Identification of transporters essential for survival of *Leishmania* promastigotes in the digestive tract of sand flies

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Abstract

Leishmania amastigotes ingested by female phlebotomine sand flies are exposed to a harsh and dynamic environment, markedly different from that of their mammalian host. Within the sand fly's alimentary tract, these parasite forms encounter shifts in temperature, pH and nutrient availability, which trigger significant morphological and physiological adaptations. Membrane transporter proteins, channels and pumps play a crucial role in facilitating the movement of solutes across eukaryotic membranes. Previously, a systematic loss-of-function screen of the *L. mexicana* "transportome" identified forty transporter deletion mutants that caused significant loss of fitness in macrophage and mouse infections. Here, using an independently generated library of over 300 barcoded gene deletion mutants, we monitored their growth fitness for seven days *in vitro* and tested which transporters are required for *Leishmania* promastigotes to successfully colonise *Lutzomyia longipalpis* sand flies for nine days. Overall, fitness scores correlated between promastigotes from long-term *in vitro* culture and *in vivo* sand fly infections. More importantly, for 34 mutants, a significant loss of fitness was observed exclusively *in vivo*. Moreover, deletion of the vacuolar H^+ ATPase (V-ATPase) proved detrimental for parasite persistence and promastigote differentiation in the sand fly, uncovering a key role for the V-ATPase at different stages throughout the *Leishmania* life cycle.

Author Summary

Leishmania parasites cause leishmaniases - a group of neglected tropical diseases that affect millions of people worldwide. These parasites must survive in two radically different environments: inside a mammalian host and within the gut of a blood-feeding sand fly. To thrive in the sand fly, *Leishmania* undergo extensive physiological changes and depend on transporter proteins to move nutrients and other molecules across their cell membranes. In this study, we focused on identifying which of these transporters are critical for the parasite's survival inside the sand fly. We used a genetically engineered library of *Leishmania* promastigotes - the parasite form adapted to the insect vector - to assess the importance of more than 300 different transporter genes. We discovered that 34 of these transporters are essential for successful colonization of the sand fly. Among them, one key protein complex - the

vacuolar H^+ ATPase (V-ATPase) pump – was found to be crucial for parasite survival in the insect vector. Our findings deepen our understanding of how *Leishmania* adapts to life within the sand fly and highlight potential molecular targets for disrupting its transmission.

Introduction

Leishmania (Kinetoplastida: Trypanosomatidae) are unicellular eukaryotic protozoa and the causative agents of leishmaniases (1), a group of neglected tropical diseases. Over 20 *Leishmania* species share a digenetic life cycle, alternating between an insect vector and a mammalian host (1). Females of more than 90 species of phlebotomine sand flies - *Phlebotomus* in the Eastern Hemisphere and *Lutzomyia* in the Western Hemisphere - serve as their primary vectors (2).

When a female sand fly takes a blood meal from an infected mammalian host, it ingests immotile amastigote forms, which are encased in a chitin-rich peritrophic matrix (PM) (2). During this stage, parasites face dramatic environmental changes, including a temperature drop (from ~37 °C to ~26 °C), pH shift (from acidic to neutral/alkaline), and altered nutrient availability (from blood components to sugar meals and microbiome metabolites). These signals trigger rapid differentiation from amastigote into promastigotes, often within 6 hours post-feeding (3,4).

Inside the sand fly gut, *Leishmania* promastigotes undergo several differentiation stages. While stage-specific transcriptional profiles have recently been described for *L. major* isolated from *Phlebotomus duboscqi* guts(4), a definitive set of molecular markers for each *L. mexicana* promastigote morphotype is currently lacking. Morphology, location and physiology are therefore still widely used to distinguish different promastigote developmental stages in the sand fly. The initial amastigote to procyclic promastigote differentiation is marked by key metabolic changes, including a ~10-fold increase in uptake of carbon sources (e.g., glucose and non-essential amino acids), and elevated secretion of glycolytic end-products (5–8).

These weakly motile, short-flagellated procyclic forms undergo binary fission for at least 48-96 hours before slowing down their replication and differentiating into highly motile elongated nectomonad promastigote forms (9).

After 72-96 hours post blood feeding, the toxic products of blood digestion cause a reduction in the number of procyclic forms (10) and enzyme-mediated PM disintegration ensures that nectomonads escape the PM-encased blood meal into the midgut lumen (9,11). There, they bind to the midgut epithelium via parasite- and vector-derived surface molecules such as lipophosphoglycans (LPGs) or mucin-like O-glycoconjugates, which prevent their expulsion during defecation (12–15).

Parasites of the *Leishmania* subgenus then differentiate into replicative leptomonad promastigotes and migrate to the anterior midgut (3). During this phase, flagellar motility is crucial for *Leishmania* parasites to migrate through the thoracic midgut toward the stomodeal valve, the structure at the junction between the sand fly foregut and midgut – a process required for transmission. For example, Beneke et al. (2019) used genetically engineered *L. mexicana* promastigotes with impaired flagellar motility to infect *Lu. longipalpis* and observed that these species require directional motility to successfully colonise the fly (16). Furthermore, Cuvillier et al. (2003) showed that overexpression of constitutively active variant of the ADP-ribosylation factor-like protein 3A (ARL-3A) in *L. amazonensis* promastigotes resulted in cells with a short, non-motile flagellum, which failed to colonise *Lu. longipalpis* sand flies (17).

When reaching the stomodeal valve, parasites undergo terminal differentiation into two morphologically distinct forms: replicative, short-flagellated, non-motile haptomonads and non-replicative, long-flagellated highly motile metacyclic promastigotes (18,19). Notably, recent single cell transcriptomic evidence from *L. major* promastigotes isolated from *Ph. duboscqi*, suggest that metacyclic promastigotes may be further divided into two transcriptionally distinct sub-forms; replicative early metacyclics and non-replicative late metacyclics (4). Moreover, the same study provides convincing evidence that in addition to metacyclics, which are traditionally viewed as the primary infective stage, haptomonads also play a significant role in transmission (4).

In mature sand fly infections, parasites secrete promastigote secretory gel (PSG) - a viscous matrix rich in filamentous proteophosphoglycans (fPPGs) - which fills the thoracic midgut (20–24). In addition, they secrete chitinase (25), which damages the insect´s alimentary canal (26). Along with fPPGs, this disruption alters sand fly feeding behavior and promotes regurgitation during subsequent blood meals

(12,27,28). Collectively, these changes enhance parasite transmission by increasing the number of parasites egested into the skin of the mammalian host (28).

Although our understanding of the *Leishmania* life cycle within sand flies still lags behind that of other vector-borne diseases, several molecular determinants of vector colonisation have been identified (3,12,16,24,29–42). Recent advances in reverse genetics and high-throughput barcode sequencing (bar-seq) in *Leishmania* sp. (43) have significantly accelerated discovery of parasite genes essential for promastigote fitness and vector colonisation (16,32,44). For instance, proteins required for flagellar assembly (IFT88, LmxM.27.1130) and motility (e.g. the central pair protein PF16, LmxM.20.1400 or the inner dynein arm protein IC140, LmxM.27.1630) (16) were found to play a crucial role in persistence in sand flies and migration to the stomodeal valve (17). In a separate screen targeting the parasite's kinome, ATM (LmxM.02.0120) and PI4K (LmxM.33.3590), were identified as atypical (aPK) and phosphatidylinositol 3' kinase-related (PIKK) protein kinases, respectively, conditionally essential for survival only in sand flies, suggesting unique pathways are involved in vector-stage survival (32). Another kinase, MPK9 (LmxM.19.0180), was identified as essential for sand fly colonisation (32). In an independent study, MPK9 was shown to influence flagellar length (45), reinforcing the importance of flagellum integrity for the parasite survival inside the vector (45). Moreover, single-cell RNA sequencing is beginning to reveal molecular markers of parasite development in insect stages (4,46). Despite these advances, the role of transporter proteins in sand fly colonisation remains largely underexplored. Exceptions include two nucleotide sugar transporters involved in lipophosphoglycan (LPG) biosynthesis. One, LPG2 (LmxM.33.3120), encodes a GDP-mannose transporter responsible for incorporating the initial and repeating mannose units into the mannose-rich LPG structure and was shown to be essential for development of *L. donovani* and *L. major* in several sand fly species, including *Ph. argentipes*, *Ph. papatasi*, *Ph. duboscqi* and *Ph. perniciosus* (31,33,35,47). Another, LPG5A/B gene array (LmxM.24.0360-65 3120), encodes a UDP-galactose transporter, results in reduced colonisation of *L. major* in *Ph. duboscqi* (35). This transporter incorporates galactose units into the same repetitive LPG backbone, highlighting the importance of glycan modifications in vector attachment and colonisation. This is further supported by ablation of additional (non-transporter) genes involved in LPG biosynthesis (15,16,31,47). Interestingly, LPG mediated binding between sand flies and *Leishmania* is

known to be crucial in parasite-vector systems where the vector is specific, i.e. each glycoconjugate presented by each different *Leishmania* sp. enables extraordinary specificity to a single vector species (48,49). In contrast, permissive vectors such as those within the genus *Lutzomyia*, have been reported to present additional binding mechanisms involving O-glycosylated proteins (50).

