellar pocket, lysosome and cytoplasm (Supplementary Data 2).

Protein kinases required for differentiation and survival in the mammalian host

In order to ascertain the role of protein kinases in differentiation across various life cycle stages, 159 protein kinase barcoded mutants (154 gene deletion mutants, five mutants retaining an extra CDS), including one non-protein kinase control (Δ*mca*), were pooled and subjected to *Leishmania* life cycle progression. We tested the ability of mutants to transition through the *Leishmania* life cycle as procyclic promastigotes (PRO), metacyclic promastigotes (META), axenic amastigotes (AXA), amastigotes in macrophages (inMAC) and amastigotes in the footpads of mice (FP), taking samples at various time points for bar-seq analysis[34] (Fig 4a). The relative growth rate of each mutant was determined by counting barcodes represented in each time point and calculating the proportion of each mutant within the population. Log-proportions were plotted as trajectories across time points for each experiment. Outputs from experimental arms 1-3 were plotted for direct comparison against one another for six example genes that had no loss of fitness, an increased relative fitness or a decreased relative fitness (Fig. 4b). The eukaryotic initiation factor kinase mutant, Δ*eif2ak3* showed a strong increase in representation for axenic amastigotes, macrophage infection and infection of mice, but may not represent gain-of-fitness due to decrease in the relative abundance of mutants that have a loss of fitness. The mitogen-activated protein kinase kinase 3 (Δ*mpkk3*) and cdc2-related kinase 10 (Δ*crk10*) mutants maintained an average level of representation across all timepoints in all three experiments. This was in contrast to the mitogen-activated protein kinase gene deletion mutants Δ*mpk1* and Δ*mpk2,* which both demonstrated a loss of fitness in both axenic and intracellular amastigote stages, a phenotype that has been reported previously [18,21,38]. Our results agree with those findings and also showed that loss of fitness is seen in the mouse footpad infection. The zinc finger kinase mutant (Δ*zfk*) also displayed a loss of fitness across the three experimental arms, but with an increased representation upon enrichment of metacyclic promastigotes. Although each barcode proportion profile has been examined individually (Supplementary Data 3 c,f,i), we opted to use an unbiased clustering algorithm to sort the mutants into groups with similar phenotypes, taking all time points into account. We used the projection pursuit method [39] to calculate the differences between the trajectories of logged-proportions, looking at both the first and second differences between each time point within the series, for each of the mutant strains. This allowed qualitative similarities and differences between trajectories to be assessed in an unbiased way. After examining multiple solutions, we set our parameters to produce six clusters for each of the experimental arms (EA1-EA3) (Supplementary Table 2). Axenic amastigote trajectories allowed for identification of mutants where differentiation and replication were impaired, without interference by host factors, which can be interpreted as parasite-independent signalling phenotypes (Supplementary Data 3). Macrophage infections *in vitro* were used for identification of mutants for genes important during infection, either differentiation and adaptation to the acidic environment of the phagolysosome or replication inside

it. The last experimental setup for *in vivo* infections took into account the mouse immune system, able to eliminate mutants less able to survive in this environment. The mouse footpad infection experiment, examining the first differences in the series, was therefore chosen as an example of the cluster analyses (Fig. 4c). Cluster 1 (green) contains 12 trajectories for mutants that exhibited an increased representation during the mouse infection time points compared to others, indicating a relative gain of fitness in the population. Cluster 2 (red) contains trajectories with an average representation throughout promastigote stages and small increase between the metacyclic enrichment and three week footpad infection stage. Cluster 3 (sky blue) contains the largest group of protein kinase mutants with fairly flat trajectories, decreasing slightly on average from the point of metacyclic enrichment. Cluster 4 (purple) contains protein kinase mutants that on average exhibit fairly flat trajectories throughout the promastigote stages, demonstrating average growth, with a decrease in fitness upon footpad infection. Cluster 5 (blue) contains mutants that show a significant increase in representation during metacyclic enrichment followed by a sharp decrease during footpad infection time points. Cluster 6 (grey) contains protein kinase mutants with trajectories which show a general loss of representation over time with a slight recovery during metacyclic enrichment. Each of the individual kinase trajectory examples (Fig 4b, Supplementary Data 3) are found in separate clusters and provide an example of the data for the axenic amastigotes and macrophage infections. The percentage barcode representations for each of the mutants are also shown in a heat map format for each of the clusters, depicting the trends for individual mutants. PCA analysis supports the relationship between the different clusters (Fig. 4d, Supplementary Data 3).

We had identified previously a STE protein kinase in *T. brucei*, RDK1, as a repressor of bloodstream form differentiation (Jones et al., 2014) and the *L. mexicana* syntenic ortholog fell into cluster 3 (Fig. 4b). We analysed the *L. mexicana* Cas9 T7 progenitor line, Δ*rdk1* mutant and an add-back Δ*rdk1::RDK1* for life cycle progression and found that Δ*rdk1* had the same proliferation profile as the progenitor promastigotes in culture, but a loss of fitness of ~40% after 72 h in the *in vitro* macrophage assay and a loss of fitness in mice that was complemented with the add-back (Supplementary Fig. 3a-c). Furthermore, Δ*rdk1* mutants had accelerated differentiation from amastigotes to promastigotes, as assessed by expression of HASPB, an amastigote specific protein (Supplementary Fig. 3d) [40]. This analysis confirms the bar-seq results, provides confidence in the pooled library data, and identifies RDK1 as a *Leishmania* repressor of differentiation.

Protein kinases important for differentiation from promastigotes to amastigotes

We took advantage of our three experimental arms for cross validation of mutants showing loss of fitness in amastigote infection, differentiation and/or replication. Clusters that identified protein kinases important for the amastigote stages were compared across the three arms for both first and second difference outputs from the projection pursuit analysis. Twenty nine protein kinase mutants were identified as important for amastigotes across at least two of the experimental arms (EA1-3) (Fig. 5a, Supplementary Table 2). These included MPK1 and MPK2 and six other protein kinase mutants that had been identified previously as being important for *in vivo* infection of *T. brucei* [41], as well as TOR3, previously identified as required for macrophage infections [22]. A further 15 protein kinases were identified with loss of fitness only in the mouse footpad infection (Supplementary Table 2), suggesting that these mutants can differentiate into amastigotes successfully and infect macrophages but cannot survive in the harsh *in vivo* environment.

Protein kinases required for survival in the sand fly.

We next addressed the ability of gene deficient mutants to survive in the sand fly vector *Lutzomyia longipalpis*. Mutants were separated into three sub-pools based on their relative growth rates of slow, average or fast. Each pool of ~50 procyclic promastigote mutants had four additional control lines [42], where a barcode was added to the ribosomal locus (Supplementary Table 1, Supplementary Fig. 4). *L. longipalpis* sand flies were allowed to feed on blood containing the three pools, then bar-seq analyses were carried out on DNA samples from day 0, 1, 5 and 8 post blood meal (Supplementary Fig. 4, Supplementary Table 2). Fifteen protein kinase mutants were identified that had a significant loss of representation after 8 days of infection (p = <0.05), whilst the four controls maintained an average ratio throughout (Fig 5b, Supplementary Table 2).

The two *in vivo* screens provided a list of protein kinases important for successful infection of mice and colonisation of the sand fly midgut. Seven protein kinases were identified as being required in both screens, indicating that they play fundamental roles and are required for differentiation and/or survival in both the insect vector and mammalian host (Fig. 5b). This list includes MPK1, MPK2, CRK7 and LmxM.06.0640, a MAP3K. Eight protein kinases, including MPK9, ATM, AMPKb and PI4K were identified as important for colonisation of the sand fly only, as these cluster with average trajectories in the amastigote study (Fig. 5c). Sixteen protein kinases were identified as being required for differentiation and/or survival in the mammalian host only (Fig. 5d), as these show no significant decrease in representation by day 8 post blood meal in the sand fly. These include CMGC family protein kinases MAPK10, MAPK15 and CRK8, as well as PKAC1, PK4 and TOR3.

Protein kinases involved in flagellum function.

We hypothesised that protein kinase mutants that had defective motility as promastigotes would have a loss of fitness in the sand fly [42], but not in the mouse. To test this we screened for non-motile mutants in a transwell assay, identifying four mutants, Δ*ulk1*, Δ*mpkk2*, Δ*02.0570* and Δ*29.0600* with significantly shortened flagella (Fig. 6a, Supplementary Fig. 5) and severely impaired motility (Fig. 6b, Supplementary Movie 1), as assessed by 2D tracking. The few motile cells present in these populations of mutants showed slow swim rates compared to the parental line (Supplementary Fig. 5). Re-expression of *ULK1* in Δ*ulk1* and *MPKK2* in Δ*mpkk2* restored the flagellum length to that of the parental line (Supplementary Fig. 6). ULK1 and MPKK2 share not only similar domain architecture with an N-terminal kinase domain and a number of ARM domains (Fig. 6c) but both are located at the flagellar tip (Fig. 6d, Supplementary Fig. 6). LmxM.02.0570 is one of only five *Leishmania* protein kinases with a predicted transmembrane domain and it was localised to both the lysosome and flagellum, a location found for just two other protein kinases in our dataset (LmxM.26.1730 and LmxM.36.1530). The trajectories for mouse infection for Δ*mpkk2* and Δ*ulk1* follow a similar trend showing increased representation after enrichment of metacyclic promastigotes and decreasing representation during mouse infection and hence group with protein kinases important for amastigote differentiation and survival in clusters 5 and 6 respectively (Fig. 6e). LmxM.29.0600 and LmxM.02.0570 on the other hand are found in clusters 1 and 2 respectively for the mouse footpad infection due to the former exhibiting a small increase in fitness and the latter a general but less significant decrease. Interestingly, MPKK2 and ULK were also identified as important protein kinases for survival in the sand fly, whereas MPK3, LmxM.29.0600 and LmxM.02.0570 were not (Fig. 6f). This suggests that these proteins are fundamental to infection for a reason independent of their short flagellum and provides evidence that not all mutants with flagellar defects as promastigotes are hindered in colonisation of the sand fly.

Discussion

Protein kinases are fundamental to orchestrating the cellular signalling required for *Leishmania* survival throughout the life cycle. Here we have taken a holistic approach to exploring the role of each protein kinase in the parasite's life cycle, first assessing whether each gene is dispensable for growth as promastigotes and then using pooled library bar-seq phenotyping to assess the ability of gene deletion mutants to progress through the life cycle. We also determined the location of each protein kinase in promastigote stage cells, allowing generation of hypotheses for potential signalling pathways associated with specific organelles. Gene deletion mutants were successfully generated for 162 of the protein kinases in promastigotes, equating to 79% of the kinome, indicating a high level of

functional redundancy. This result is consistent with the level of essentiality for the kinome also seen using RNAi in bloodstream form trypanosomes [43]. In agreement with previous studies, we were unable to generate gene deletion mutants for six protein kinases previously characterised as being essential for promastigotes (CRK1, CRK3, CRK12, MPK4, TOR1 and TOR2) [14,22,30,44,45]. We were however able to successfully generate a gene deletion mutant for LmxM.01.0750 and LmxM.32.1710, previously published as essential in *L. major* and *L. infantum* promastigotes respectively [17,23] and failed to isolate a gene deletion mutant for DYRK1, despite this being achieved in *L. infantum* [28]. These may be due to different gene deletion approaches taken allowing time for compensatory mutations or differences between the species. When we consider essentiality within the different protein kinase families, the AGC and CMGC kinases host a high proportion of essential genes. This is perhaps anticipated given that AGC protein kinases mediate diverse and vital cellular functions in eukaryotes and mutations in AGC protein kinases are associated with human disease such as cancer [46]. In addition CMGC protein kinases include the cyclin dependent kinases and MAP kinases, which are fundamental to cell cycle progression [47]. In comparison the NEK protein kinases, which have an expanded family in *Leishmania* [10] are relatively dispensable in *Leishmania* promastigotes with 18/20 gene deletion mutants successfully generated. We noted that protein kinases located in some organelles were more likely to have essential functions than in others, for example, 9/20 (45%) in the nucleus were found to be essential; many of these are CDKs (CRK9, CRK11, CRK12) or other protein kinases likely to be involved in essential nuclear functions such as cell cycle control and transcription (KKT2, KKT3, AUK1, TLK, PKAC2b, LmxM.24.0670)[43,48,49]. In contrast, as the flagellum is not essential for promastigote growth *in vitro* [13,42], it is not surprising that only 2/13 (15%) protein kinases found in the flagellum are essential. The basal body is another organelle whose correct duplication and segregation is crucial for cell division [50,51] yet, surprisingly, only 2/23 (9%) of protein kinases localising to the basal body are essential (PLK and LmxM.14.0830).

In order to pool our library of protein kinase gene deletion mutants, and test requirements through life cycle stages, we utilised bar-seq, a powerful technique previously used for genome-wide screens in multiple human cell types, as well as parasites such as *Toxoplasma* and *Plasmodium* [52,53,54]. This approach has now been applied to a small group of *Leishmania* flagellar proteins [42], ubiquitination pathway enzymes [34,55] and the protein kinases of this study. Whilst the plot of barcode proportions as trajectories across experimental time points provided information on life cycle progression for individual mutants, projection pursuit cluster analyses provided a non-biased way to group the experimental outcomes into clusters of mutants with comparable trends. The largest proportion of

mutants were clustered with flat trajectories, indicating no change in fitness and whilst it may be that many of these protein kinases are not important for differentiation or survival, other possible explanations include redundancy of protein kinases regulating the same pathways or their roles in other mechanisms are not related to differentiation, replication and survival. For example, Δ*clk1* and Δ*clk2* promastigotes are viable, however, double *CLK1*/*CLK2* gene deletion mutants could not be isolated, indicating that they might have redundant functions in regulating the kinetochore, as has been described in trypanosomes [48,56]. Five protein kinases have signal peptides and two of the casein kinase family, CK1.4 and CK1.2 are secreted [26,57] and potential virulence factors, however, they are essential in promastigotes and therefore were not present in the pooled libraries. The loss of fitness phenotype for gene deletion mutants of secreted protein kinases may also be masked if they are secreted by other mutants in the pool, still influencing the host-parasite interaction and immune response.

Successful infection of the mouse was the most challenging phenotype assessed for the gene deletion mutants, as the cells required the ability to differentiate from promastigote to amastigote, infect and survive within the macrophage, as well as withstand the host immune response. Using cluster analysis we identified 29 genes required across at least two of the differentiation specific experimental arms and a further 15 only required for infection of the mouse. Using these data protein kinases were identified that are part of intrinsic pathways required for intracellular cell growth as amastigotes and also protein kinases that act in extrinsic signalling pathways influencing host-parasite interaction. For example, CRK4 is required for infection of the mouse, but is not required for axenic amastigote growth, suggesting an extrinsic role. On the other hand, MPK10 clusters as important across all three experimental arms implying an intrinsic requirement, supported by research showing it to have stage-specific activity important in promastigote and amastigote differentiation [11]. A careful consideration is required when interpreting the data from enriched metacyclic promastigote cells, where a cluster of protein kinases was identified that have an increased trajectory, yet has a loss of fitness during infection of macrophages or mice. Whilst some mutants may become *bona fide* metacyclic promastigotes yet not infect macrophages, it is also possible that enrichment occurred for repressors of differentiation mutants, which have made an early transition towards amastigote morphology and alternative life cycle stages such as haptomonads. An example is *RDK1,* a regulator of amastigote to promastigote differentiation, and a homologue of *T. brucei* RDK1, which has previously been reported to be involved in differentiation of the bloodstream to procyclic form [43]. Additionally, a mutant that had an impaired proliferation rate as a promastigote would not reach stationary phase by day 7, leading to less infective metacyclic

forms present in the pooled culture. AMPKa is an example of this, with a consistent loss of fitness throughout the procyclic promastigote stages and metacyclic enrichment, leading to poor representation in macrophage and mouse infections. Phenotyping of selected individual mutants, rather than a pool, will allow more detailed analyses.