Recently, we systematically assessed the fitness contribution of 312 predicted *L. mexicana* membrane transporters, channels and pumps in promastigotes in culture (*in vitro*) during exponential phase of growth, in amastigotes in human induced pluripotent stem derived macrophages (iMACs) and over 6 weeks in mice (*in vivo*) (44). Using bar-seq, we showed that deletion of at least 40 transporters compromised amastigote survival *in vivo* (44). The vacuolar H^+ ATPase (V-ATPase) emerged as a crucial proton pump for the survival of parasites *in vivo*, and *in vitro* under conditions of low external pH (44). Here, we extend that work by conducting an independent comprehensive systematic loss-of-function screen targeting 316 single putative transporter-encoding genes and 17 gene arrays. We assessed mutant fitness over a one-week time course *in vitro* and in a sand fly model of infection *in vivo*. This screen revealed some mutants that show gain-of-fitness phenotypes and many with loss-of-fitness phenotypes. While there was a positive correlation between mutant fitness *in vitro* and in the flies, these results indicate a vital function for ion pumps, sugar nucleotide transporters, and transporters of some other classes, notably several mitochondrial carrier proteins, for survival and fitness within their sand fly vector. Moreover, the V-ATPase is required for effective completion of the developmental cycle from promastigotes to metacyclics *in vivo*.

Results and Discussion

An expanded gene deletion screen of the L. mexicana *transportome reveals that most transporters are dispensable for promastigote survival* in vitro

We previously reported the identification *L. mexicana* transportome, comprising of 312 putative membrane transporters, channels and pumps, and their functional evaluation in the mammalian host parasitic stage in a gene deletion fitness screen (44). Here we expanded on this by studying the fitness phenotypes of promastigote forms under prolonged *in vitro* culture and in sand fly infection assays *in*

vivo. For this screen, we generated arrayed CRISPR/Cas9 mutant libraries targeting the previously identified "transportome" (44) plus four newly identified genes: two from the Acetate Uptake Transporter (AceTr) family, one with similarity to the Selenoprotein P Receptor (SelP-Receptor) family, and one Multidrug/Oligosaccharidyl-lipid/Polysaccharide (MOP) Flippase, as classified by the Transporter Classification Database (TCDB) (51), thus expanding the number of proteins in the *L. mexicana* "transportome" to 316 (Supplementary Table 1) (16,43,44,52). This approach successfully generated 304 viable mutant populations, each resistant to both Blasticidin and Puromycin selection drugs (Supplementary Tables 2,3), broadly organised by TCDB families into four sub-libraries (Supplementary Table 4). Diagnostic PCR confirmed the successful deletion of all copies of the targeted genes for 154 lines (null mutants); the remaining 132 lines where the targeted gene was still detectable were classified as 'refractory to deletion' (Supplementary Table 3). Of the confirmed single-gene deletions, 46 had not been successfully generated in our previous screen (44). This new mutant library also identified three additional single-member superfamilies whose transporters appear dispensable *in vitro*, namely the mitochondrial EF hand Ca^{2+} uniporter regulator (MICU; *LmxM.07.0110*), the Proton-dependent Oligopeptide Transporter (POT; *LmxM.32.0710*) and the Selenoprotein P Receptor (SelP-Receptor; *LmxM.28.2380*) in addition to the eight superfamilies that were previously shown to be dispensable (44).

Deletion of genes arranged in tandem arrays

This screen also expanded the analysis of transporter genes in tandem arrays, targeting a total of 17 arrays, including nine arrays not previously targeted, from the following families: AceTr (1 array), Amino Acid/Auxin Permease (AAAP, 4 arrays), Cyclin M Mg^{2+} Exporter (CNNM, 1 array), Equilibrative Nucleoside Transporter (ENT, 1 array), Major Facilitator (MFS, 2 arrays), Mitochondrial Carrier (MC, 4 arrays), P-type ATPase (P-ATPase, 1 array), Voltage-gated Ion Channel (VIC, 2 arrays), Zinc (Zn^{2+})-Iron (Fe^{2+}) Permease (ZIP, 1 array) (Supplementary Table 1). Upon analysis of the 13 drug resistant mutants that survived the selection, only one AAAP array mutant (*LmxM.34.5350* and *LmxM.34.5360*) was found to be null (Supplementary Table 1 and 3). For the *LmxM.18.1290* and *LmxM.18.1300* array where a null mutant was previously achieved (44), only double puromycin,

blasticidin resistant mutant populations retaining at least one copy of the targeted gene were recovered (Supplementary Table 1 and 3, Supplementary Figure 2). As discussed previously (44), technical challenges such as high sequence similarity and underestimated gene copy numbers can hinder the isolation of null mutants from tandem arrays. For instance, at first, we failed in the isolation of a null mutant for the glucose transporter array which harbours three genes (LmGT1-LmGT3), despite its successful deletion in *L. mexicana* using a strategy of gene knockout by homologous recombination (53,54). In this case, we repeated transfections using both puromycin and blasticidin selection, but only double drug-resistant populations emerged that had retained at least some LmGT genes. It was only after a third round of transfection and subsequent selection of clonal cell lines that we successfully isolated three clones lacking the entire array (Supplementary Figure 5A-C). The doubling times of two null mutant clones were measured and found to be significantly increased (8.73 and 8.24 h) compared to that of the parental cells (5.14 h) (Supplementary Figure 5D-E). This suggests that although the GT-array null mutants are viable, mutants that somehow retained one or several of the genes from the targeted array may have a significant growth advantage in mixed populations. These data show that while it is possible to achieve null array mutants, the technical challenges in identifying and isolating array mutants precludes phenotype screens at scale with this bar-seq method.

Less than 30% of the Leishmania *transportome is essential for promastigote survival*

Consolidating data across the two independently generated libraries indicates that 225 (~71%) transporter-encoding genes are dispensable for promastigote survival in standard *in vitro* laboratory cultures (Figure 1; Supplementary Table 3; Supplementary Figure 1) (44). The successful deletion of these genes is positive proof that they are not essential for cell proliferation under the tested conditions, although they may still contribute to fitness. Conclusive statements cannot be made however about the importance of genes where a deletion attempt failed. For the 91 genes refractory to deletion in two independent screens (Supplementary Table 3), further attempts at gene deletion may yet prove successful. Data released from the LeishGEM genome wide gene deletion screen (https://browse.leishgem.org/) (55) already reports several transporter gene deletion mutants that were

not retrieved in the current screen. Examples include transporter-encoding genes located on supernumerary chromosomes like the putative acidocalcisome inositol 1,4,5-triphosphate receptor/Ca^{2+} release channel (LmxM.16.0280), one of the four amino acid transporters of the AAT1 locus, AAT1.4 (LmxM.30.0350), a glycosomal ABC transporter, GAT1 (LmxM.30.0540) and a major facilitator (LmxM.30.0720); on diploid chromosomes a porphyrin transporter (LmxM.17.1430), the amino acid transporter, AAT23.2 (LmxM.27.0680) and a mitochondrial carrier (LmxM.29.2240) (Supplementary Table 3). Conditional gene knockout strategies would be required for conclusive functional validation and stronger support for a claim of "essentiality" (56).