Differentiation in the sand fly is far more complex than that observed in *in vitro* tissue culture and involves procyclic promastigote leptomonad, haptomonad, retro-leptomonad and metacyclic promastigote stages [58]. Fifteen protein kinases were required for successful colonisation of the sand fly midgut and seven of these were also required in the amastigote stage, suggesting a stage-independent role in growth and differentiation. LmxM.25.1560, a cytoplasmic CMGC kinase, is notable in being both a likely pseudokinase and the only *Leishmaniinae* unique kinase (LUK) where the mutant had a phenotype. Eight protein kinases, including the aPK ATM (LmxM.02.0120), recently associated with a novel DNA damage-induced checkpoint in *Leishmania* [59], were identified as dispensable for full life cycle progression in the mouse but found to be important for development in the sand fly.

The transwell screen identified four mutants that had a short flagellum with severely defective motility, and allowed a comparison between the phenotypes *in vitro* and in the sand fly. Motility is required for survival in the sand fly [42] and indeed we found that Δ*ulk1* and Δ*mpkk2* had loss of fitness for sand fly colonisation, as anticipated. In contrast, Δ*29.0600* and Δ*02.0570* were non-motile yet were able to colonise the sand fly and did not diminish even after defecation of the blood meal. Further investigation will be required to determine if the short flagellum provides sufficient binding capacity for attachment to epithelium and colonisation of the midgut or that the non-motile mutants associate with others in the pool. The data on ULK1 and MPKK2 provide an example of how localisation and life cycle progression data can be used for signalling pathway hypothesis generation. ULK and MPKK2 have very similar infection profiles, localisation and their orthologues were also identified in a flagellar attachment proteome in *T. brucei* [60] providing further evidence that they act together in *Leishmania* and other trypanosomatids.

In summary, we have identified fifteen protein kinases that are required for colonisation of the sandfly, eight of which play an intrinsic role and seven of which are extrinsic kinases required for survival in the sandfly only. We also identified twenty nine protein kinases that are required for successful differentiation from metacyclic promastigote to amastigote and survival in macrophages and the mouse (Fig. 7). A large databank of information is now available for each of the protein kinases that make up the *Leishmania* kinome, providing an opportunity to decipher signalling

pathways and link protein kinases involved in regulating important aspects of the parasite's biology such as replication, differentiation and responses to stress. Perhaps more importantly, these data present an opportunity to characterise and focus experimental effort on those pathways which are divergent to humans, and which potentially can be targeted in new drug and vaccine therapies.

Methods

Cell culture *Leishmania mexicana* (MNYC/BZ/62/M379) promastigotes were routinely cultivated in HOMEM medium (modified Eagle's medium) supplemented with 10% (v/v) heat-inactivated fetal calf serum (HIFCS – Gibco) and 1% Penicillin/Streptomycin solution (Sigma-Aldrich) at 25°C. For differentiation assays promastigotes were grown in Grace's insect medium (Sigma-Aldrich) with 10% HIFCS (Gibco), 1% Penicillin/Streptomycin solution (Sigma-Aldrich) and 1% BME vitamins (Sigma), pH5.5 at 25°C. Parasites were subcultured at 1.5 x 10^5 parasites mL^{-1}, reaching mid-log phase at 72 h with approximately 5 x 10^6 parasites mL^{-1}, and stationary-phase at 168 h with approximately 2 x 10^7 parasites mL^{-1}. Axenic amastigote differentiation and proliferation was achieved by incubating log-phase promastigotes in Schneider's *Drosophila* Medium (Sigma-Aldrich) supplemented with 150 µg mL^{-1} of Hemin and 20% HIFCS at the concentration of 1 x 10^6 parasites mL^{-1} for 120 h at 35°C, 5% CO_2. Axenic amastigotes were subcultured by transferring cells into fresh medium at the same initial concentration. Bone marrow-derived macrophages (BMDM) were differentiated using committed myeloid progenitors isolated from BALB/c mice and incubated with macrophage colony-stimulating factor secreted by L929 cells as previously described [61]. Macrophages were infected with metacyclic promastigotes isolated by centrifugation over Ficoll 400 (Sigma-Aldrich) gradient [62] and quantified on a hemocytometer. Metacyclic promastigotes were incubated with BMDM (1:1) for 4 h and washed before incubation at 35°C and 5% CO_2 for 12h and 72 h in RPMI supplemented with 10% HIFCS and 1% Penicillin/Streptomycin solution.

Generation of the CRISPR-Cas9 mutants and bar-seq screens The protein kinase gene deletion library and N- or C-terminal tagged cell lines were generated and validated as described [34] using http://leishgedit.net/ to design primers in the *L. mexicana* Cas9 T7 line [32]. Primers used to generate sgRNA expression cassettes and donor cassettes, as well as the ones used for diagnostic PCRs are available in Supplemental table 1.

Life cycle phenotyping of gene deletion mutants *in vitro* and *in vivo* was carried out as described [34] but with the following modifications. Mid-log phase promastigote mutants were grown separately and mixed in equal proportions in six replicates (4 x 10^4 mL^{-1} for each cell line) into Grace's media supplemented with 20% of FCS, 1% Penicillin/Streptomycin solution and 1% BME vitamins, pH5.5 at 25°C. Samples were collected at 0 h, 24 h, 48 h, 72 h and 168 h. Axenic amastigotes were differentiated from 168 h promastigote cultures and grown for 120 h. Metacyclic promastigotes from six independent 168 h promastigote cultures were purified from Ficoll 400 gradients and samples were collected for time point 0 h. These purified metacyclic promastigotes were used for infection of BMDM at a 1:1 ratio (samples collected at 12 h and 72 h) and 12 mice were also infected with 10^6 parasites in the left footpad (two animals for each replicate, and time points collected at 3 and 6 weeks). DNA was extracted using DNeasy Blood & Tissue (Quiagen) following manufacturer's instructions for animal cells (promastigotes, axenic amastigotes and macrophage infections) or animal tissue (mice lesions).

For *Lutzomyia longipalpis* infection, promastigote growth data collected in experimental arms 1-3 at 72 h was ranked according to barcode representation and split into Fast, Average and Slow groups accordingly (Supplementary Table 1). Three pools of protein kinase mutants plus controls were mixed in equal proportions using mid-log procyclic promastigotes. For this experiment, three heterozygous protein kinase mutants were removed and an additional five protein kinase gene deletion mutants that had not been included in the mammalian infection screen were added (LmxM.03.0210, LmxM.29.3580, LmxM.29.3050, LmxM.20.1340 and LmxM.36.2630; Supplementary Table 1). The pool final density was counted and 50 ml cultures were set up at 2 x 10^5 cells ml^{-1} in HOMEM media. At 72 h, cells were washed three times in PBS and resuspended in defibrinated heat-inactivated sheep blood at a concentration of 2 x 10^7 promastigotes mL^{-1} [63]. The *L. longipalpis* colony was maintained at 26°C and high humidity on 50% sucrose solution and a 12 h light/12 h dark photoperiod, as described previously [64]. The three pools were each fed to a cage of 200 female flies between 3-5 d old, through a chick skin membrane. Each fly took ~300 of each mutant in the library. DNA samples were prepared from the pools prior to sand fly feeding (72 h after pooling, referred to as Time 0) and also from duplicate sets of 25 whole sand flies taken at 1, 5 and 8 d post blood meal. DNA was extracted using a Tissue Lysis Buffer and PCR template preparation kit (Roche).

Preparation of DNA, PCR and sequencing of barcodes was performed as described previously [34]. Amplification of the barcodes was carried out using F primer OL8684 (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAgatgatgattacTAATACGACTCACTATA ACTGGAAG) and R primer OL11799 (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACACTCGTTTTCATCCGgcag). Q5 polymerase was used to amplify the barcodes in a 20 ul reaction containing 100 ng of DNA, with 28 cycles of amplification, 60°C annealing temperature and 10 s extension time. Nextera adapters were then added to the resulting DNA before being analysed on an Illumina HiSeq 3000. A Python script was used to search each Illumina read for the 12bp sequence preceding each barcode. Total counts for each unique barcode sequence were generated by summing the counts of the following unique 12bp sequences per sample. Total counts for each sample, including replicates are in Supplemental table 2.

Animal work All experiments were conducted according to the Animals (Scientific Procedures) Act of 1986, United Kingdom, and had approval from the University of York Animal Welfare and Ethical Review Body (AWERB) committee. To assess virulence of the *Leishmania mexicana* Cas9 T7 progenitor line, *Δrdk1* mutant and an add-back *Δrdk1::RDK1* groups of 5 female BALB/c mice (4-6 weeks) were infected subcutaneously at the left footpad with 1 x 10^6 purified metacyclic promastigotes. Animals were culled after 6 weeks and the parasite burden determined with a limiting dilution assay essentially as described previously [65]. Footpads were digested with 4 mg ml^{-1} collagenase D for 2 h at 37°C before dissociation through 70 µm cell strainers (BD Biosciences). Homogenates were resuspended in HOMEM supplemented with 20% FCS and serial dilutions (2-fold) performed in 96-well clear flat-bottom plates. Dilutions were performed in duplicate, distributed in at least 4 plates and incubated for 7-10 days at 25°C. Wells were analysed for the presence of parasites, and number of parasites calculated by multiplying the dilution factors. Results are shown as total parasites per tissue. Two-tailed unpaired *t*-test was performed as a statistical analysis.

Bar-seq statistical analyses For the purposes of clustering, we characterised mutant phenotypes in terms of the changes over time of the logged-proportion of each mutant subpopulation relative to the total population. For each mutant these changes constitute a time series to which we apply a differencing operator that removes the time series' average heights and slopes so that attention is

instead focused on their curvature. The differenced times series are then treated as high-dimensional Euclidean vectors. These vectors are clustered using a Projection Pursuit Clustering algorithm, adapted from [39] and coded up in R (R: The R Project for Statistical Computing, n.d.). The algorithm, for which pseudo-code is provided in Supplementary Equation 1, involves iteratively projecting and re-clustering the vectors. This method is used to find a plottable two dimensional space in which the phenotype vectors are maximally separated. Once projected into this space, proposed clustering solutions for the vectors can be scrutinised and checked against prior knowledge of genetic characteristics. Such scrutinising led to the decision to choose a 6-cluster solution from the algorithm, whose output is illustrated in Supplementary Data 3.

Whole genome sequencing of protein kinase mutants Sequence reads generated from an Illumina HiSeq 3000 were trimmed with Cutadapt version 2.5 [66] and mapped to the *L. mexicana* Cas9 T7 genome assembly [67] and resistance cassettes with BWA-MEM algorithm as part of bwa version 0.7.17 [68]. Mosdepth 0.2.6 [69] was used to calculate the median coverage of each chromosome and scaffold in the assembly, as well as the mean coverage of each gene of interest. The ratio of gene coverage to chromosome coverage was used to assess gene deletion.

DNA cloning Expression of protein kinases either for facilitated gene deletion mutant or addback generation was achieved by using the pNUS system [70] modified for expression with a C-terminal 6xMYC fusion. Gibson Assembly (NEBuilder® HiFi DNA Assembly) was used. The pNUS backbone plasmid was digested using AvrII and gel extracted (Qiagen). The required CDS was amplified from *L. mexicana* genomic DNA using primers designed on NEBuilder assembly online tool, with a 5' 20 nt overlap with the pNUS backbone. Fragments were purified (Qiagen) and Gibson Assembly was performed as suggested by the manufacturer and transformed into *E. coli* DH5α chemo competent.

Live cell imaging Endogenous tagged cell lines in log-phase were centrifuged at 1000 x *g* for 10 min, washed in phosphate saline buffer pH 7.2 (PBS) and re-suspended in 10 μg mL^{-1} of Hoechst 33342 (Thermo Scientific) before incubation for 15 min at room temperature protected from light. To remove Hoescht 33342 the cells were washed with PBS, re-suspended and immobilised in 50 μL of ice-chilled CyGEL™ (Biostatus). Samples were transferred to slides, covered with a coverslip and immediately imaged using a Zeiss LSM880 confocal microscope, with 488 and 405 nm lasers, at room temperature. Images were processed using Zen Black (Zeiss).

Western blotting Cells were re-suspended in NuPAGE sample buffer supplemented with 50 mM of dithiothreitol (DTT) and loaded on 4-12% NuPAGE Bis-Tris Protein Gels and electrophoresed at 180V for 1 h. Gels were transferred to Nitrocellulose membranes using wet transfer system XCell II Blot Module (Invitrogen) at a constant amperage of 350 mA for 2 h. Membranes were blocked with 5% skimmed milk in Tris Buffered saline-Tween for 1 h before incubation with primary antibody anti-myc tag monoclonal clone 4A6 (Millipore) diluted to final concentration of 200 ng mL^{-1} in 3% skimmed milk in TBST for 1h at room temperature. After washing membranes proceeded to incubation with secondary antibody anti-mouse IgG (H+L) conjugated to HRP (Molecular Probes) at 300 ng mL^{-1} in 3% skimmed milk in TBST for 1h at room temperature. Membranes were washed, incubated with Clarity Max substrate (BioRad) and developed in ChemiDoc MP (BioRad).

Motility screen and assays The six replicate pooled library cultures were grown in HOMEM media for 12 h in 20ml culture. A 2ml culture was added to the bottom of each well of a 8μm transwell plate (Costar #3428 24mm Diameter insert, 8.0 μm Pore size 6 well plate) and additional media was added to the top of each well before incubating at 25°C for 4 h to allow motile cells to cross the membrane and equilibrate on both sides. The culture was collected from the top and bottom of each

well. Cells were pelleted, DNA extracted and bar-seq analyses completed. Statistical analyses included two-tailed unpaired *t*- test and Discovery using the Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli, with Q = 50%. Each row was analysed individually, without assuming a consistent SD. Outputs with a *p* value <0.005 were chosen for further phenotypic characterisation. Motility mutants were grown individually to a mid-log culture. Cells were fixed in a final concentration of 1% paraformaldehyde (Alfa Aesar 16% W/V #43368) for 15 min. Fixed cells were washed in PBS by pelleting at 1000 x g for 1 min. The remaining pellet was gently re-suspended in 20ul PBS. 10 µl of suspension was added to a glass slide (Thermo Superfrost plus #J1800AMNZ) with 5 µl of mounting solution and coverslip. Cells were imaged on an inverted Ziess axioObserver with a 63x lens. Multiple FOV images were taken for each line. Cell morphology measurements were made using Zen-2.6 (blue-edition). For consistency, cell width was always measured across the nucleus, while flagellum length was measured from the tip to the edge of the cell body.

For tracking, cells were loaded into glass sample chambers measuring approximately 5mm × 20mm × 0.5 mm constructed from glass slides and UV-curing glue. The samples were imaged under dark field illumination on a Nikon Eclipse E600 upright microscope using a ×10 magnification objective lens with a numerical aperture of 0.3. Five video microscopy sequences were acquired from different areas of the sample chamber for each mutant using a CMOS camera (Mikrotron MC-1362). Movies were acquired at 50 Hz for 40 s, at a resolution of 1024 × 1024 pixels, corresponding to a field of view of approximately 1.4 mm× 1.4 mm within which cells were tracked. Minimum images were constructed, composed of the lowest values at each pixel in each video sequence. A rolling ball filter with a radius of 30 pixels was applied to each minimum image before using them to correct the video sequences and remove static background features. The corrected movies were then thresholded to highlight cells moving by either swimming or Brownian motion. The cell coordinates in each frame were extracted, and these coordinates were assembled into cell tracks using custom-written software routines [71]. To smooth short-time fluctuations associated with camera noise, the cell tracks were fitted using piecewise cubic splines. This allowed reliably extracted instantaneous cell velocities. This technique also allows separation of motile and non-motile cells based on their mean-squared displacements. The spline-smoothed trajectory of a non-motile particle has a mean-squared displacement that scales with time, whereas a swimming cell's mean squared displacement scales with time squared. By setting a threshold for the slope of each cell's mean-squared displacement at short times, and a threshold for the total mean squared displacement after 2 s, motile and non-motile particles were effectively separated and the motile fraction estimated. The instantaneous speed of each swimming cell was averaged over the course of the cell track, and a distribution of average swimming speeds made.

Author Contribution. J.C.M. conceived the study. N.B., C.C.M.C-P., R.N., E.V.C.A.F., V.G., J.B.T.C made the kinome libraries. N.B., C.C.M.C-P., R.N. A.J. carried out the bar-seq screens. B.P. carried out statistical analysis. N.B., C.C.M.C-P., B.P., K.N., J.W.P analysed data. J.S., B.V., P.V did sand fly experiments. P.B.W and L.G.W did motility analysis. N.B., C.C.M.C-P. R.N.,V.G., A.M. did phenotype analysis. N.B, C.C.M.C-P., J.C.M prepared and wrote the manuscript. All authors reviewed, edited and approved the paper. J.C.M. obtained funding.

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Declaration of Interests. The authors declare no conflict of interest

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Figure Legends

Fig. 1 *L. mexicana* kinome and conservation in other pathogenic trypanosomatids. (a) Representation of eukaryotic (ePK) and phosphatidylinositol 3' kinase-related kinases (PIKK) protein kinase families, separated by colours defined by primary protein sequence conservation. Image for illustrative purpose only with no phylogenetic significance. Red dots represent protein kinases for which gene deletion mutants could not be generated and are therefore potentially essential genes in procyclic promastigotes. (b) Phylogenetic tree of trypanosomatids with the number of conserved protein kinases indicated by blue circles and the percentage of potentially essential conserved protein kinases indicated within a red rectangle. (c) Pie-charts show dispensable (non-essential) and required (potentially essential) protein kinases in procyclic promastigotes, separated into families.

Fig. 2 Generation of the *L. mexicana* kinome gene deletion library**.** (a) Schematic representation of gene editing by CRISPR-Cas9 in the *L. mexicana* progenitor cell line showing integration of repair

cassettes containing 30nt homology sites, a unique barcode and antibiotic resistance genes for puromycin (*PAC*) and blasticidin (*BSD*). Diagnostic PCRs for the presence of the CDS with Oligo 1 and Oligo 2, (depicted in blue), correct integration of the repair cassettes using Oligo 3 and Oligo 4 (depicted in red), and the gene in the endogenous locus with Oligo 5 and Oligo 6 (depicted in green). (b) Diagnostic PCR for (i) *LmxM.25.0853* and (ii) *PKAC1* (*LmxM34.4010*) using primer pairs as above in *L. mexicana* Cas9 T7 (WT) and Populations A and B (c) Heat map indicating the presence or absence of target genes after whole genome sequencing. The ratio of gene coverage to chromosome coverage was used as a measure of gene copy number in selected mutants. A value of 1 represents two alleles of a given gene. (d) Diagnostic PCR for facilitated gene deletion mutant *LmxM.04.0650* using Oligo 1 and 2 for the CDS, Oligo 5 and 6 for the endogenous locus and Oligo 3 and 4 for correct integration. Western blot using anti-myc confirmed episomal expression of LmxM.04.0650 at the anticipated size of 140 kDa.

Fig. 3 Endogenous tagging of *L. mexicana* protein kinases. (a) Schematic representation of process for N-terminal tagging. Transfection of the sgRNA (PCR1) directs the double stranded break immediately upstream of the target CDS and the repair template (PCR2) contains 30nt at 5'and 3' of the fragment for homologous recombination on the target locus to insert the fluorescent tag and an antibiotic-resistance gene. (b) Bar graph representing the localisation of dispensable and required protein kinases for each organelle (c) Pictorial illustration of the 10 localisation groups. An exemplar live cell image of protein kinases tagged with mNeonGreen for each localisation. Procyclic promastigotes were incubated with Hoechst 33342 for DNA labelling (pink). Scale bar: 5 µm.

Fig. 4 Identification of protein kinases involved in *L. mexicana* differentiation and infection. (a) Schematic illustration of the bar-seq screen design with three experimental arms (EA1, blue; EA2 , black; EA3, red) depicting investigation of differentiation to amastigote stages and investigation of protein kinase mutants in sand fly infection (EA4, green). A pool of gene deletion mutants were generated in procyclic promastigotes (PRO), which were grown in Graces Media at pH 5.5 for 168 h. Cells were then either diluted into amastigote media at 35°C and grown as axenic amastigotes (AXA) for 5 d or enriched for metacyclic promastigotes. Metacyclic promastigotes (META) were used to either infect macrophages (inMAC) or inoculate mouse footpads (FP). DNA was taken at the indicated time points and the unique barcodes amplified by PCR to apply bar-seq analyses. (b) Proportion of barcodes across 8 time points, as indicated in A. The trajectories of six exemplar protein kinase mutants have been plotted for the three experimental arms. Values are mean +/- S.D., N = 6. (c) Projection pursuit cluster analysis was applied to the trajectories from each experimental arm, grouped into six clusters, each consisting of trajectories with a similar trend. Only clusters resulting from the mouse footpad infection are shown. The image for each cluster shows gene trajectories overlaid with average trends in bold. Trajectories plotted using logged % barcode representation data, normalised to time 0. Heat maps below show % barcode representation data and depict the trend for each individual gene. Gene IDs in red (MCA, MPK1 and MPK2) have documented phenotypes in *Leishmania* and serve to benchmark the dataset. (d) The relationship between the clusters is shown on a two-dimensional PCA plot. Colours match the clusters in C.

Fig. 5 Identification of protein kinases involved in colonisation of *Lutzomyia longipalpis*. (a) Heat map of 29 protein kinase mutants that cluster in groups important for the amastigote stage in two of the three experiments using both 1 and 2 difference clustering. In bold are the genes that have been identified as important for mouse infection in a previous trypanosome study [41]. (b) Summary showing the protein kinases important for sand fly infection. Control plot (top) shows the relative barcode representation for four control lines with a barcode inserted in the ribosomal locus at three timepoints post blood meal ((PBM), Day 1, Day 5 and Day 8). Protein kinases important for *L. longipalpis* infection plot (bottom) shows the 15 mutants with significant loss of representation by

Day 8 identified using a multiple *t*-test (n=2). 7 protein kinases identified as important for both sand fly infectivity and for survival in amastigote stages indicated in bold. (c) Heat map showing the amastigote stage data for the 8 protein kinase mutants that are important for sand fly infection only. (d) Plot showing the infectivity of the protein kinase mutants important for survival of amastigotes only.

Fig. 6 Identification of protein kinases involved in motility. (a) Bar chart showing flagellar length: cell body length ratio for 5 protein kinase deficient mutants identified with significant loss of representation at the top of the transwell, indicating a motility defect. Error bars indicate standard deviation (n = >50). Mutants ordered according to cell body width measurement. Widest cells shown to the right. * protein kinase mutants with short flagellar phenotype. (b) Graph showing the fraction of the population for each promastigote protein kinase mutant progressively swimming. The motile fraction of cells in the control (*T7Cas9,* N=2532) and four mutant strains: Δ*ulk1* (N=2936), Δ*mpkk2* (N=4896), Δ*29.0600* (N=2623), and Δ*02.0570* (N=3532). The error bars represent standard error of the mean/95% confidence intervals and the data points denote results from individual technical replicates. (c) Schematic showing the protein structures of the four protein kinase mutants identified in the motility screen. (d) Localisation of the proteins tagged with mNeonGreen. (e) Bar-seq trajectories for the four protein kinase mutants for footpad infection (f) and normalised bar-seq reads for these four protein kinase mutants during development in the sand fly.

Fig. 7 Schematic representation of *L. mexicana* differentiation cycle. Fifteen protein kinases were identified as required for colonisation of the sandfly. Twenty nine protein kinases were identified as required for differentiation into amastigote stages *in vivo* and *in vitro*.

Supplementary Information

Supplementary Table 1 Kinome data overview and primers

Supplementary Table 2 Kinome wide library outputs

Supplementary Equation 1 Projection pursuit cluster analysis methodology and script.

Supplementary Data 1 Diagnostic PCRs to test for gene deletion mutants. The DNA prepared from populations (Pop) recovered after transfection was used to test for the presence/ or absence of the protein kinase coding sequence (CDS) and integration (INT) of the resistance cassette at the correct locus. Exemplar gel explained in Fig 1b. All primers used listed in Supplementary Table 2.

Supplementary Data 2 Localisation of mNeonGreen tagged protein kinases. An example image is shown for each of the protein kinase localisations. Multiple images are included for protein kinase localisations that were stage specific or for which N- and C- terminal tagging differed. Images are ordered by the protein kinase family. Scale bar: 2 µm

Supplementary Data 3 Heat maps and cluster analysis for amastigotes. Data for the three experimental arms (EA1, 2 and 3 (see Fig 4a)) sorted via cluster analysis. Heat maps show % abundance of barcodes. Cluster trajectories plotted using the logged % of barcodes, normalised to time 0 (relative abundance (Log10)). (a) Axenic amastigote data clustered by 1 difference (b) Axenic amastigote clustered by 2 differences. (c) Individual plots for axenic amastigote data (coloured according to 1 difference clustering). (d) Macrophage infection data clustered by 1 difference. (e) Macrophage infection data clustered by 2 differences. (f) Individual plots for macrophage infection data (coloured according to 1 difference clustering). (g) Mouse footpad

infection data clustered by 1 difference. (h) Mouse footpad infection data clustered by 2 differences. (i) Individual plots for mouse footpad infection data (coloured according to 1 difference clustering). Procyclic promastigotes (PRO), axenic amastigotes (AXA), metacyclic promastigotes (META), infected macrophages (inMAC) and mouse footpads (FP).

Supplementary Fig. 1 Putative pseudokinases of *L. mexicana*. (a) Schematic of a conserved protein kinase structure. The highly conserved lysine and two aspartic acid residues required for an active kinase are highlighted. (b) List of all protein kinases missing one or more of these residues. Boxed K, D, D letters with diagonal line indicate missing residues. The 11 pseudokinases are indicated in bold text. Green star denotes protein kinases that are specific to the *Leishmaniinae* family. Solid red circle indicates that the protein kinase is required for promastigote survival.

Supplementary Fig. 2 Redundancy of *CLK1* and *CLK2*. **(**a) Gene deletion (circle) for *CLK1* (LmxM.09.0400) and *CLK2* (LmxM.09.0410). Attempts were made to delete *CLK1*, *CLK2* or both *CLK1* and *CLK2*. Successful deletions are indicated after antibiotic selection. (b) Diagnostic PCRs are shown for gene deletion mutants. WT; *L. mexicana* M379, T7/Cas9; *L. mexicana* cell line expressing Cas9-T7 in populations (Pop) and clones (C). (c) Integrative genomics viewer of the illumina whole genome sequencing reads from Δ*clk1* and Δ*clk2* mutants are aligned against the reference genome of the Cas9 T7 progenitor cell line generated by Nanopore sequence. Reads absent at the N-terminal end of the *CLK1* and *CLK2* indicate a gene deletion mutant as the remainder of the CDSs are identical between both genes. The multiple alignments are indicated by the coloured white reads (mapping quality zero).

Supplementary Fig. 3 Characterisation of *RDK1* (a) Proliferation of promastigotes comparing *L. mexicana* Cas9 T7 progenitor line and Δ*rdk1* clones 1 and 2 (b) Bone-marrow macrophage infections using purified metacyclic promastigotes of *L. mexicana* Cas9 T7 progenitor line and Δ*rdk1* clones 1 and 2 analysed for total number of amastigotes infecting 200 macrophages (MΦ) at early (12 h) and late (72 h) time points. (c) Parasite burden determined by limiting dilution of 5 BALB/c footpad lesions infected with *L. mexicana* Cas9 T7 progenitor line, Δ*rdk1* mutant and an add-back Δ*rdk1::RDK1* for six weeks. Values plotted with the mean and 95% SEM interval. Statistic analysis are two-tailed unpaired student *t*-test. (d) HASPB expression was analysed in metacyclic promastigote enriched cultures, amastigotes (12 h and 120 h after induction of differentiation) and procyclic promastigotes (24 h after induction of differentiation). Expression of tubulin is shown as loading control. HASPB/Tubulin ratio values are shown below each lane, calculated for each time point using Cas9 T7 cell line expression as a control.

Supplementary Fig. 4 Fitness plots for protein kinase mutants during sand fly colonisation. Data are shown for Day 1 (Black), 5 (Dark grey) and 8 (light grey). Top plot shows mutants included in the slow pool, middle plot indicates average growth pool and the bottom plot indicates fast growth pool. Fitness was calculated using normalised reads at each time point against the normalised reads at time point 0. Mutants contributing to less than 0.2% of the reads on the day of the infected blood meal are highlighted in grey and were excluded from the loss of fitness analyses. Mutants showing a significant loss of fitness are highlighted in red. Corresponding raw data can be found in Supplementary Table 2.

Supplementary Fig. 5 Speed histograms for protein kinase mutants. These panels show the distribution of swimming speeds in the parental line (T7Cas9, N=1149) and four protein kinase mutants: Δ*ulk1* (N=57), Δ*mpkk2* (N=144), Δ*29.0600* (N=6), Δ*02.0570* (N=145). The instantaneous swimming speed of each cell was averaged across the duration of its swimming track to produce one count in the histograms.

Supplementary Fig. 6 Protein kinases involved in flagellum function. (a) Phenotypic analysis measurements for protein kinase mutants important for motility. (b) Additional images supporting the exempla protein kinase localisations in Fig. 6d and short flagellum null mutants in Fig. 6b (c) Phase images of Δ*ulk1* and Δ*mpkk2* mutants (top) and Δ*ulk1::ULK1 and* Δ*mpkk2:MPKK2* add-back mutants (bottom).

Supplementary Movie 1 Motility of protein kinase mutants. These movies show the effect of mutations on the flagellar beat of *L. mexicana*. The movies were acquired at a frame rate of 500 Hz using a phase-contrast imaging and a 60x magnification microscope objective. The scale is the same for all panels, and the movie is played back at 1/5 th of the true speed so the high-speed flagellar motion can be more easily seen.