Prolonged culturing of transporter deletion mutants identifies novel growth fitness phenotypes in vitro

We next asked whether gene disruption had any effect on the relative growth rates of the surviving promastigotes in standard laboratory cultures, over a one-week time course. To assess their relative fitness, all 304 viable isolated barcoded transporter mutants and 13 array mutants, were combined into a single masterpool. To this pool we added five barcoded parental control lines (SBL1-5), eight non-transporter null mutants with previously characterised phenotypes [three independently barcoded Δ*LPG1* (*LmxM.25.0010*, normal growth, important for sand fly colonisation), two independently barcoded Δ*PF16* (*LmxM.20.1400*, normal growth, essential for sand fly colonisation), three independently barcoded Δ*IFT88* (*LmxM.27.1130*, very slow growth, essential for sand fly colonisation) (17), and one non-transporter null mutant Δ*LmxM.15.0240* (nonspecific lipid-transfer protein) (44) (Supplementary Table 4). This masterpool was split into three separate flasks and grown in standard M199 culture medium for seven days. Cultures were diluted into fresh medium twice during this period (Figure 2A) to maintain the populations in the exponential phase of growth (Figure 2B). DNA was sampled at baseline (0 hours), and after 24, 48, and 144 hours (Figure 2) and each mutant's relative representation over time was assessed by measuring DNA barcode abundance at the sampled time points and calculating the proportion of each barcode at a given time point relative to its representation in the starting population (Supplementary Table 5). This showed that the parental control cells and a majority of the mutants maintained a flat trajectory, indicating that they proliferated in the population at similar

rates. There were a small number of mutants with an upwards trajectory, indicating their proportion within the pool increased over time, and a larger set where barcode proportions sharply decreased over time (Figure 2C, Supplementary Table 5, Supplementary Figure 3,4). To quantify this, fitness scores were calculated by comparing the change in barcode proportions for a given mutant to that of the mode of the cell line change distribution (Supplementary Table 5). After 144 hours, seven mutants displayed enhanced fitness in the promastigote *in vitro* pool (fitness score above 2 and $p < 0.05$, Figure 2D): two mutants lacking a mitochondrial carrier (Δ*LmxM.15.0120* and Δ*LmxM.34.3330*), two ABC transporters (Δ*LmxM.06.0100* and Δ*LmxM.11.1290*), two amino acid permeases (Δ*LmxM.30.1820* and Δ*LmxM.27.0680*) and one hypothetical protein of the MSF family (Δ*LmxM.34.2810b*). In contrast, 107 transporter mutants exhibited significantly reduced fitness (score below 0.5 and $p < 0.05$, Figure 2D), with two barcodes dropping below the detection limit at 48 h and eleven at 144 h (zero read counts in all three replicates, Supplementary Table 5). Amongst the mutants disappearing rapidly from the population was a confirmed deletion of ABCB3, which acts in heme and cytosolic iron/sulfur clusters biogenesis and is required for *L. major* virulence (57). This severe loss of fitness in culture may explain why previous attempts to generate null mutants for this transporter were unsuccessful (44,57). Still detectable at the lowest levels were confirmed null mutants for a predicted sodium/hydrogen exchanger of the CPA1 family (Δ*LmxM.14.0980* score 0.014, p=0.0001) and a putative calcium-transporting P-ATPase (Δ*LmxM.32.1010*, score 0.009, p=0.004). Also significantly depleted were mutants for predicted ADP/ATP carrier proteins: Δ*LmxM.07.0530* (MCP15, refractory to deletion, MCP nomenclature taken from (58)) and the tandem array *LmxM.19.0200_LmxM.19.0210* (MCP5, refractory to deletion). The deletion of the fourth predicted ADP/ATP carrier predicted in the *L. mexicana* genome (LmxM.14.0990, MCP16) resulted in a less severe but also significant loss of fitness (score 0.17 and $p = 0.0016$), indicating that these mitochondrial carrier proteins perform vital non-redundant functions in promastigotes.

The culture conditions were designed to provide ample nutrients, a buffered environment and constant temperature. However, over the 144 hours, cells experienced changes in population density and two culture dilutions, thereby possibly being exposed to a variety of stresses, including microenvironmental pH shifts, nutrient depletion, and waste metabolite accumulation. These data show that, although viable,

many of the null mutants were less fit than the parental cell lines and would be outcompeted over time in mixed populations.

Loss and gain of fitness in transporter deletion mutants in vivo

Leishmania promastigotes naturally live in the alimentary tract of phlebotomine sand flies and we next asked which mutants would be able to tolerate this more varied and harsher environment. To evaluate the relative fitness of all viable transporter mutants *in vivo*, we created four sub-pools (named P1-P4) which were added separately to blood feeds presented to female *Lutzomyia longipalpis* sand flies. Each pool contained five barcoded parental control lines (SBL1-5), three non-transporter null controls with established *in vitro* and *in vivo* promastigote phenotypes (ΔLPG1, ΔPF16, ΔIFT88), and on average 86 barcoded mutants per pool. We reasoned that this smaller pool size would reduce the chance of mutants being lost at random considering the small volume ingested by the sand flies. Assuming that in experimental conditions each *Lu. longipalpis* female feed 0.8-1 µl of blood(59), each fly would be expected to ingest 16´000-20’000 if the blood-cell suspension was prepared at 2 x 10^7 parasites/ml, guaranteeing an average of 186-233 parasites per mutant line, from a pool of 86 barcoded mutant lines – a level within the range used in comparable bar-seq studies (16,32). DNA was collected from the parasite-blood mixture at 0 hours (pre-infection) and from infected sand flies after 2 days (48 h) and 9 days (216 h) post blood meal (PBM) (Figure 3A). The barcode proportions for each mutant at each time point were quantified by sequencing. In each cohort, the parental control lines remained stable over the 9-day infection (Supplementary Figure 4C-F), with many of the mutant barcodes following the same trajectory as the parentals, indicating no fitness change. The trajectories of the control mutants Δ*IFT88*, Δ*LPG1*, and Δ*PF16* indicated depletion of these mutants, as expected (16), albeit with some variation between the different pools (Supplementary Figure 4C-F). To quantify these changes, fitness scores were calculated to identify mutants that became significantly depleted or enriched in the flies (Supplementary Table 5). The fitness scores of the mutants 9 days PBM in flies showed a positive correlation (Pearson r = 0.6055) with the fitness scores of promastigotes measured after 144 h in culture (Figure 3B). Across all sub-pools, 80 barcoded transporter mutants displayed significantly reduced

fitness (score < 0.5, $p < 0.05$), while nine mutants showed increased fitness (score > 2, $p < 0.05$) in the sand fly (Figure 3C-F).

There was a small number of mutants that showed a loss-of-fitness phenotype only in the fly (Figure 3B), including a UDP-Gal nucleotide sugar transporter (LmxM.22.1010 HUT1L), one amino acid transporter (LmxM.32.1420), two ABC transporters (LmxM.32.3260 ABCI4 and LmxM.32.1800 ABCD3, the ortholog of the *T. brucei* glycosomal transporter GAT2(60), one uncharacterised MSF transporter (LmxM.01.0440), a putative calcium motive p-type ATPase (LmxM.34.2080) and a subunit of the V-ATPAse (discussed below). While the substrates of most of these transporters remain to be defined, they may contribute to the parasite's metabolic adaptation to changing environments in the fly, or to the secretion of glycoconjugates (e.g. the HUT1L mutant). The importance of glycoconjugate secretion *in vivo* is supported by the phenotype of the control mutant Δ*LPG1*, which became strongly depleted in the flies. Loss of the Golgi GDP-Man transporter LPG2, which lacks a broader range of glycoconjugates including LPG (33,35,47,61), also resulted in mutants with low fitness scores in flies (16), as well as in *in vitro* cultures, although these did not pass the statistical significance test.

The gain-of-fitness mutants included three ABC transporters; ABCG3 (LmxM.06.0100), ABCH1 (LmxM.11.0040) and ABCA6 (LmxM.11.1290), one MFS protein (LmxM.34.2810), one UDP-galactose transporter (LmxM.24.0365), aquaglyceroporin 1 (AQP1, LmxM.30.0020), two folate-biopterin (FBT) transporters; FT1 (LmxM.10.0400) and LmxM.19.0920 and one voltage-gated calcium channels (VGCC) of the VIC family (LmxM.17.1440). The latter encodes one of two L-type VGCCs in *Leishmania*, previously shown to be sensitive to VGCC inhibitors (62). Interestingly, Δ*LmxM.17.1440* promastigotes also exhibited significantly increased fitness *in vitro* after 24h and 144 h (this study and (62)) while their abundance decreased in macrophages at 120 h (score = 0.5, $p < 0.05$ (62)). In contrast, the second VGCC (LmxM.33.0480) showed consistently reduced fitness both *in vitro* and *in vivo*, suggesting that these VGCCs have distinct roles in calcium homeostasis across life cycle stages. Similarly, stage-specific phenotypes were observed for the two predicted magnesium transporters of *L. mexicana*. Mutants where MGT2 (LmxM.25.1090) was targeted, but only double drug-resistant populations, refractory to gene deletion were isolated, resulted in decreased fitness in sand flies (score 0.01, p=0.008), while the MGT1 mutant (Δ*LmxM.15.1310*, null) was enriched in the flies 9 days PBM.