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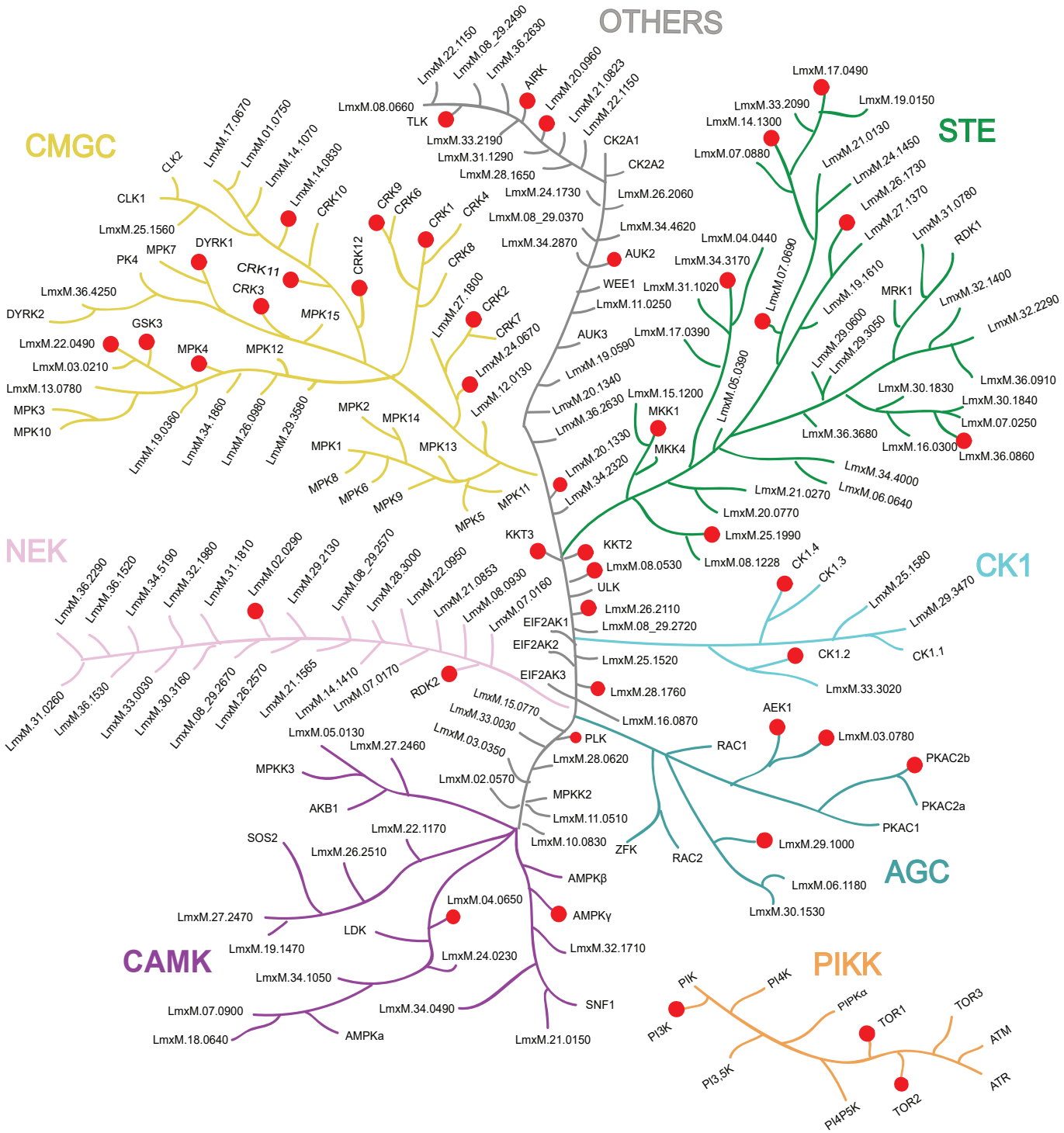

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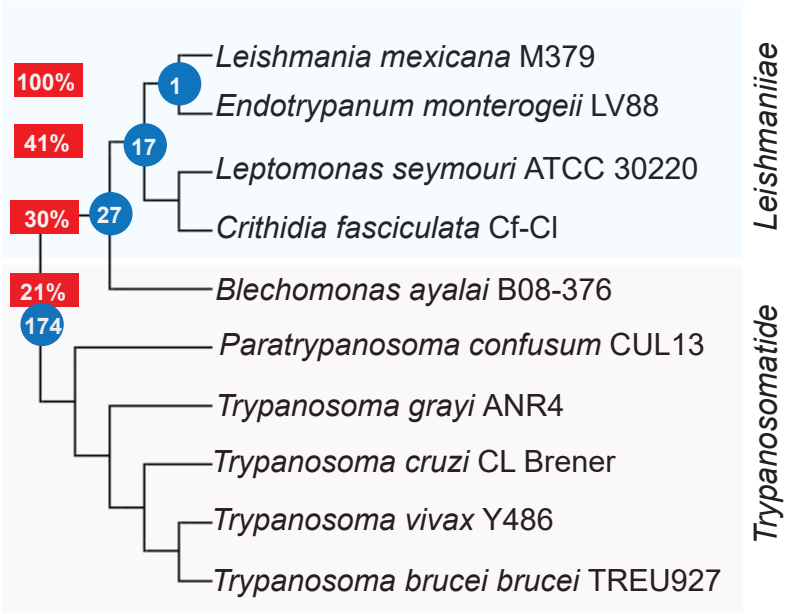

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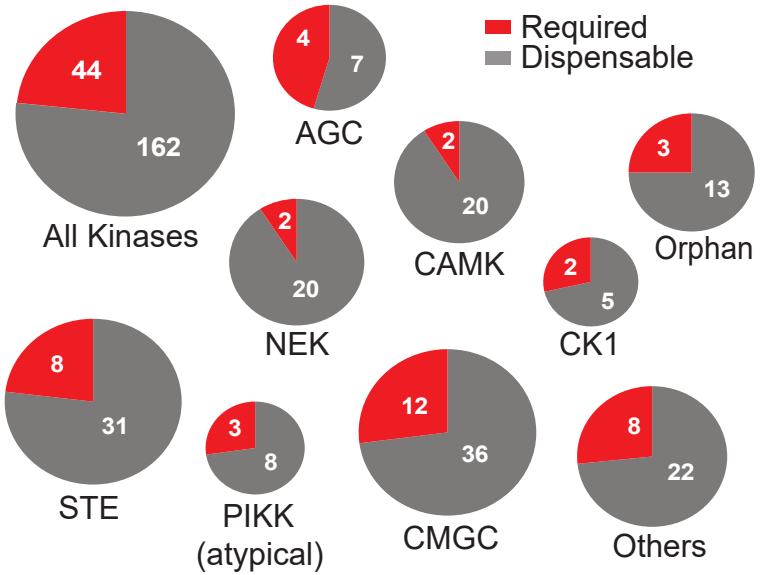

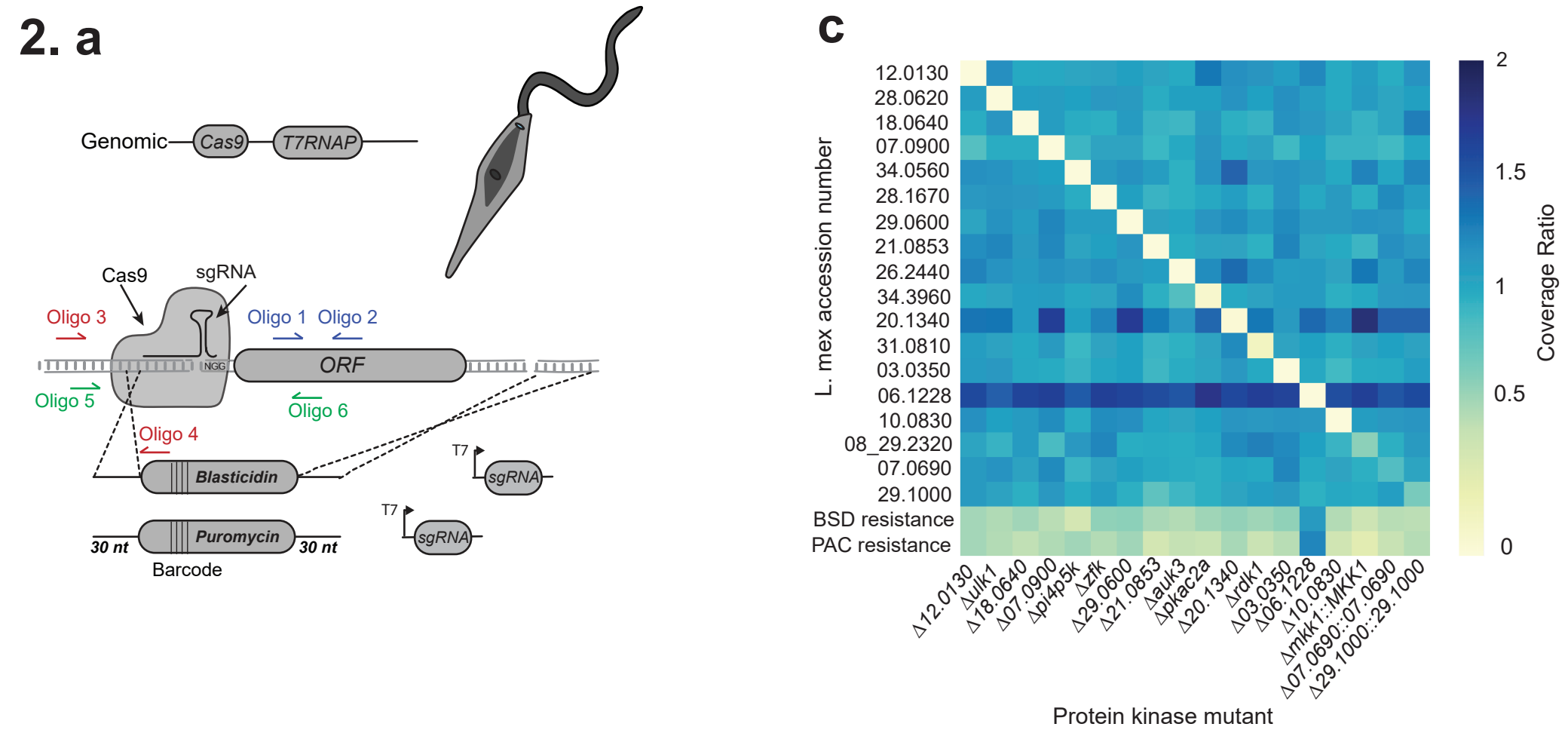
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Oligo 4
Blasticidin
T7
sgRNA
T7
sgRNA
Puromycin
30 nt
30 nt
Barcode
c
L. mex accession number
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07.0900
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29.0600
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Δ29.0600
Δ21.0853
Δauk3
Δpkac2a
Δ20.1340
Δrdk1
Δ03.0350
Δ06.1228
Δ10.0830
Δmkk1::MKK1
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Δ29.1000::29.1000
Protein kinase mutant
Coverage Ratio
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1
0.5
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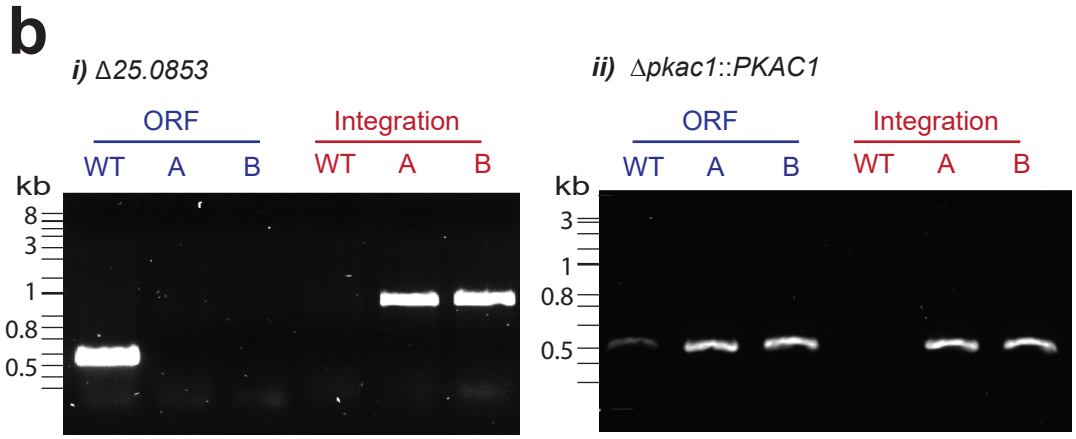
b
i) Δ25.0853
ORF
Integration
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kb
8
3
1
0.8
0.5
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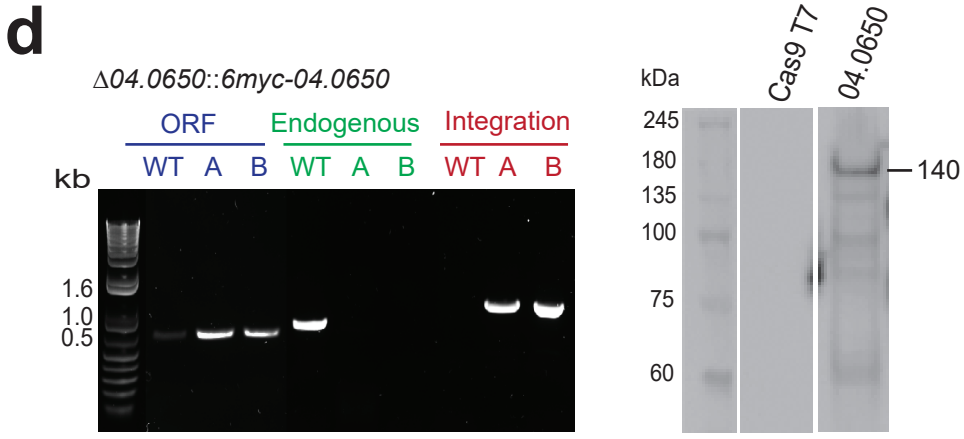
ORF
Integration
WT A B WT A B
kb
3
1
0.8
0.5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Integration
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kDa
Cas9 T7
04.0650
245
180
135
100
75
60
140

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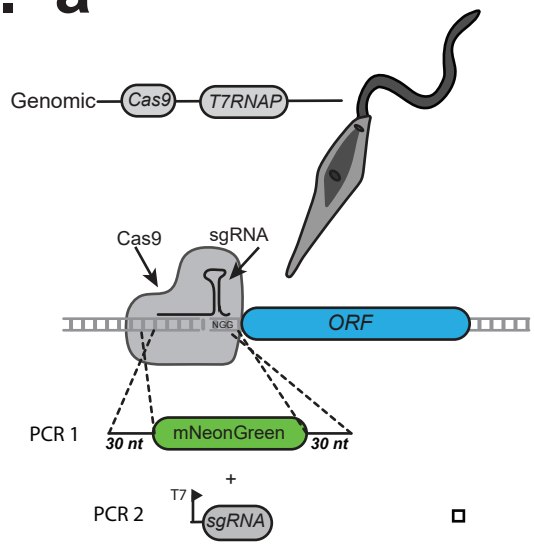
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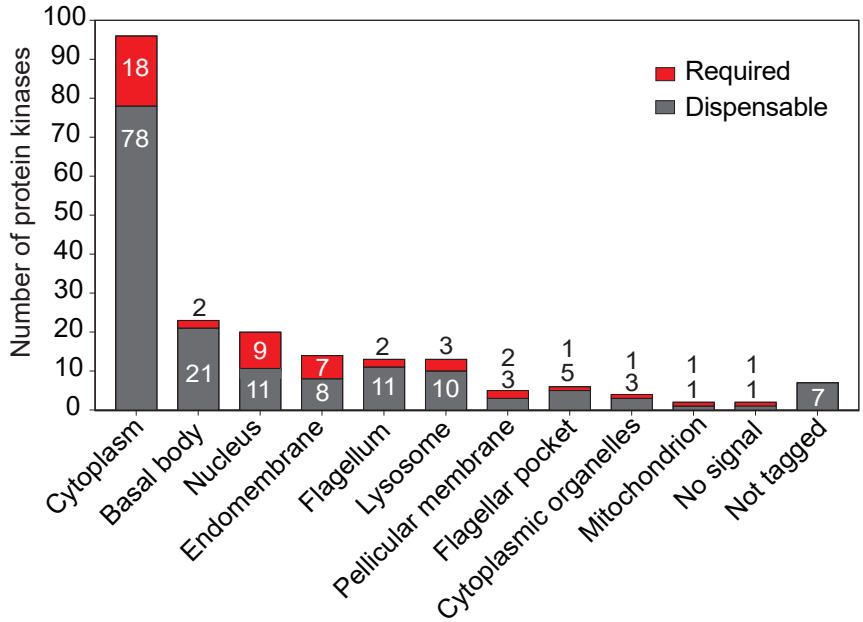
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80
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50
40
30
20
10
0
Required
Dispensable
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78
2
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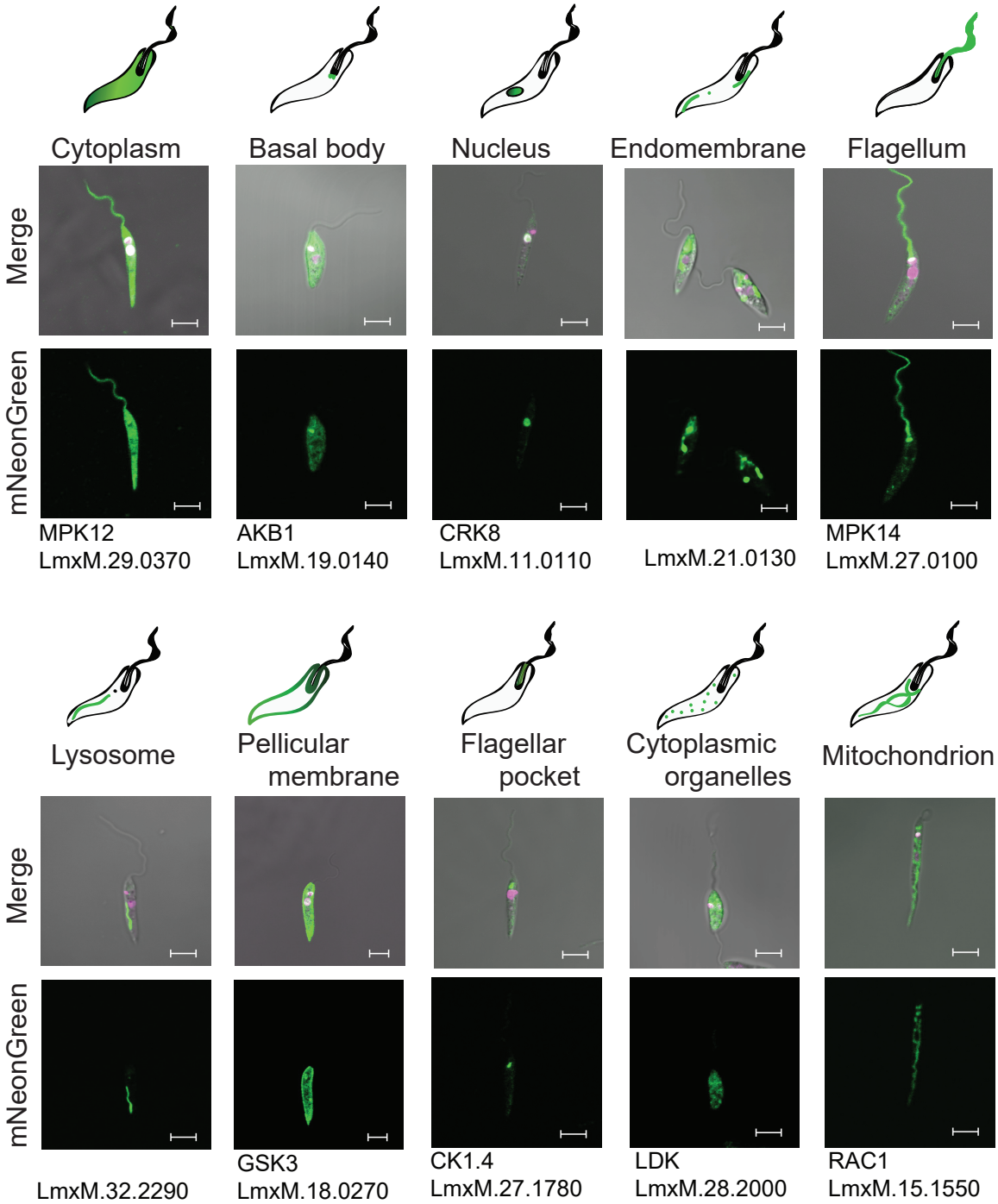
9
11
7
8
2
11
3
10
2
3
1
5
1
3
1
1
1
1
7
Cytoplasm
Basal body
Nucleus
Endomembrane
Flagellum
Lysosome
Pellicular membrane
Flagellar pocket
Cytoplasmic organelles
Mitochondrion
No signal
Not tagged

c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Basal body
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Endomembrane
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CRK8
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MPK14
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Lysosome
Pellicular membrane
Flagellar pocket
Cytoplasmic organelles
Mitochondrion
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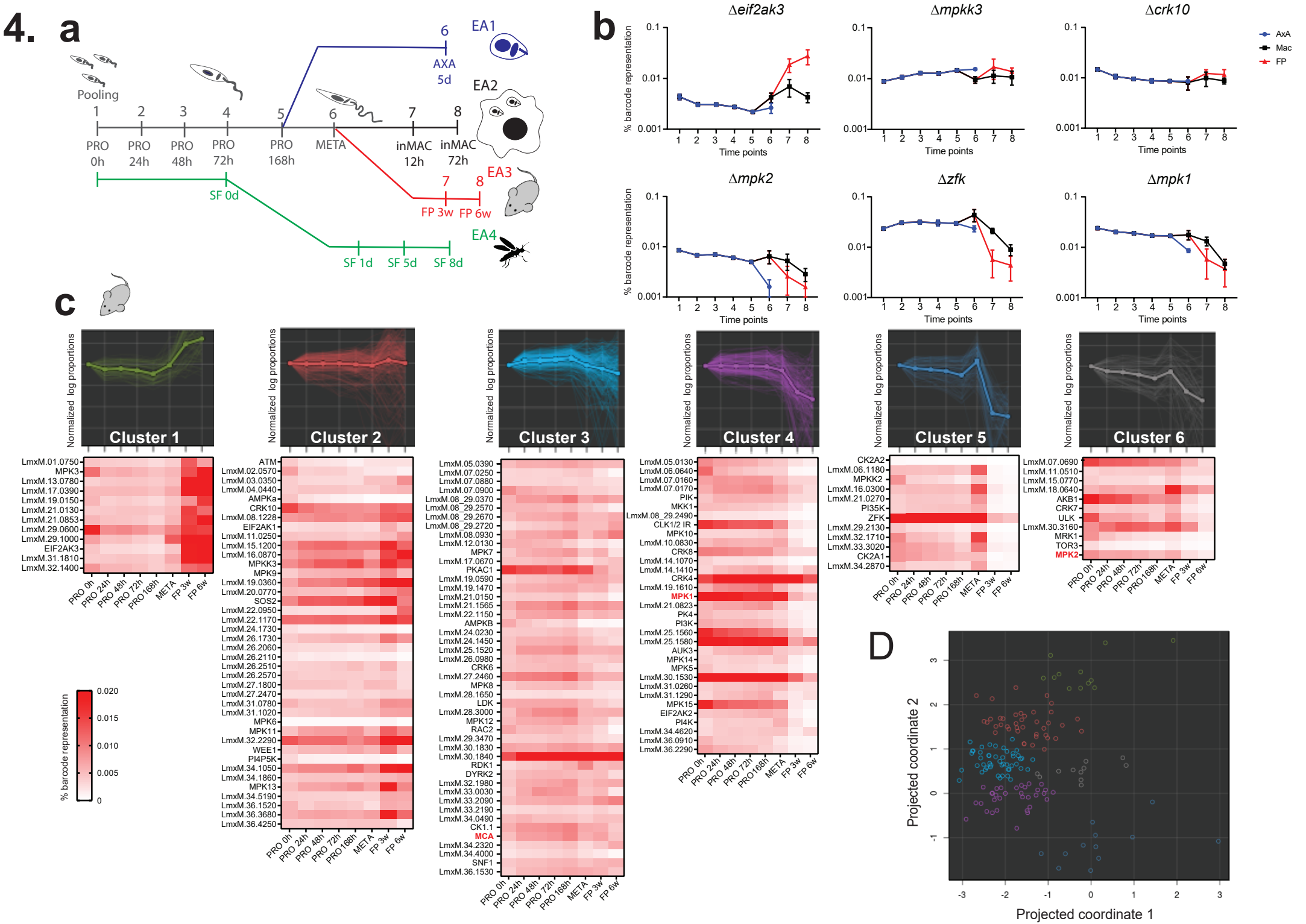
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2
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5
6
7
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META
inMAC 12h
inMAC 72h
SF 0d
SF 1d
SF 5d
SF 8d
FP 3w
FP 6w
AXA 5d
EA1
EA2
EA3
EA4
b
Δeif2ak3
Δmpkk3
Δcrk10
Δmpk2
Δzfk
Δmpk1
% barcode representation
Time points
AxA
Mac
FP
c
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Cluster 6
Normalized log proportions
% barcode representation
D
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Projected coordinate 2

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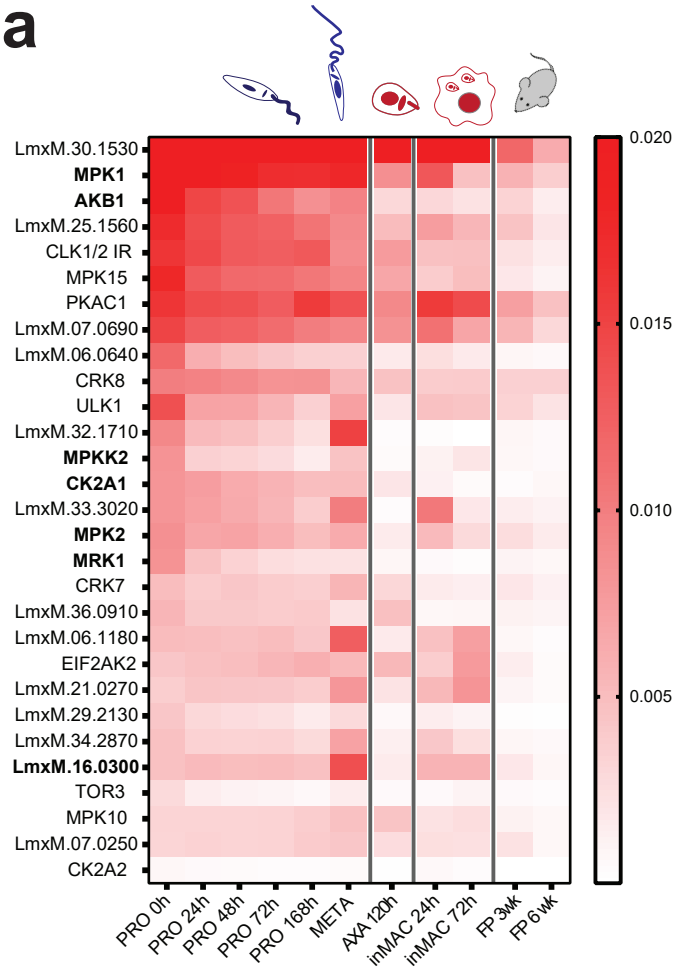
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CRK8
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TOR3
MPK10
LmxM.07.0250
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PRO 24h
PRO 48h
PRO 72h
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0.010
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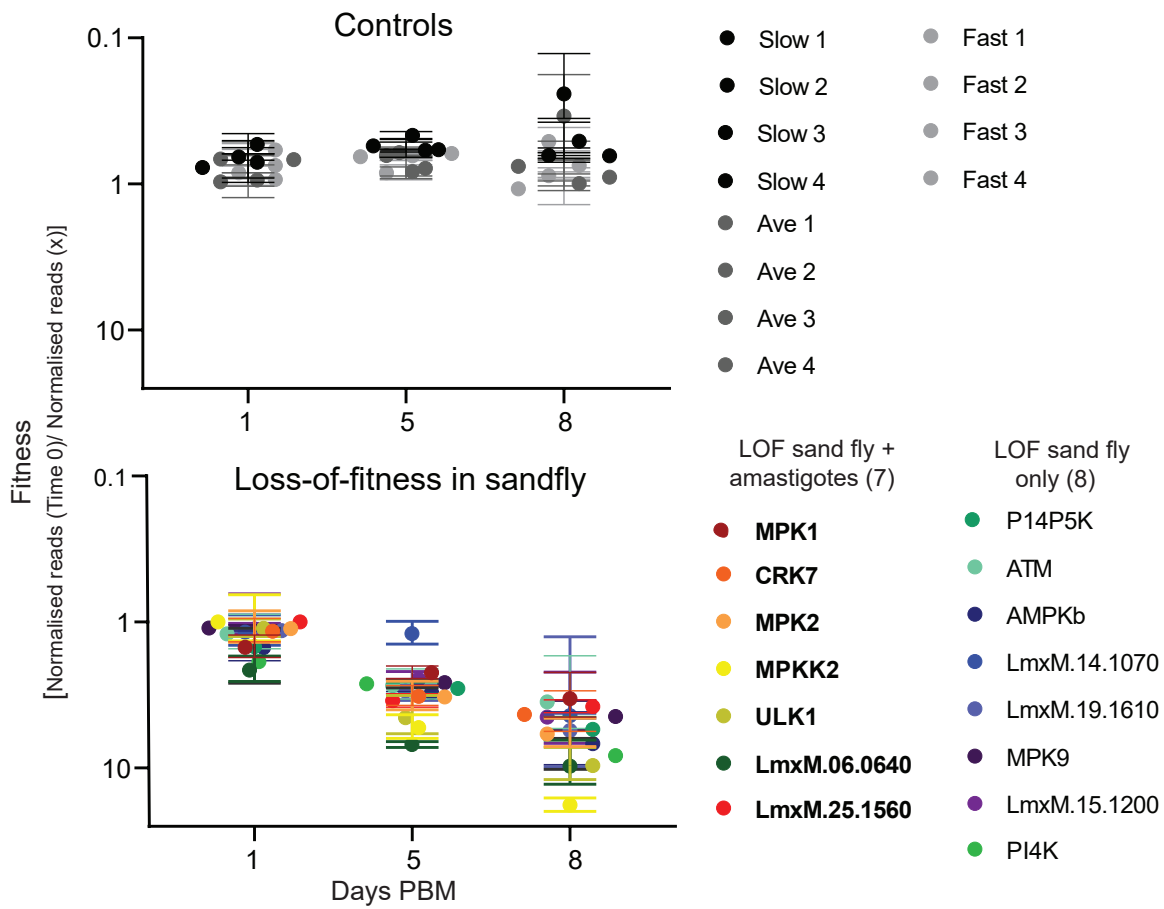
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Loss-of-fitness in sandfly
Fitness
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0.1
1
10
1
5
8
Days PBM
Slow 1
Slow 2
Slow 3
Slow 4
Ave 1
Ave 2
Ave 3
Ave 4
Fast 1
Fast 2
Fast 3
Fast 4
LOF sand fly + amastigotes (7)
MPK1
CRK7
MPK2
MPKK2
ULK1
LmxM.06.0640
LmxM.25.1560
LOF sand fly only (8)
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AMPKb
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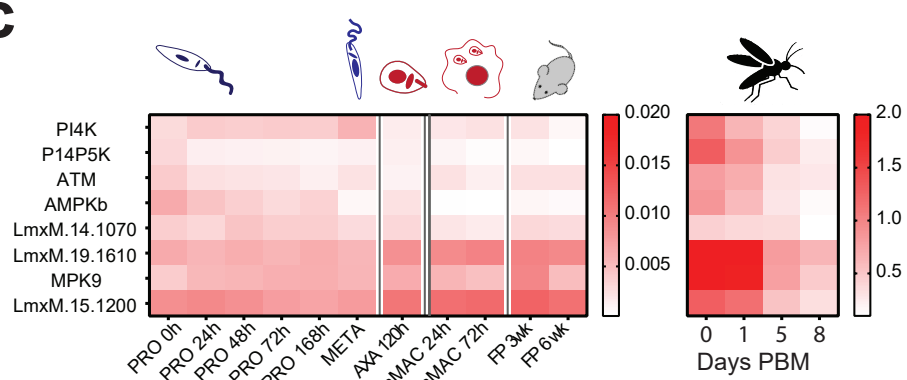
LmxM.19.1610
MPK9
LmxM.15.1200
PI4K

c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PRO 48h
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PRO 168h
META
AXA 120h
inMAC 24h
inMAC 72h
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FP 6wk
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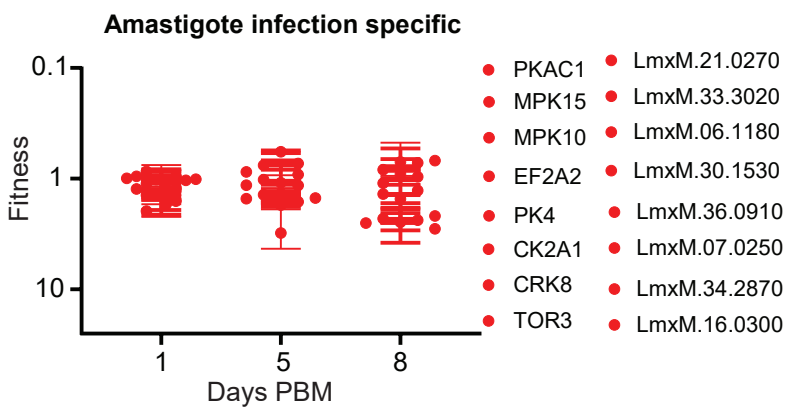
5
8
Days PBM