An MGT1 null mutant was previously found to have significantly reduced fitness in mice (44). Prior work suggests AQP1 may function in volume regulation, osmotaxis and antimony [Sb(III)] uptake (63), although its broader biological role remains unclear. While direct measurements sand fly gut or macrophage phagolysosome osmolality are unavailable, estimates suggest values range from 85-448 mOsm/kg in a hematophagous insect gut (64) and 275-295 mOsm/kg in mammalian blood (65). The phagolysosome is likely mildly hyperosmotic due to ion influx, digestion and acidification. How these osmotic shifts across host environments are buffered in the absence of AQP1 remains to be investigated. Similarly, the phenotypes of the FBT family mutants requires further study. Folate transporter FT1 (LmxM.10.0400) is known to be highly expressed in actively dividing promastigotes (66,67). *Leishmania*, like many other eukaryotic cells, are folate (vitamin B9) auxotrophs, requiring external folate uptake. Host folate availability can vary widely depending on nutritional status and microbiota, implying that *Leishmania*'s 13 FBTs (Figure 1B) may be specialised for stage-specific roles. The mutant for the FBT family biopterin transporter BT1 (Δ*LmxM.34.5150*) was significantly depleted from flies 9 days PBM (score 0.003, p=0.02) and completely lost from the promastigote *in vitro* culture.

Whether the higher fitness scores reflected more rapid proliferation or better survival or persistence in the fly following excretion of the digested bloodmeal cannot be deduced from these data. Similarly, a loss of barcodes from the population could indicate a higher death rate or slower proliferation. In the *in vitro* assay, exponential growth of the population was precisely measured, showing a rate of 20.3 doublings over the 144 h time course (Supplementary Table 6). In the fly, the promastigotes normally progress through a series replicative and non-replicative developmental stages. How many exact doublings a wild type *L. mexicana* promastigote is expected to undergo during 9 days in *Lu. longipalpis* is not precisely determined, but it is likely to be fewer than in the constant environment of a culture flask (12). Previous reports, suggest that promastigotes may take ~ 29 h (Supplementary Table 6) to double in the digestive tract of a female sand fly (12). However, this is a very rough estimate, since factors like parasite death or loss from the fly during defecation of bloodmeal remnants, are likely the dominant reasons for severe dropouts in the sand fly. Furthermore, the used standard M199 culture medium in this study, is a high glucose medium routinely used for *in vitro* growth to maximise the growth rate and density of *Leishmania* promastigotes, likely differing significantly from the natural sand fly gut

environment and its energy sources. Thus, small differences in growth rate would be hard to detect in the fly, but easily detectable in culture (12). Likely as a result of that, we observed a greater number of mutants with small but significant changes in fitness *in vitro* (107/304) compared to *in vivo* in sand flies (80/304) (Figure 3B, Supplementary Table 5). We can speculate that this reflects differences between the assays, where the *in vitro* populations underwent a larger number of doublings, combined with the technical advantage of sampling larger amounts of DNA from the *in vitro* cultured cells, allowing for the reliable detection of small differences in replication rate, which may not be detectable in the fly assay.

The V-ATPase of L. mexicana *is critical for survival and parasite differentiation in the sand fly gut*

Vacuolar proton ATPase (V-ATPase) pumps are multi-subunit protein complexes that acidify intracellular organelles by translocating protons across membranes. This complex was already shown to be important for the regulation of endocytosis in *T. brucei* bloodstream forms (68). We have recently demonstrated, that V-ATPases in *Leishmania* localise to a crescent-shaped region near the flagellar pocket (44) and while the loss of V-ATPase subunits had little effect on the growth of promastigotes in standard culture medium at neutral pH, it proved detrimental to promastigotes in acidified culture medium, and caused significant loss of fitness in macrophages and mice (44).

Here, the barcode trajectories of mutants lacking various V-ATPase subunits indicated a moderate decline after 48 hours of promastigote *in vitro* growth (Figure 4A) when the cells had reached a density of $>1 \times 10^7$ cells ml^{-1} (Figure 2B); at that density, dilution into fresh medium was required to maintain the population in log phase. Despite the decline in abundance, the barcodes of V-ATPase mutants were still represented within the pool after 144 h of continued exponential growth. In the sand flies, the decline was more pronounced within just 48 h PBM (Figure 4B), when the infected bloodmeal was still surrounded by peritrophic matrix, and declined further at 9 days PBM, resulting in lower fitness scores than observed *in vitro* (Supplementary Table 5).

To investigate this phenotype further, we introduced an ectopic copy of the V-ATPase subunit E (V_1E, Figure 4C) into the V_1E null mutant cell line (Δ*LmxM.36*.3100, KO) to generate an addback control

(AB) and compared the growth profiles of the three cell lines *in vitro* and *in vivo* (Figure 4D). The growth rates of the KO, AB and parental cell lines were measured over 6 days, with one dilution at the end of log phase on day 3. All three cell lines grew at a comparable rate for the first three days; after dilution the null mutant cell line continued exponential growth but with a slightly longer doubling time. The growth of the AB cell line was indistinguishable from that of the parental control (Figure 4D). We next infected female *Lu. longipalpis* flies with the KO, AB or the parental *L. mex* Cas9 T7 cell line to measure parasite abundance, location, and developmental stages over time (Figure 4 E-G). At 48 h PBM, 100% of flies infected with parental and AB lines showed heavy infections (> 1000 promastigotes/gut, Figure 4E) and parasites were located within the endoperitrophic space surrounded by the peritrophic matrix (Figure 4F). In contrast, the KO persisted in only 40 % of flies, and infections were mostly moderate (100-1000 promastigotes/gut). By day 9 PBM, most flies infected with parental and AB lines developed heavy infections with colonization of the stomodeal valve in 97 % of cases. In contrast, the KO only established moderate or light infections, which were confined to the abdominal or proximal thoracic midgut, and did not reach the cardia (Figure 4E,F).

Quantitative analysis of promastigotes at 9 days PBM showed significant differences in the distribution of parasite morphotypes between KO, AB and parental cell lines. Elongated cells morphometrically classified as “nectomonads” predominated in the KO, whereas leptomonads were less abundant, and metacyclic promastigotes were completely absent when compared to AB and parental lines (Figure 4G, Supplementary Table 7). Additionally, the KO exhibited a significantly longer body and flagella (16 µm, p=0.000; 16.48 µm, p=0.005) compared to the parental cells (10.67 µm; 14.91 µm) (Supplementary Table 7). Expression of an episomal copy of the deleted gene in the KO, significantly restored body length (11.57 µm, p=0.000), but not flagellum length (16.11 µm, p=0.747).

In laboratory cultures, promastigotes in stationary-phase culture may encounter metabolic stress as cultures grow dense, including waste accumulation, nutrient depletion, and cell crowding, which may cause the longer doubling times of the V-ATPase mutants (Figure 4A,D). In the sand fly, these challenges are intensified by competition with the host and its microbiota for limited resources and change of the external pH in the sand fly gut, which shifts from ~6 in unfed or sugar-fed insects to ~8.15 following a blood meal (69). Blood ingestion also triggers diuresis, a process in which hematophagous

insects, including sand flies, expel excess water and concentrate ingested blood, facilitating more blood uptake. This process is mediated by water absorption in the midgut and urine production by the malpighian tubules (69,70). Our results suggest that the V-ATPase may contribute to parasite fitness under these different stresses, beyond adaptation to a low pH environment. This could occur by regulating endocytosis, endo-/lysosomal trafficking, vesicle fusion, protein degradation, and autophagy (71–73) a pathway that has been shown to be important for the differentiation to amastigotes and metacyclic promastigotes *in vitro* (74). Here we show that the differentiation of V-ATPase mutants was delayed or prevented in the insect vector. Taken together, the mutant phenotypes *in vitro*, in the insect vector and in a mammalian host (44) identify the V-ATPase as being key to the parasite's ability to adapt to changing environments at every stage in its life cycle.

Materials and Methods

Leishmania parasites

Promastigote forms of the *L. mexicana* cell line *L. mex* Cas9 T7 (52) and all generated mutants in this study were either grown in T25 cm^2 flasks at 27 °C or flat bottom well plates at 27 °C + 5 % CO_2 in filter-sterilised M199 medium (Life Technologies) supplemented with 2.2 g/L $NaHCO_3$, 0.005% hemin, 40 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) pH 7.4 and 10 % FCS [referred in the text as "standard M199"]. 50 µg/ml Nourseothricin Sulphate and 32 µg/ml Hygromycin B were added to the medium for the maintenance of the *sp*Cas9 and T7 RNA polymerase transgenes (52).