d


Amastigote infection specific
Fitness
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1
10
1
5
8
Days PBM
PKAC1
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MPK10
EF2A2
PK4
CK2A1
CRK8
TOR3
LmxM.21.0270
LmxM.33.3020
LmxM.06.1180
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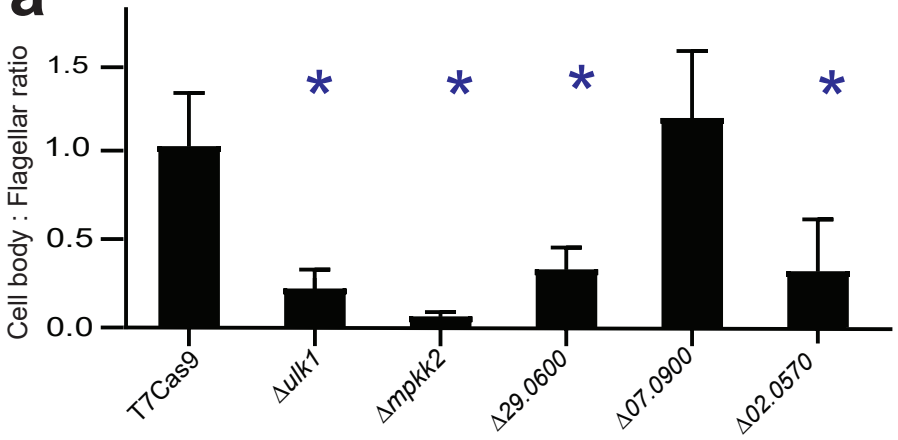
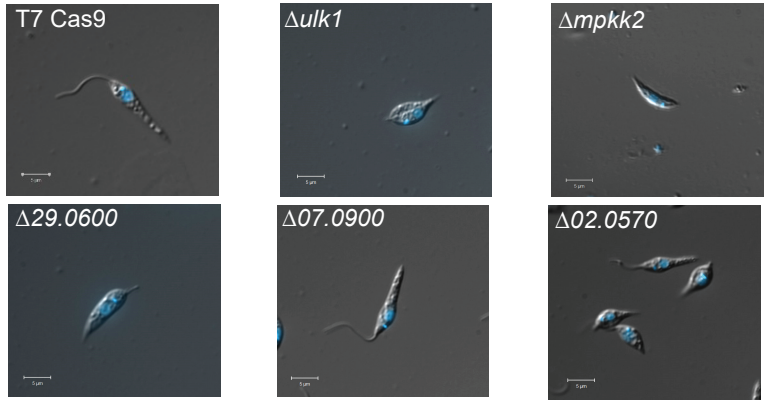

Cell body : Flagellar ratio
1.5
1.0
0.5
0.0
*
*
*
*
T7Cas9
Δulk1
Δmpkk2
Δ29.0600
Δ07.0900
Δ02.0570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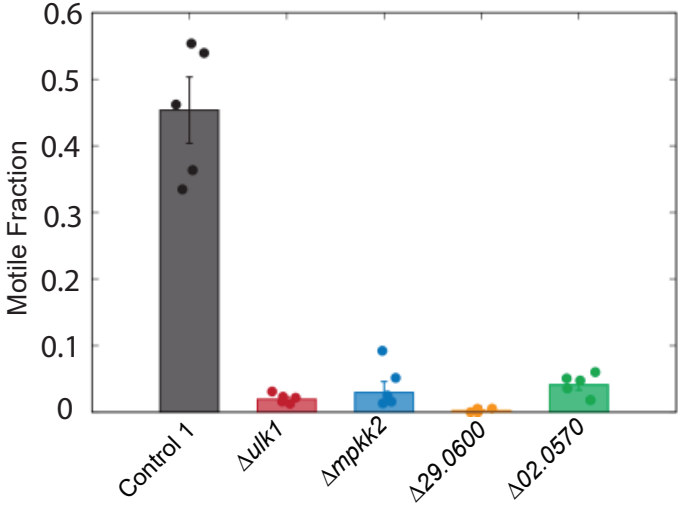
Δ02.0570

b


Motile Fraction
0.6
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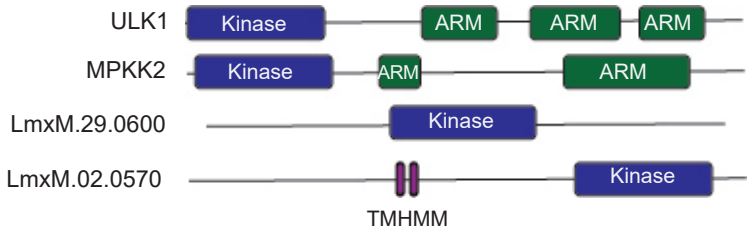
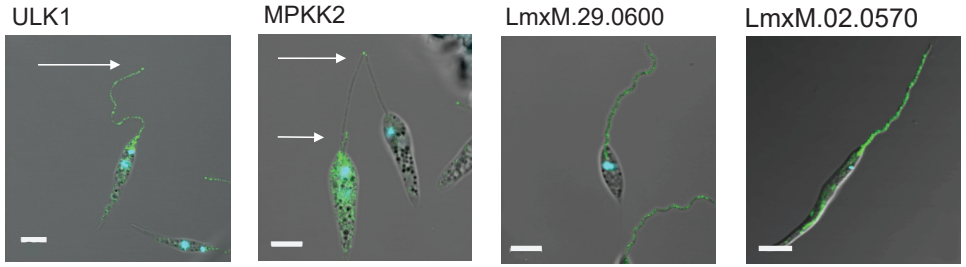
0.3
0.2
0.1
0
Control 1
Δulk1
Δmpkk2
Δ29.0600
Δ02.0570

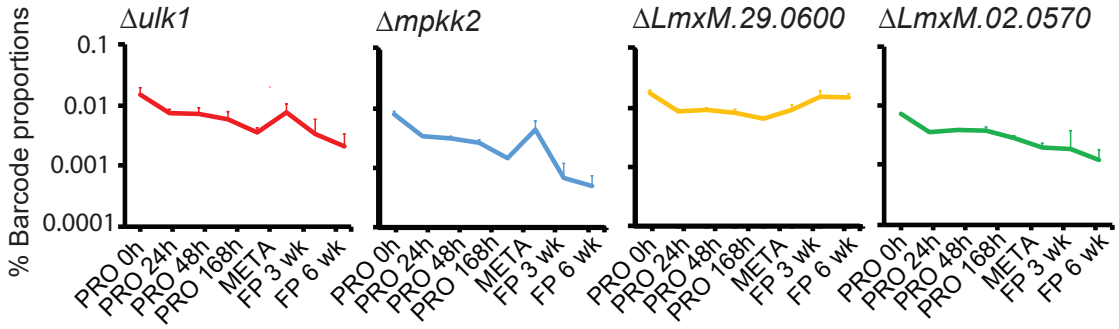
c


ULK1
Kinase
ARM
ARM
ARM
MPKK2
Kinase
ARM
ARM
LmxM.29.0600
Kinase
LmxM.02.0570
Kinase
TMHMM

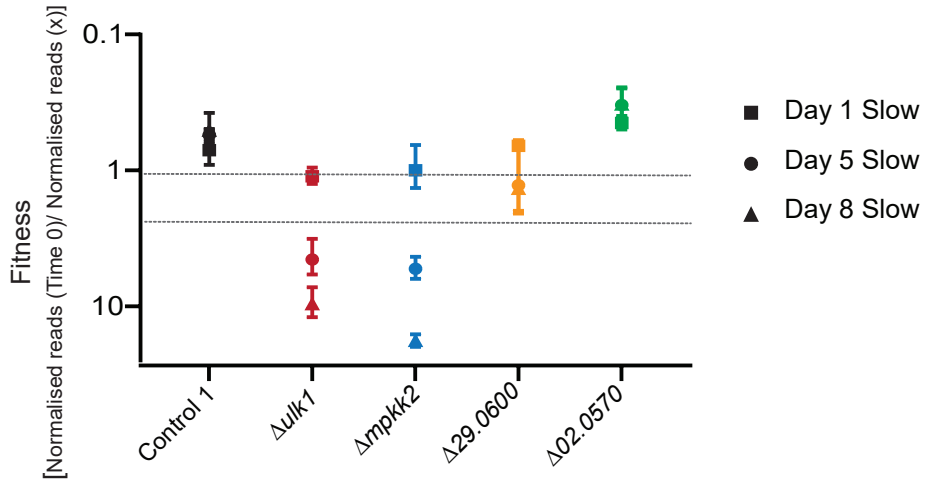
d


ULK1
MPKK2
LmxM.29.0600
LmxM.02.0570

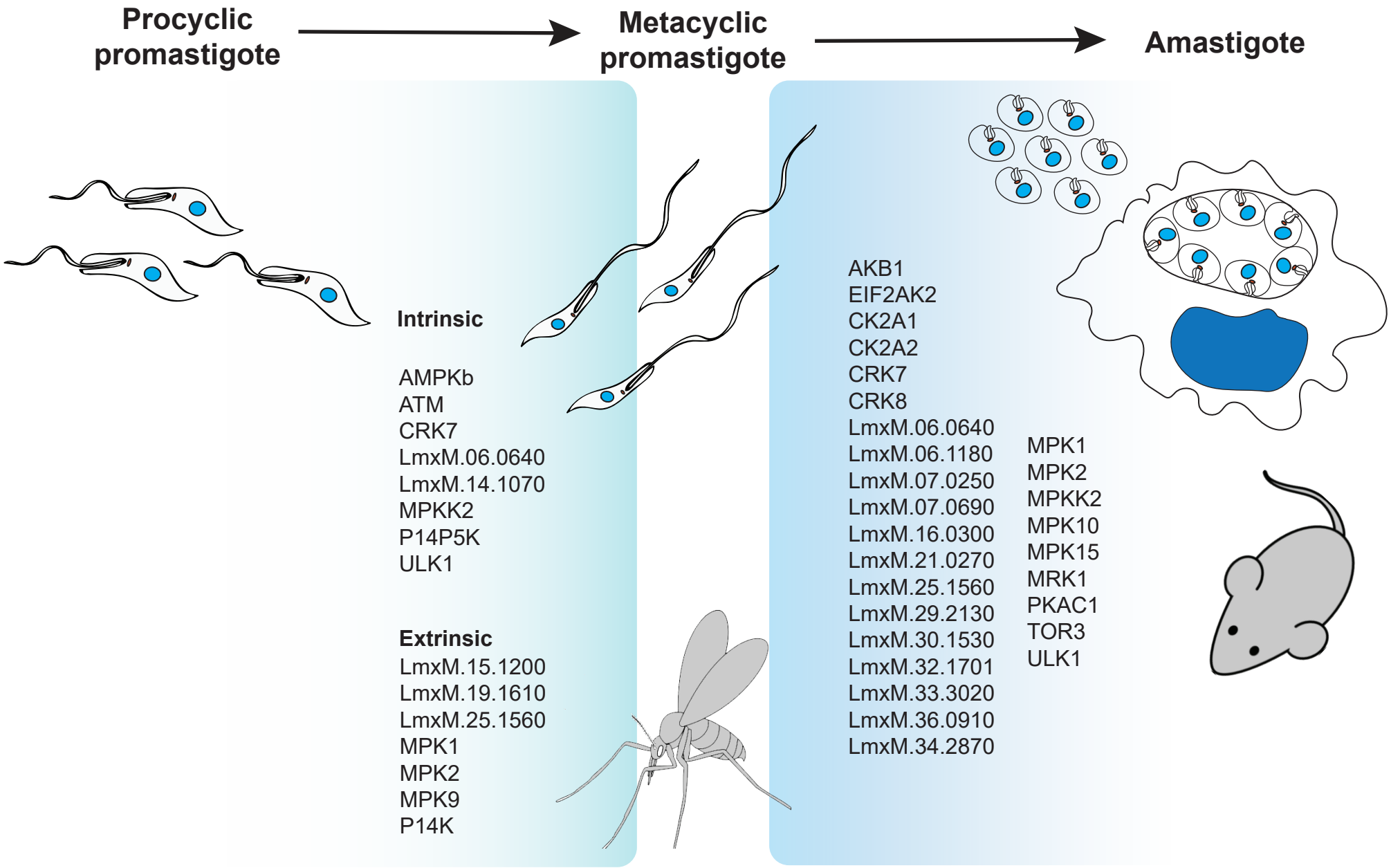
e


% Barcode proportions
Δulk1
Δmpkk2
ΔLmxM.29.0600
ΔLmxM.02.0570
0.1
0.01
0.001
0.0001
PRO 0h
PRO 24h
PRO 48h
PRO 168h
META
FP 3 wk
FP 6 wk

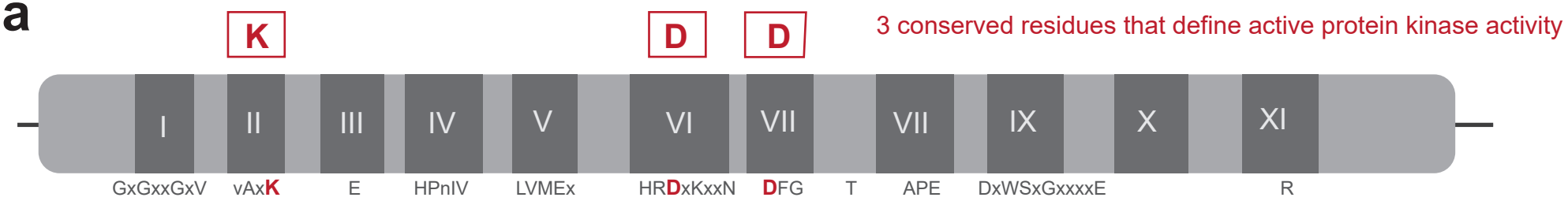
f


Fitness
[Normalised reads (Time 0)/ Normalised reads (x)]
0.1
1
10
Control 1
Δulk1
Δmpkk2
Δ29.0600
Δ02.0570
Day 1 Slow
Day 5 Slow
Day 8 Slow

7.