Phlebotomine sand flies

A laboratory colony of *Lutzomyia longipalpis* (originating from Jacobina, Brazil) was maintained in the insectary of the Charles University (Prague, Czechia) under standard conditions (at 26 °C fed on 50% sucrose solution with a 14 h light/10 h dark photoperiod) as described previously (75). The use of laboratory mice for sand fly breeding has been approved by the Ministry of Education, Youth and Sports number MSMT-25062/2023-6. Mice were kept in the animal facility of Charles University in Prague in

accordance with institutional guidelines and Czech legislation (Act No. 246/1992 and 359/2012 coll. on protection of animals against cruelty in present statutes at large), which complies with all relevant European Union and international guidelines for experimental animals.

Revised transportome of Leishmania mexicana

This study included the genes that were previously reported in the 'TransLeish' mutant screen (44), plus four newly identified putative membrane transporter genes: LmxM.03.0370 and LmxM.03.0390, encode for proteins of the The Acetate Uptake Transporter (AceTr) Transporter Classification Data Base (TCDB) family (51), LmxM.28.2380, belonging to the Selenoprotein P Receptor (SelP-Receptor) family and LmxM.28.2410, encoding a protein of the Multidrug/Oligosaccharidyl-lipid/Polysaccharide (MOP) Flippase TCDB family, thus updating the current size of the *L. mexicana* transportome to a total of 316 putative members.

CRISPR-Cas9 gene knockouts

Gene deletions were done using the CRISPR-Cas9 barcoding method previously described (52). Diagnostic PCRs for the validation of gene deletions was done as reported in (44) using ORF_Fw and ORF_Rv primers (Supplementary Table 2). In addition to targeting each gene individually, a total of 17 tandem arrays were targeted and 8 non-transporter null mutant control cell lines were produced [three independently barcoded Δ*LPG1*, two independently barcoded Δ*PF16*, and three independently barcoded Δ*IFT88* (Supplementary Table 1). Fitness screens were done with populations for which gene deletions were assessed by diagnostic PCR, without further subcloning. Exceptionally, for the deletion of the glucose transporter array (LmGT1-GT3), new primers were designed that captured the dissimilar UTR regions flanking the GT array. Drug resistant mutants were cloned by limiting dilution and ORF verification primers for validation of resulting null mutant clones were also redesigned, so that all three copies could be recognised (Supplementary Figure 5).

Generation of V-ATPase subunit E add-back cell line

The LmxM.36.3100 gene was cloned into the pT-add plasmid via restriction digestion cloning (Supplementary File 1). Restriction sites *Spe*I and *EcoR*I were inserted into the 5' and 3' end of the gene, respectively, with the following primers:

SubE_forward_SpeI 5' TCAAGACTAGTATGAGCGAGGCACGCCAAAT 3'

SubE_reverse_EcoRI 5' AATACAGAATTCTTACAGTGGCGCCTCGGTGT 3'

The ΔLmxM.36.3100 cell line was transfected with 5 µg of the newly generated pTadd-LmxM.36.3100 plasmid as described elsewhere (76). After approximately 15 hours following transfection, drug resistant cells were selected by addition of phleomycin at a final concentration of 25 µg/ml and cells were kept in presence of drug for the following 3 passages.

Pooling of cells for bar-seq experiments

The barcoded mutant and parental cell lines were combined in mixed pools, adding similar numbers of each individual cell line. For the *in vitro* screen, a total of 290 individually targeted transporter mutants, 13 array transporter mutants, 5 barcoded parental lines (SBL1-5; barcodes introduced into the SSU locus (16), and 9 non-transporter knock-out mutants, of which 8 acted as controls; 3 Δ*IFT88* (LmxM.27.1130), 3 Δ*LPG1* (LmxM.25.0010) and 2 Δ*PF16* (LmxM.20.1400) different barcoded mutants, were combined into a pool of $1x10^5$ cells/ml (Supplementary Table 4), which was split into three aliquots for replicate measurements of *in vitro* growth.

For sand fly infections, four separate experiments were conducted using distinct pools (Supplementary Table 4, Pool membership). Pool 1 contained 75 individually targeted transporter mutant and one array mutant, Pool 2 contained 71 individual transporter mutants and three array mutants, Pool 3 contained, 77 individual transporter mutants and 5 array mutants and Pool 4 contained 76 individual transporter mutants and 4 array mutants. Each pool also contained five barcoded parental lines (SBL1-5) and three non-transporter knock-out control mutants (Δ*IFT88*, Δ*LPG1*, Δ*PF16*). Each of the four pools was split into three equal aliquots (replicates) in preparation for the infection of female *Lutzomyia longipalpis* sand flies.

Growth curves in vitro

For the *in vitro* growth assay, the mixed pool was split into 3 equal aliquots (replicates), which were left to grow in separate flasks for 48 h, diluted to 1 x 10^6 cells/ml, grown for an additional 24 h, diluted to 1x10^5 cells/ml and grown for an additional 72 h (total 144 hours) at 27 °C + 5 % CO_2 in standard M199. For the growth curves of individual cell lines, log phase promastigotes (between 2 and 4 x 10^6 parasites/ml) of V-ATPase Subunit E (LmxM.36.3100) knockout, add-back (AB) and *L. mex* Cas9 T7 (C9T7) cell lines were seeded in standard M199 at a density of 1 x 10^5 cells/ml. Parasites were left to grow at 27 °C for 3 consecutive days and on day 3, were diluted back to 1 x 10^5 cells/ml density and left to grow at 27 °C. Growth was assessed by counting the cells every 24 hours with a CASY® cell counter (Cambridge Bioscience) using a 60 µm capillary and measurement range set between 2 and 15 µm. For each condition measurements from three replicate flasks were recorded.

Sand fly infections

For infections with pooled barcoded mutant populations, each pool was seeded at a density of 2 x 10^6 parasites/ml and grown for 24 h at 26 °C in standard M199 with 250 µg/ml of Amikacin (Amikin). On the day of infection, a total of either 3 x 10^7 (Pools 1-3), or 1.8 x 10^7 (Pool 4) logarithmic growing parasites were washed three times with sterile 0.9% NaCl saline solution (Braun) and then resuspended in 300 µl of saline, which were then mixed with 2.7 ml of ram's defibrinated blood (LabMedia), previously heat inactivated at 56 °C for 35 minutes. For each separate pool, three groups of 120-180 female sand flies, 4-5 days old, were allowed to feed on the parasite-blood mixture, through a skin membrane from a 1-day old chick, as previously described (62). Fully engorged females were separated and maintained at 26 °C with free access to 40% sucrose solution. Infected sand flies were dissected at days 2 (48 h) and 9 (216 h) post blood-meal (PBM) (Supplementary Table 8). At day 2 PBM, a total of 3 to 9 female sand fly guts were checked to qualitatively assess the progress, localisation and intensity of infection by light microscopy. Parasite abundance was graded into three qualitative categories: negative, light (<100 parasites/gut), moderate (100-1000 parasites/gut) and heavy (>1000 parasites/gut), as described elsewhere (59). For infections with individual promastigote cell lines; female sand flies (5–

9 days old) were infected by feeding through a chick-skin membrane parasites from log-phase cultures (3–4 day cultures), washed twice in sterile saline solution and resuspended in heat-inactivated ram blood at concentration of 1 x 10^6 promastigotes/ml. Engorged sand flies were maintained as described above. Female flies were dissected at days 2 and 9 PBM and the abundance and localisation of *Leishmania* promastigotes in the sand fly digestive tract was examined as described above. Experiments were performed in duplicates.

Sampling of DNA for sequencing

For the promastigote *in vitro* cultures, gDNA was extracted at 0 h, 24 h, 48 h and 144 h of growth from approximately 1x10^7 cells from each replicate culture, using the Wizard® SV Genomic DNA Purification System (Promega) according to the manufacturer's instructions, eluting in 40 µl of bi-distilled water (Ambion). For the *in vivo* experiments, gDNA was extracted from the pool after mixing in standard M199 and from the parasite-blood mixture used for the infection (time point 0). For extraction of genomic DNA from whole infected sand flies, a total of 17 to 82 specimens were collected from each batch at 2- and 9-days post blood meal (PBM) (Supplementary Table 5). For both cells and tissues, the High Pure PCR Template Preparation Kit (Roche) was used and all samples were eluted in 100 µl of VWR Life Sciences PCR grade water as previously described (16).

Bar-seq library preparation and sequencing

The preparation of bar-seq amplicon libraries was done as previously reported (44), with minor changes. For the initial bar-seq amplicon PCR, 100 ng of gDNA isolated from promastigote cultures, 250 ng of gDNA isolated from *Leishmania*-blood meal mix and 500 ng gDNA isolated from whole *Leishmania*-infected female sand flies, were used. To account for the excess of host gDNA present in blood and sand fly derived samples, the number of cycles for the same PCR was increased from 31 for *Leishmania* culture derived samples to 35 for blood and sand fly samples. Raw sequencing files (fastq) for all samples generated during this study were deposited in the European Nucleotide Archive (ENA) study accession PRJEB90861 (ERP173867).

Quantification of barcoded cell line fitness

For each sample, we grouped and counted raw sequencing reads containing barcode sequences, normalised this to the sample total reads and calculated the change in the proportion of reads of each cell line in each sample compared to the zero time point, as previously reported (45). The majority of cell lines in a pool do not exhibit a fitness change, therefore we identified the mode (peak) of the distribution of changes in proportion as the reference to compare to in each replicate. For each time point and cell line we assigned fitness scores by dividing the median of the cell line's change in proportion over all replicates with the median over all modes. A fitness score above one indicates that proportion of barcodes from a particular cell line have increased faster relative to the bulk of the pooled cell lines, corresponding to faster growth and/or better survival than the bulk of the pooled cell lines from the start of the assay up to that time point. A fitness score below one indicates the inverse. P-values were calculated using a paired t-test of the log-transformed cell line changes in proportions against the corresponding reference values, testing the null hypothesis that the cell line change in proportion from all replicates of a particular cell line in a given time point cannot be distinguished from the change of the bulk of the pooled cell lines in all replicates of the same time point. Cell lines were labelled as having a strong fitness phenotype in a given time point if their p-value was below 0.05 and their fitness score was either below 0.5 (deleterious phenotype) or above 2 (beneficial phenotype).

In vivo *parasite morphometry*

Midgut smears of infected sand flies were fixed with methanol, stained with Giemsa, examined by light microscopy with an oil immersion objective and photographed (Olympus DP70) (Supplementary Figure 6, Supplementary Table 7). Body length, flagellar length and body width of 200 randomly selected promastigotes were measured on day 9 PBM using Fiji (65). Four morphological forms were distinguished, based on criteria previously described (10,28). Briefly; elongated nectomonads (EN), body length ⩾14 μm; leptomonads (LE) body length <14 m and flagellar length ⩽2 times body length and metacyclic promastigotes (MP), flagellar length >2 times body length and body length < 14 μm.

Haptomonads were not distinguished in this study as they are often remaining attached to the gut and can be underrepresented on gut smears. Differences in proportion of morphological forms and measurements were compared by a Tukey's HSD (honestly significant difference) test, using the software SPSS version 27.

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Data availability statement

All data supporting the findings of this study are available within the article and its supplementary materials, which have been deposited to Figshare (https://figshare.com/) under Doi: 10.6084/m9.figshare.29481254 (Supplementary Figure 6 and Supplementary Table 7).

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"Identification of transporters essential for survival of *Leishmania* promastigotes in the digestive tract of sand flies"

Figure Legends

Figure 1. Consolidated summary of gene deletion results for the transportome of *Leishmania mexicana*.

(A) Top, pie-charts showing the numbers of successful gene deletions (cyan) and non-successful deletion attempts (magenta), across two independent screens (44 and this study). Bottom, break-down of non-successful deletions attempts into two sub-categories: (i) Double drug-resistant populations where ORF is still detected (or PCR inconclusive) (yellow); (ii) Attempts where no drug resistant populations were ever recovered, or populations where resistant cells could only be recovered with single drug selection and ORF was still detected (dark pink). (B) Summary of gene deletion results separated into TCDB families (Supplementary Table 3); colours as for A.

Figure 2. Fitness of promastigote mutants *in vitro*.

(A) Overview of the experiment timeline for *in vitro* growth of promastigotes in culture. DNA sampling time-points are indicated by yellow arrows. Dark pink arrows denote dilutions, to (a) 1 x 10^6 parasites/ml and (b) 1 x 10^5 parasites/ml. (B) Growth profile of the masterpool of promastigote mutants over time. Data points are the average of three measurements; yellow dots indicate where gDNA was sampled. The dotted line indicates dilution of the cultures. (C) Trajectories of the average of normalised reads of the promastigote masterpool, relative to time-point "0 hours" (T_0). Red dotted line highlight relative barcode abundance of 1. Controls are shown in colour: dark blue, SBL1-5 parental cell lines; Cyan, ΔLPG1; Magenta, ΔIFT88; Yellow, ΔPF16. Grey, all other barcoded cell lines. (D) Volcano plot showing fitness scores against p-values of mutants from the promastigote masterpool after 144 hours of growth. Dashed lines demarcate fitness score thresholds of < 0.5 and > 2, and a significance threshold of $p < 0.05$. Barcodes meeting both threshold criteria are coloured black, non-significant values are grey. Controls are coloured as in D.

Figure 3. Fitness of promastigote mutants *in vivo*.

(A) Overview of the experiment timeline for sand fly infections. DNA sampling time-points are indicated by yellow arrows. (B) Fitness scores from the promastigote masterpool after 144 hours *in vitro* growth plotted against the fitness scores from all mutants 216 hours after infection of sand flies. Black dashed lines mark fitness score thresholds of < 0.5 and > 2. (C-F) Volcano plots showing fitness scores against p-values after 216 h (9 days PBM) in sand flies, separated by sub-pools (P1-P4). Dashed lines demarcate fitness score thresholds of < 0.5 and > 2, and a significance threshold of $p < 0.05$. Barcodes meeting both threshold criteria are colored black, non-significant are grey. Controls are shown in colour: Dark blue dots, SBL1-5 parental cell lines; Cyan, ΔLPG1; Magenta, ΔIFT88; Yellow, ΔPF16. Black numbers denote the abbreviated GeneIDs (LmxM.xx.xxxx) of selected mutants with the highest fitness changes.

Figure 4. V-ATPase is required for differentiation and colonisation in the sand fly vector.

(A) Trajectories of the average of normalised reads for V-ATPase deletion mutants from the promastigote masterpool (grey lines), relative to T_0. Dark blue lines, SBL1-5 parental controls. (B) Barcode trajectories for V-ATPase deletion mutants in sand flies, normalised to the start of the experiment. Colour code as in A. (C) Schematic of V-ATPase pump with each subunit labelled. Subunit E, for which the null mutant was individually characterised, is highlighted in magenta. (D) Growth of parental (PAR, dark blue), V-ATPase V_1E null mutant (KO, magenta) and V-ATPase V_1E add-back (AB, grey) mutant promastigotes *in vitro*. After 3 days of continuous growth, cultures were diluted back to 1 x 10^5 parasites/ml and monitored for an additional 3 days. (E) Parasite abundance in the digestive tract of dissected sand flies infected with PAR, AB or KO parasite lines, assessed at 2 days PBM (left) and 9 days PBM (right). (F) Location of PAR, AB or KO parasite lines at 2 days PBM (left) and 9 days PBM (right). NI, non-infected; ES, endoperitrophic space; AMG, abdominal midgut; TMG, thoracic midgut; CAR, cardia; SV, stomodeal valve. (G) Promastigote morphotypes of PAR, AB or KO parasite lines observed in infected gut smears after 9 days PBM.