Procyclic promastigote
Metacyclic promastigote
Amastigote
Intrinsic
AMPKb
ATM
CRK7
LmxM.06.0640
LmxM.14.1070
MPKK2
P14P5K
ULK1
Extrinsic
LmxM.15.1200
LmxM.19.1610
LmxM.25.1560
MPK1
MPK2
MPK9
P14K
AKB1
EIF2AK2
CK2A1
CK2A2
CRK7
CRK8
LmxM.06.0640
LmxM.06.1180
LmxM.07.0250
LmxM.07.0690
LmxM.16.0300
LmxM.21.0270
LmxM.25.1560
LmxM.29.2130
LmxM.30.1530
LmxM.32.1701
LmxM.33.3020
LmxM.36.0910
LmxM.34.2870
MPK1
MPK2
MPKK2
MPK10
MPK15
MRK1
PKAC1
TOR3
ULK1

S1 a



b

Leishmaniinae specific

Required kinase

Pseudo kinase - missing all three residues

K̸ D̸ D̸

1. LmxM.21.0150 (CAMK)
2. LmxM.22.1170 (CAMK)
3. LmxM.01.0750 (CMGC)
4. LmxM.30.1830 (STE)
5. LmxM.30.1840 (STE)
6. LmxM.34.4620 (Pseudo orphan)
7. LmxM.08.0660 (Pseudo orphan)
8 LmxM.26.2060 (Pseudo orphan)
9. LmxM.28.1650 (Pseudo orphan)
10. LmxM.34.2870 (Other)
11. LmxM.22.1150 (Other)

Kinases missing 1 or more important resisdues

K̸ D̸ D

12. LmxM.17.0670 (CMGC)

K̸ D D̸

13. LmxM.04.0650 (CAMK)
14. LmxM.26.2110 (Orphan)
15. LmxM.20.1340 (Other)

K D D̸

16. MPK15 (CMGC)
17. LmxM.36.4250 (CMGC)
18. LmxM.19.1610 (STE)
19. LmxM.16.0870 (Orphan)
20. LmxM.20.1330 (Other)
21. LmxM.15.0770 (Other)

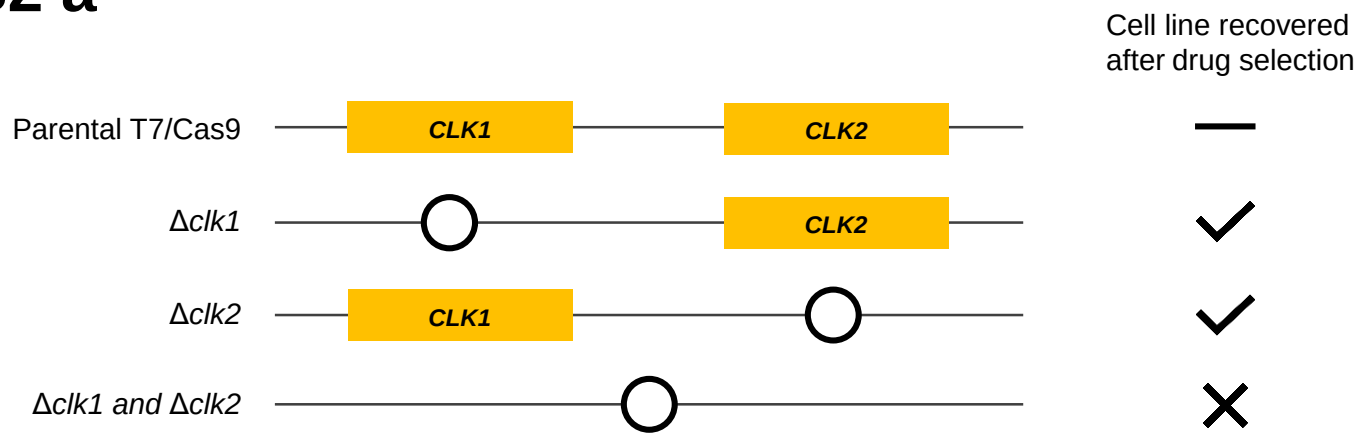
K D̸ D

22. LmxM29.1000 (AGC)
23. LmxM.33.2019 (Pseudo orphan)
24. AUK3 (Other)

K̸ D D

25. LmxM.34.0490 (CAMK)
26. CK1.4 (CK1)
27. LmxM.25.1580 (CK1)
28. LmxM.33.3020 (CK1)
29. CRK10 (CMGC)
30. CRK11 (CMGC)
31. LmxM.25.1560 (CMGC)
32. LmxM.19.0360 (CMGC)
33. LmxM.22.0490 (CMGC)
34. LmxM.03.0210 (CMGC)
35. LmxM.13.0780 (CMGC)
36. LmxM.12.0130 (CMGC)
37. LmxM.31.1020 (STE)
38. LmxM.04.0440 (STE)
39. LmxM.14.1300 (STE)
40. LmxM.32.1400 (STE)
41. LmxM.07.0250 (STE)
42. LmxM.36.2290 (Other)
43. LmxM.02.0570 (Orphan)
44. LmxM.03.0350 (Orphan)
45. LmxM.10.0830 (Orphan)
46. LmxM.31.1290 (Pseudo-orphan)
47. LmxM.11.0230 (Other)
48. LmxM.28.0620 (Other)
49. LmxM.28.1760 (Other)
50. LmxM.21.0823 (Other)
51. LmxM.36.2630 (Other)

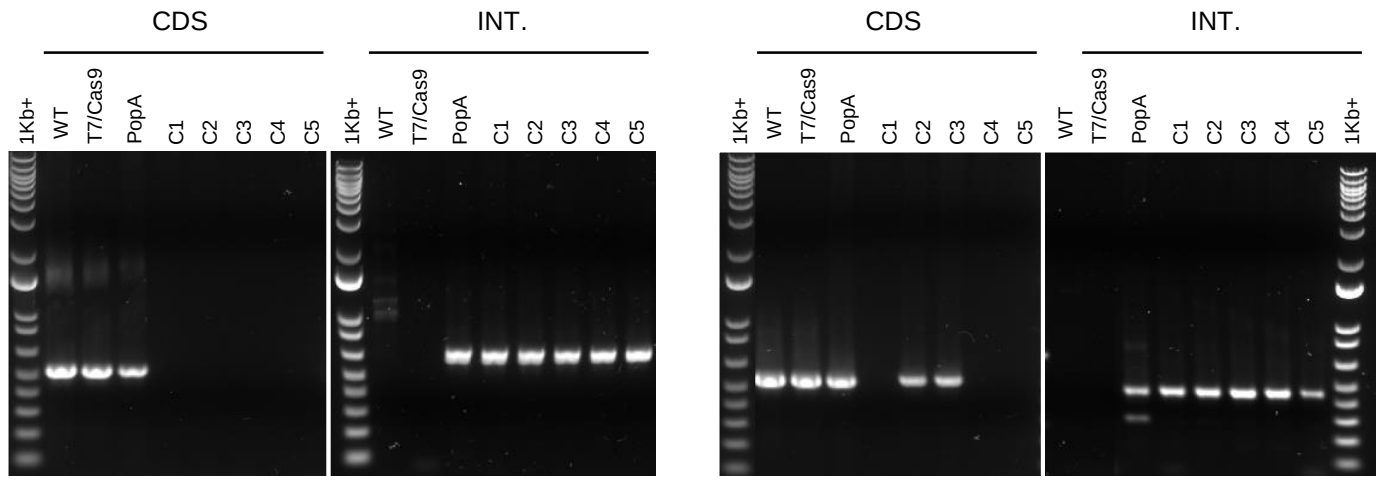
S2 a



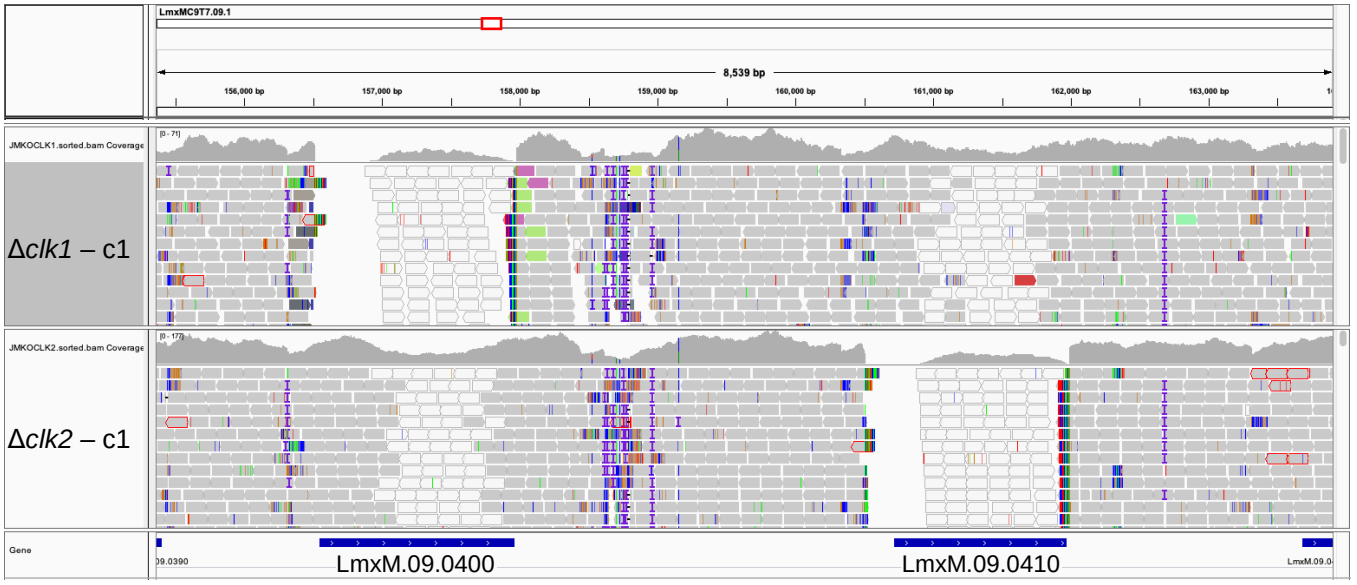
b

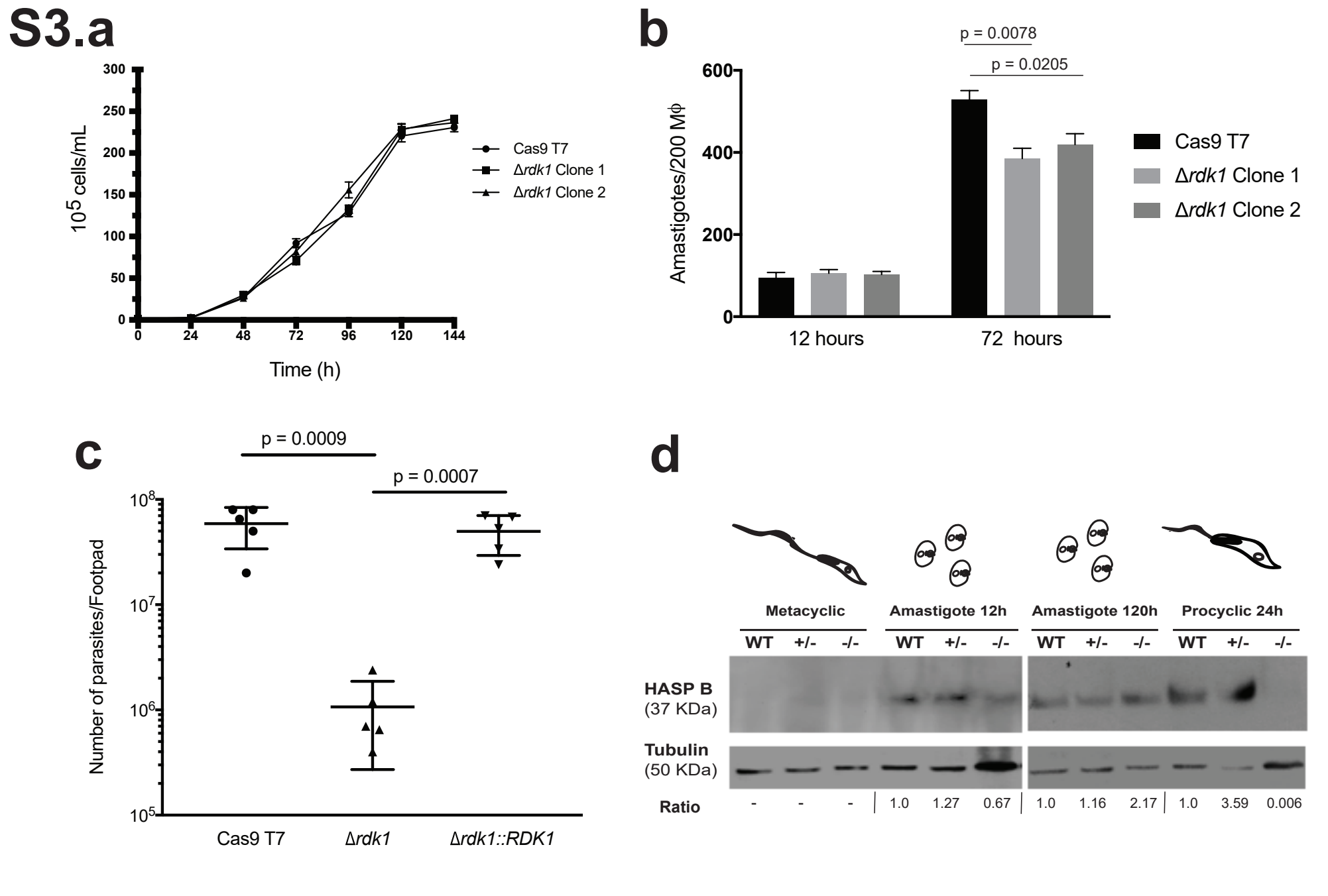
LmxM.09.0400 [CLK1]

LmxM.09.0410 [CLK2]