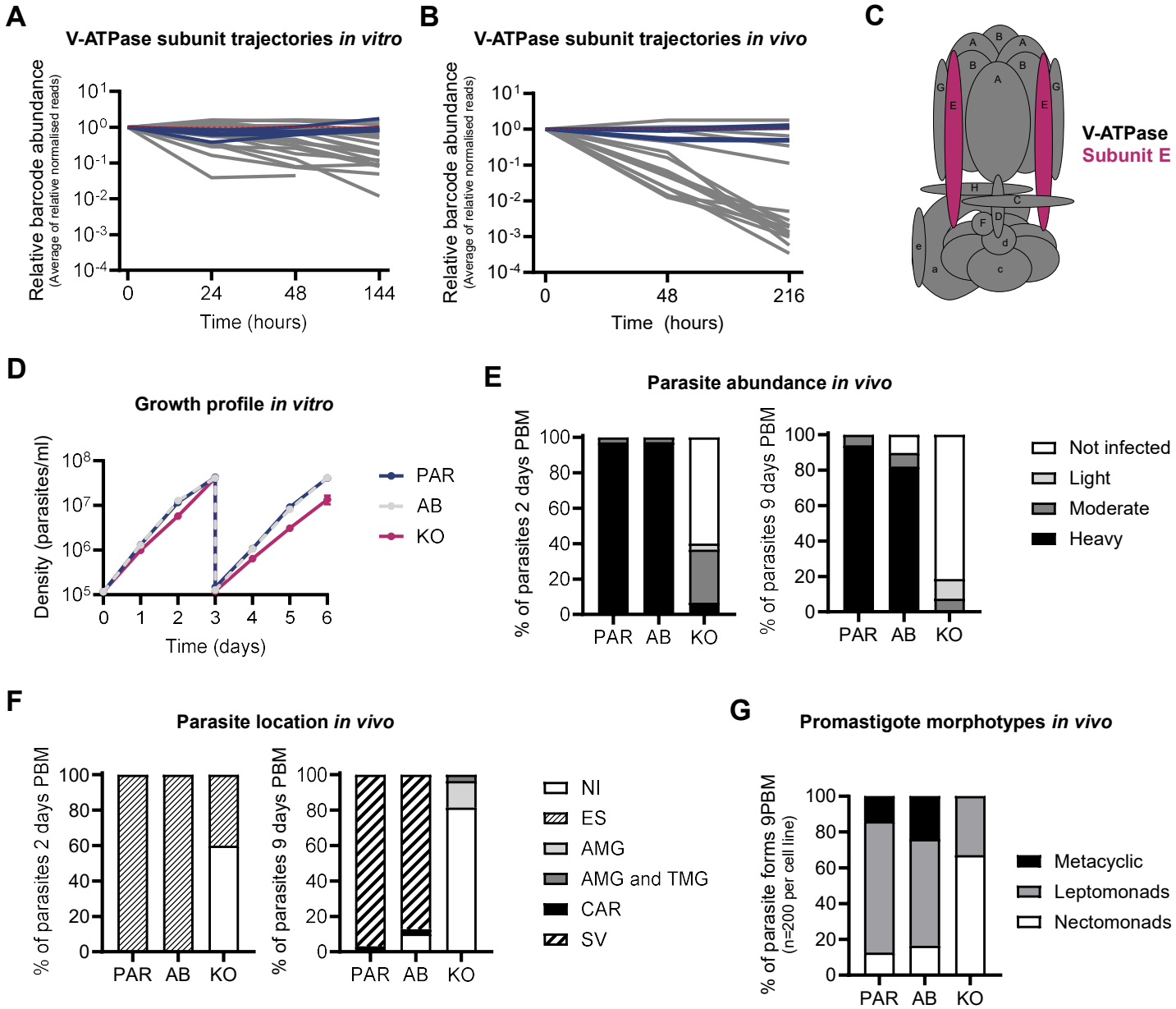
A
V-ATPase subunit trajectories in vitro
Relative barcode abundance
(Average of relative normalised reads)
Time (hours)
B
V-ATPase subunit trajectories in vivo
Relative barcode abundance
(Average of relative normalised reads)
Time (hours)
C
V-ATPase
Subunit E
D
Growth profile in vitro
Density (parasites/ml)
Time (days)
PAR
AB
KO
E
Parasite abundance in vivo
% of parasites 2 days PBM
% of parasites 9 days PBM
PAR AB KO
Not infected
Light
Moderate
Heavy
F
Parasite location in vivo
% of parasites 2 days PBM
% of parasites 9 days PBM
NI
ES
AMG
AMG and TMG
CAR
SV
G
Promastigote morphotypes in vivo
% of parasite forms 9PBM
(n=200 per cell line)
Metacyclic
Leptomonads
Nectomonads

Figure 4

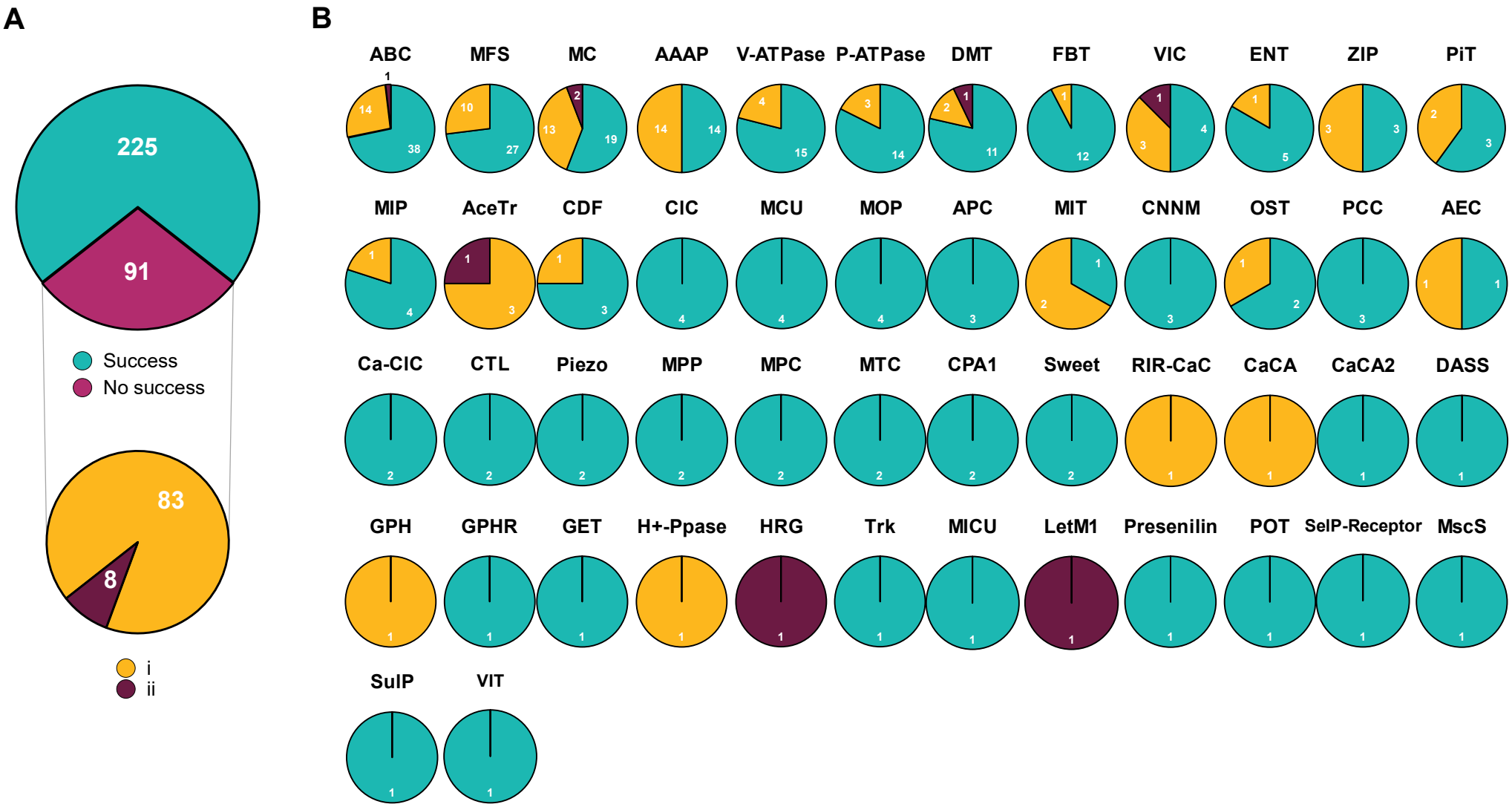
A
225
91
Success
No success
83
8
i
ii
B
ABC
MFS
MC
AAAP
V-ATPase
P-ATPase
DMT
FBT
VIC
ENT
ZIP
PiT
MIP
AceTr
CDF
CIC
MCU
MOP
APC
MIT
CNNM
OST
PCC
AEC
Ca-CIC
CTL
Piezo
MPP
MPC
MTC
CPA1
Sweet
RIR-CaC
CaCA
CaCA2
DASS
GPH
GPHR
GET
H+-Ppase
HRG
Trk
MICU
LetM1
Presenilin
POT
SelP-Receptor
MscS
SulP
VIT

Figure 1

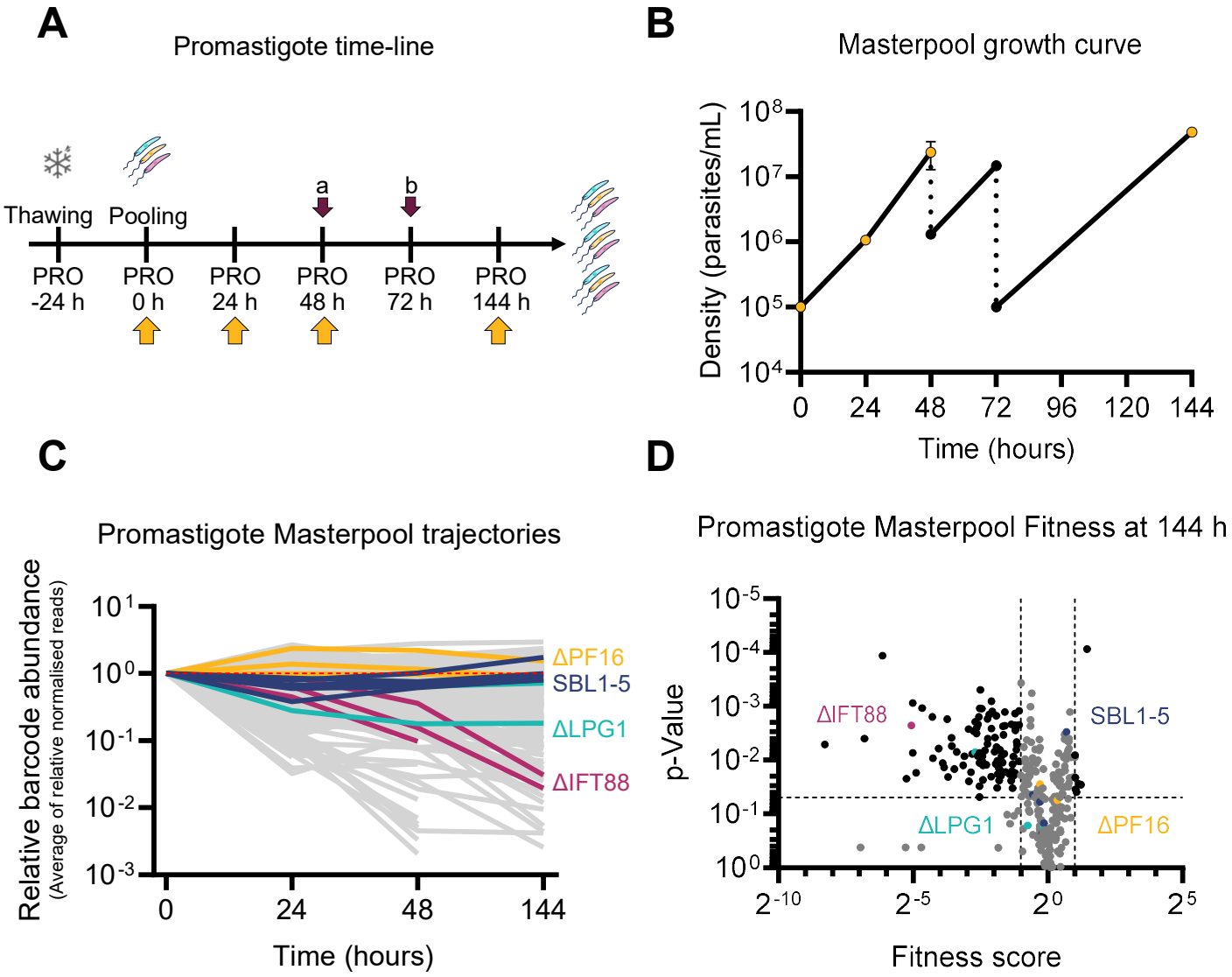
A
Promastigote time-line
Thawing
Pooling
a
b
PRO -24 h
PRO 0 h
PRO 24 h
PRO 48 h
PRO 72 h
PRO 144 h
B
Masterpool growth curve
Density (parasites/mL)
Time (hours)
C
Promastigote Masterpool trajectories
Relative barcode abundance
(Average of relative normalised reads)
ΔPF16
SBL1-5
ΔLPG1
ΔIFT88
Time (hours)
D
Promastigote Masterpool Fitness at 144 h
p-Value
ΔIFT88
SBL1-5
ΔLPG1
ΔPF16
Fitness score

Figure 2

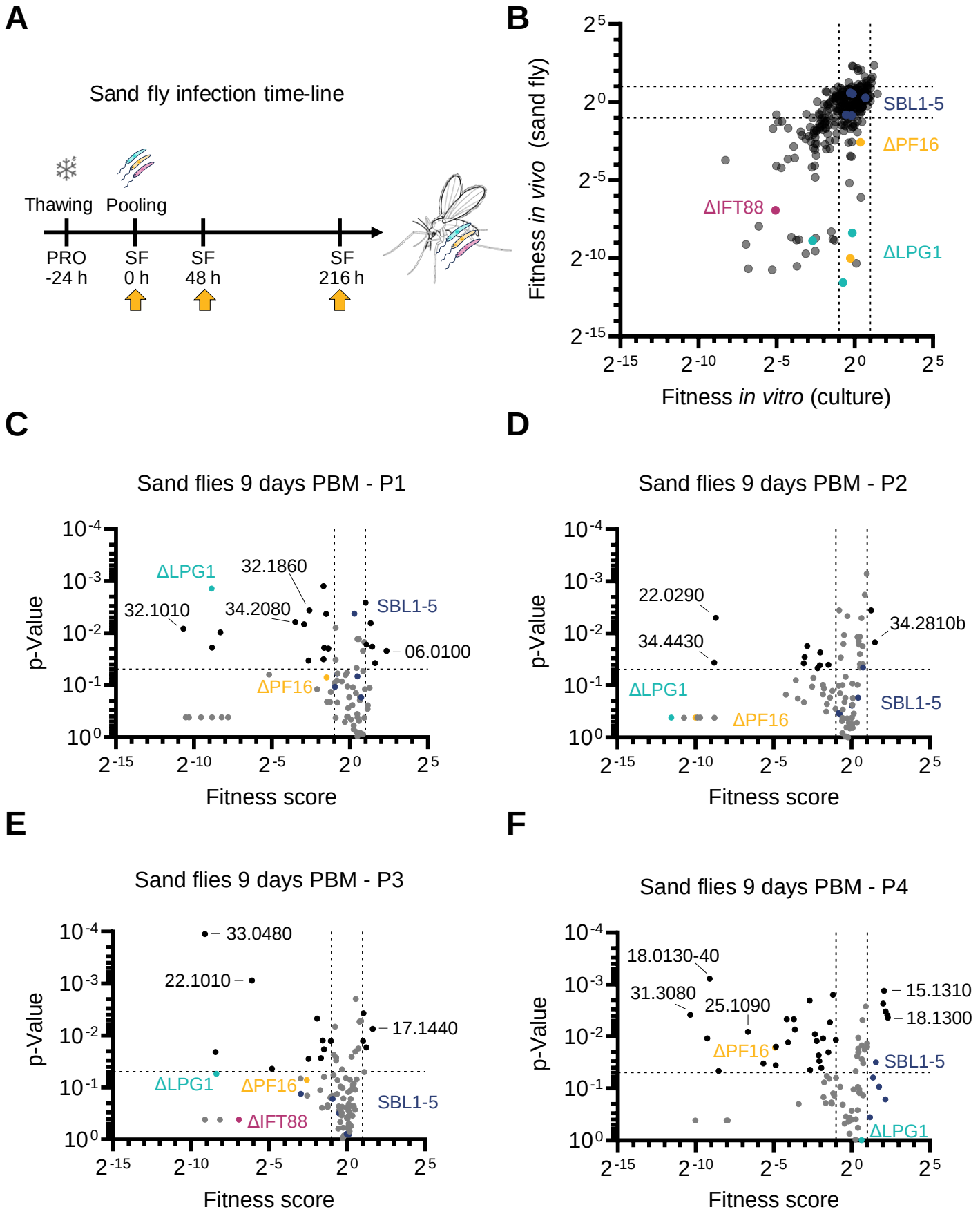
A
Sand fly infection time-line
Thawing
Pooling
PRO -24 h
SF 0 h
SF 48 h
SF 216 h
B
Fitness in vivo (sand fly)
Fitness in vitro (culture)
SBL1-5
ΔPF16
ΔIFT88
ΔLPG1
C
Sand flies 9 days PBM - P1
p-Value
Fitness score
ΔLPG1
32.1860
32.1010
34.2080
SBL1-5
06.0100
ΔPF16
D
Sand flies 9 days PBM - P2
22.0290
34.2810b
34.4430
ΔLPG1
ΔPF16
SBL1-5
E
Sand flies 9 days PBM - P3
33.0480
22.1010
17.1440
ΔLPG1
ΔPF16
SBL1-5
ΔIFT88
F
Sand flies 9 days PBM - P4
18.0130-40
31.3080
25.1090
15.1310
18.1300
ΔPF16
SBL1-5
ΔLPG1

Figure 3