c

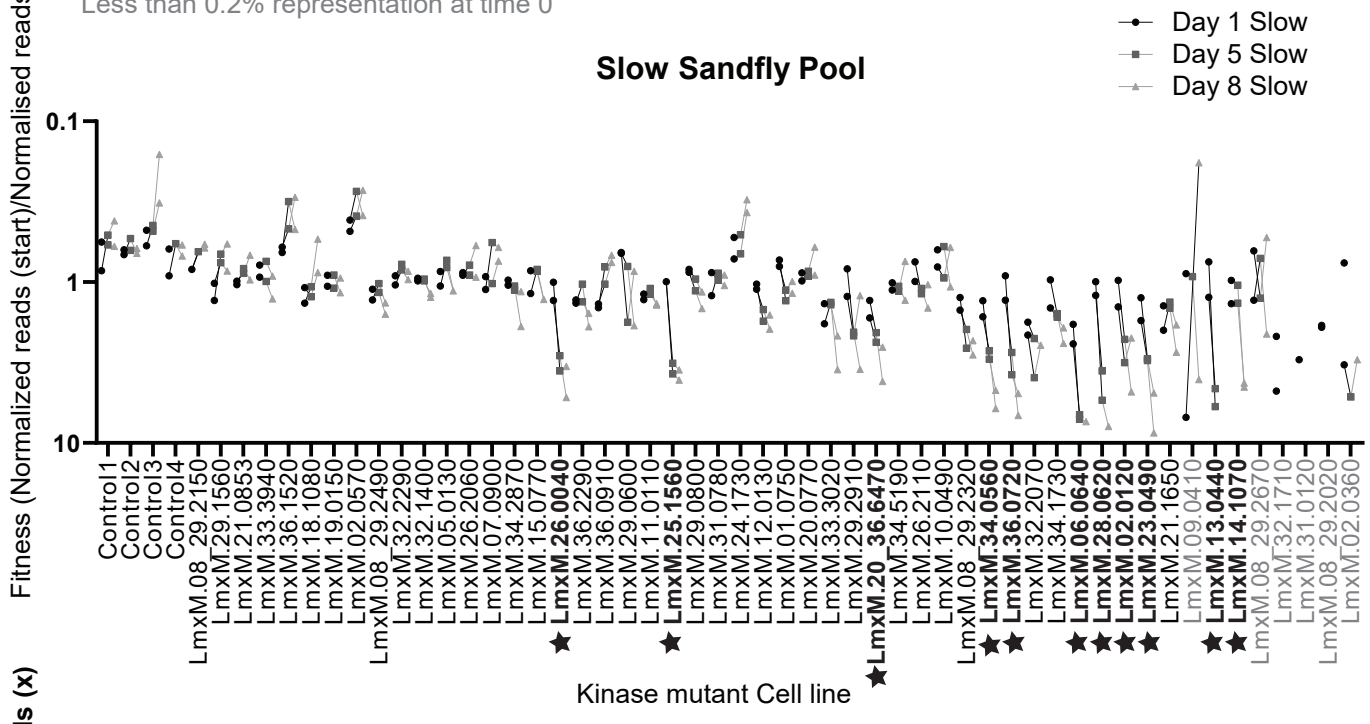


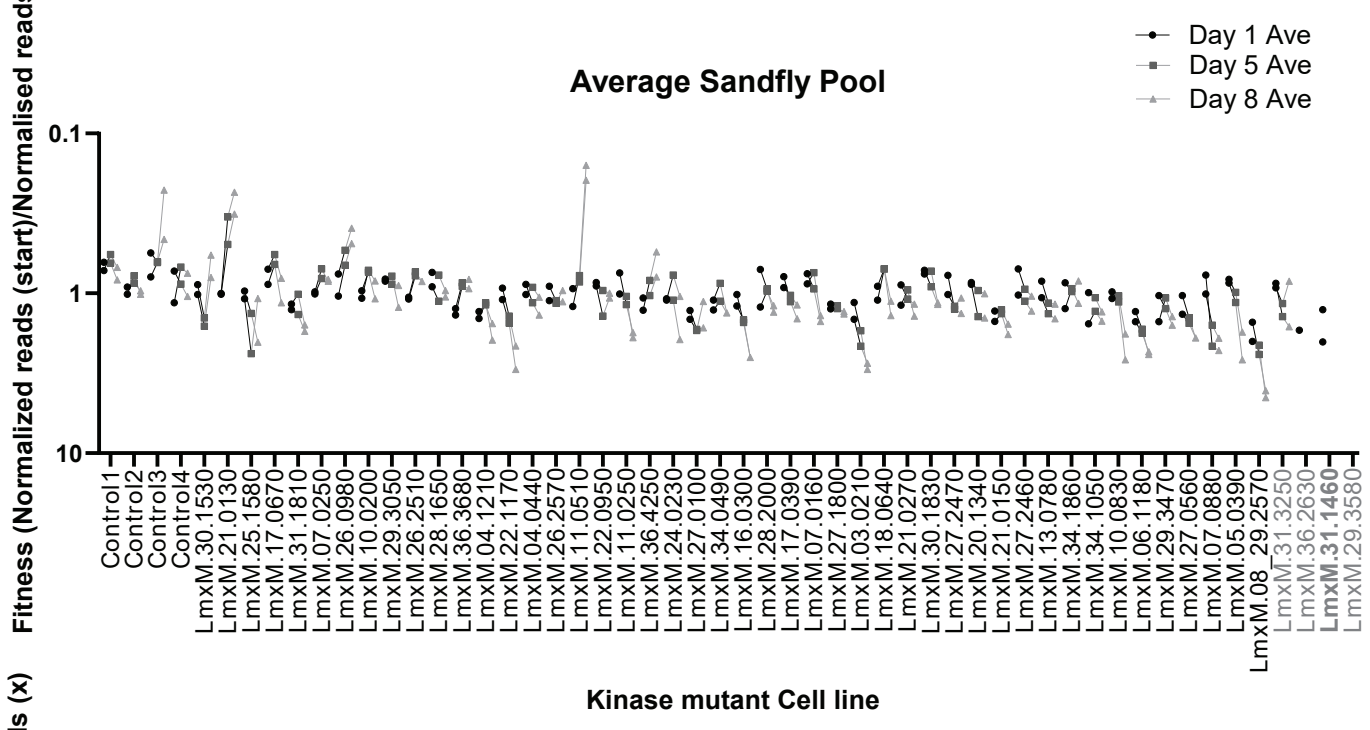

S3.a
300
250
200
150
100
50
0
10^5 cells/mL
0
24
48
72
96
120
144
Time (h)
Cas9 T7
Δrdk1 Clone 1
Δrdk1 Clone 2
b
p = 0.0078
p = 0.0205
600
400
200
0
Amastigotes/200 Mϕ
12 hours
72 hours
Cas9 T7
Δrdk1 Clone 1
Δrdk1 Clone 2
c
p = 0.0009
p = 0.0007
10^8
10^7
10^6
10^5
Number of parasites/Footpad
Cas9 T7
Δrdk1
Δrdk1::RDK1
d
Metacyclic
Amastigote 12h
Amastigote 120h
Procyclic 24h
WT +/- -/-
WT +/- -/-
WT +/- -/-
WT +/- -/-
HASP B
(37 KDa)
Tubulin
(50 KDa)
Ratio
- - -
1.0 1.27 0.67
1.0 1.16 2.17
1.0 3.59 0.006

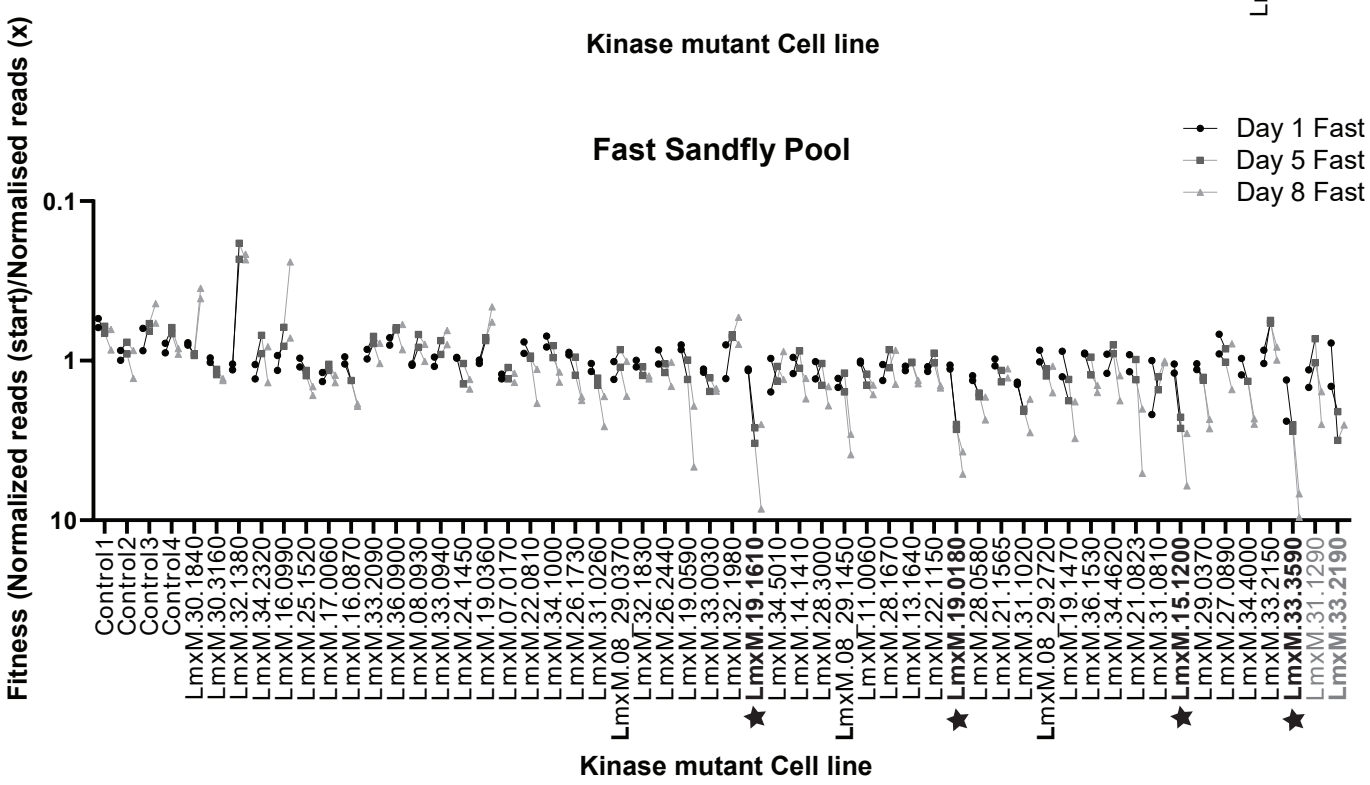
S4.

Significant decrease in fitness ★

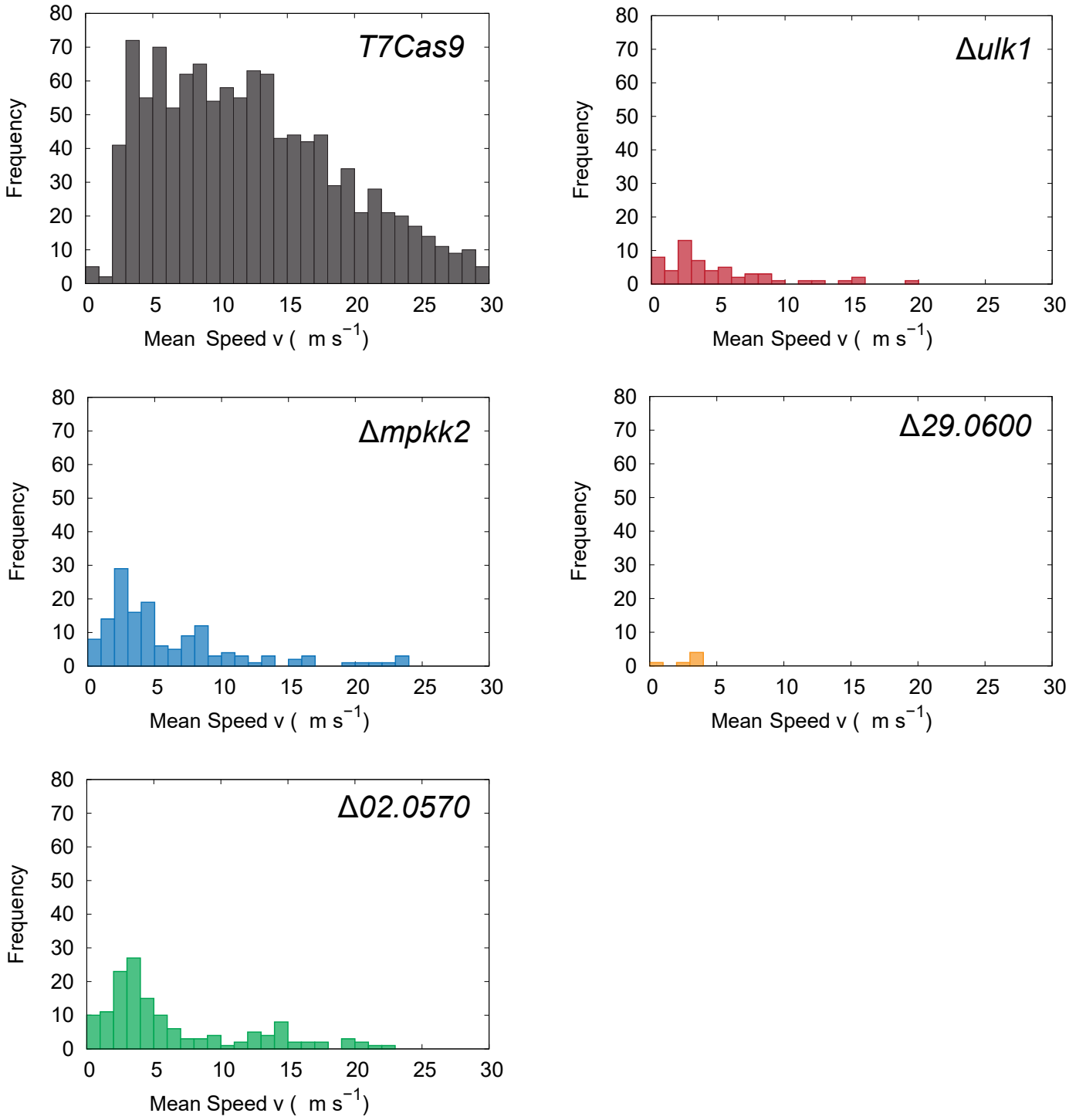
Less than 0.2% representation at time 0







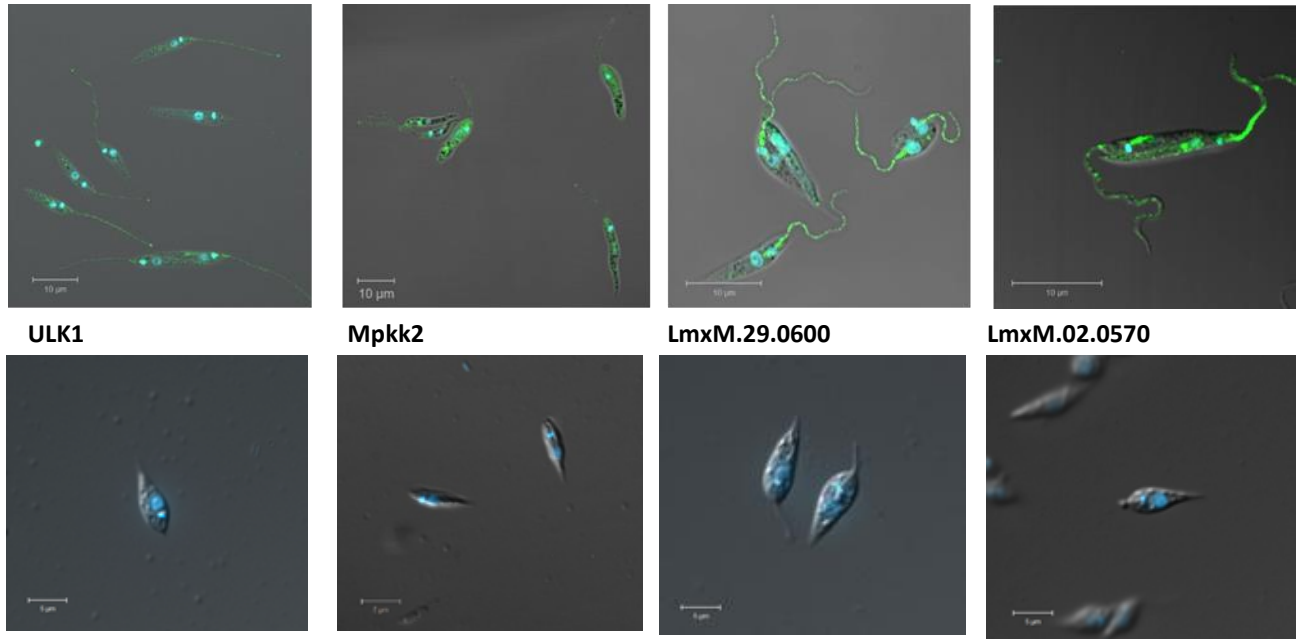
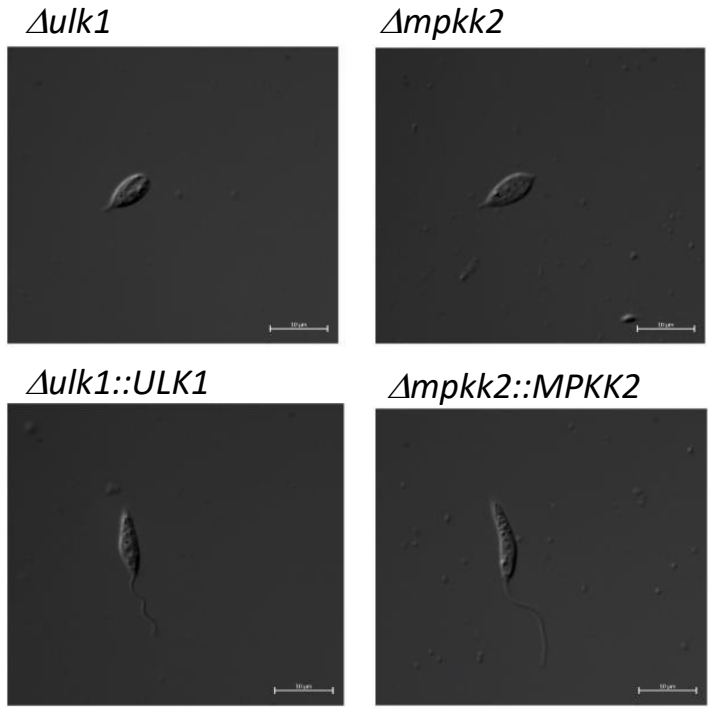
S5.


T7Cas9
Δulk1
Δmpkk2
Δ29.0600
Δ02.0570
Frequency
Mean Speed v (m s^{-1})
0
10
20
30
40
50
60
70
80
0
5
10
15
20
25
30

S6 a.

Accession	Gene Name	Mean Flagellar:Body ratio (n=50)	ST dev of Flagellar: body ratio	Mean cell body width (µm)(n = 50)	ST dev of cell body width	Phenotype	Exp identified	Significant LOF in sand fly?
T7Cas9		1.023	0.302	1.978	0.329			No
LmxM.28.0620	ULK1	0.218	0.109	1.875	0.427	Short flagellum	Both	Yes
LmxM.13.0440	MPKK2	0.061	0.027	2.287	0.347	Short flagellum	Both	Yes
LmxM.26.0600		0.327	0.126	2.36	0.337	Short flagellum	Both	No
LmxM.07.0900		1.182	0.380	3.276	0.746	Paralysed	Both	No
LmxM.02.0570		0.316	0.296	3.472	0.428	Short flagellum	Both	No
LmxM.36.1520		0.914	0.378	4.838	0.779	Uncoordinated	Exp. 1 only	No
LmxM.29.1000		1.901	0.490	2.249	0.421	Long flag, paralysed	Exp. 1 only	Not in pool 2

b



c