‘Transient’ genetic suppression facilitates generation of hexose transporter null mutants in Leishmania mexicana

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Summary

The genome of Leishmania mexicana encompasses a cluster of three glucose transporter genes designated LmxGT1, LmxGT2 and LmxGT3. Functional and genetic studies of a cluster null mutant (Δlmxgt1-3) have dissected the roles of these proteins in Leishmania metabolism and virulence. However, null mutants were recovered at very low frequency, and comparative genome hybridizations revealed that Δlmxgt1-3 mutants contained a linear extrachromosomal 40 kb amplification of a region on chromosome 29 not amplified in wild type parasites. These data suggested a model where this 29-40k amplicon encoded a second site suppressor contributing to parasite survival in the absence of GT1-3 function. To test this, we quantified the frequency of recovery of knockouts in the presence of individual overexpressed open reading frames covering the 29-40k amplicon. The data mapped the suppressor activity to PIFT3, encoding a component of the intraflagellar transport pathway. We discuss possible models by which PIFT3 might act to facilitate loss of GTs specifically. Surprisingly, by plasmid segregation we showed that continued PIFT3 overexpression was not required for Δlmxgt1-3 viability. These studies provide the first evidence that genetic suppression can occur by providing critical biological functions transiently. This novel form of genetic suppression may extend to other genes, pathways and organisms.

Introduction

Glucose and other hexoses are critical nutrients for many cells, and uptake of these hexoses through selective permeases has been widely studied in a broad range of organisms (Manolescu et al., 2007). Parasitic protozoa of the genus Leishmania take up and metabolize glucose and other sugars throughout their life cycle. These parasites exist as flagellated, extracellular promastigotes that live in the gut of the sandfly vector and as non-motile amastigotes that reside within acidified phagolysosomal vesicles of vertebrate macrophages. Promastigotes are exposed to very high concentrations of sugars, especially sucrose and its cleavage products glucose and fructose, when the sandfly takes a meal of plant honeydew (Schlein, 1986), but the levels of sugars drop as this meal is digested, and metabolism of proline that is abundant within the insect haemolymph (Mazareb et al., 1999) provides a postulated alternate energy source for promastigotes when they experience hexose-limited conditions (ter Kuile, 1993). While no direct measurements are available for the nutrient composition of the parasitophorous vacuole within mammalian host macrophages, it is thought that this environment is relatively carbohydrate-poor (Burchmore and Barrett, 2001; McConville et al., 2007; Naderer and McConville, 2008). Nonetheless, uptake and metabolism of hexoses occurs within intracellular amastigotes (Burchmore and Hart, 1995), albeit at levels that are reduced compared to promastigotes (Hart and Coombs, 1982; Mottram and Coombs, 1985). Indeed, several studies have revealed that uptake or metabolism of various hexoses is of central importance to viability of intracellular amastigotes (Burchmore et al., 2003; Naderer et al., 2010).

The genomes of Leishmania parasites encode a cluster of three glucose/hexose transporter genes designated LmxGT1, LmxGT2 and LmxGT3 for Leishmania mexicana (Burchmore and Landfear, 1998), encoding permeases of the SLC2 or GLUT transporter family (http://www.bioparadigms.org/slc/menu.asp). While the sequences of these permeases are closely related within the membrane-associated ‘core’ of the transporter that encompasses
transmembrane domains 1–12, the N-terminal and C-terminal hydrophilic domains outside of this core differ considerably and are accompanied by functional divergence. Thus, LmxGT1 is localized largely to the flagellar membrane, whereas LmxGT2 and LmxGT3 are excluded from the flagellar membrane and reside in the pellicular plasma membrane that surrounds the cell body (Burchmore et al., 2003). The mRNAs encoding LmxGT1 and LmxGT3 are expressed at similar levels in promastigotes and intracellular amastigotes, but the level of LmxGT2 mRNA is ~15-fold higher in promastigotes. All three permeases transport the hexoses glucose, fructose, mannose and galactose as well as the pentose ribose (Naula et al., 2010).

Hexose uptake and metabolism is critical for viability of intracellular amastigotes. Thus, deletion of the entire cluster of LmxGT1-LmxGT2-LmxGT3 genes [null mutant previously designated Δmgt (Burchmore et al., 2003) but referred to hereafter for clarity as Δlmxgt1-3, Fig. 1A] (Burchmore et al., 2003) strongly impairs the ability of amastigotes to replicate and survive inside primary murine macrophages (Burchmore et al., 2003). The inability of this null mutant to thrive in macrophages results from multiple phenotypic deficiencies that undermine the viability of parasites when they enter the environment of the macrophage. Thus, the null mutants (i) experience an ~8-fold reduction in the storage carbohydrate and virulence factor mannogen that is critical for amastigote viability (Ralton et al., 2003), (ii) become highly susceptible to oxidative stress, which they experience inside macrophages (Stafford et al., 2002) and (iii) become more susceptible than wild type parasites to increased temperature (shift from 26°C to 33°C) and to nutrient restriction. All of the above deficiencies diminish the ability of the null mutants to survive stresses they encounter upon entry into mammalian macrophages (Rodriguez-Contreras and Landfear, 2006; Rodriguez-Contreras et al., 2007).

In contrast to amastigotes, the Δlmxgt1-3 null mutants are viable as promastigotes, provided that the alternative energy source proline is available (Burchmore et al., 2003). The current studies pursue the observation that the null mutants were initially very difficult to generate, requiring years of effort to obtain two independent null mutants, despite the fact that once obtained they were viable in culture. This suggested that an unanticipated ‘second site’ suppressor may have arisen at low frequency during generation of the Δlmxgt1-3 null mutant and that the rare null mutants that had simultaneously acquired this suppressor mutation were more readily retrieved than those that had not. Second site suppressors are not uncommon in the genetic literature, and a candidate for such a suppressor arose in comparative genomic hybridization studies, in which Δlmxgt1-3 DNA was compared to that of wild type L. mexicana (Feng et al., 2008) using a DNA microarray.

**Fig. 1.** Flow diagrams demonstrating the origins and genotypes of null mutant lines generated and the relative ease (facile) or difficulty (difficult) with which each could be obtained (see Fig. 2B for quantifications).

A. Generation of the Δlmxgt1-3 null mutants beginning with wild type parasites. The first heterozygous knockout line is designated +/-Δlmxgt1-3 and had undergone facile targeted gene replacement of one allele of the entire cluster of LmxGT1, LmxGT2 and LmxGT3 genes. Subsequent replacement of the second allele of this gene cluster to generate the Δlmxgt1-3 homozygous null mutant was relatively difficult. The grey boxes indicate drug resistance cassettes that replaced the targeted alleles (PAC, puromycin acetyl transferase; SAT, streptothricin acetyl transferase conferring resistance to the drug nourseothricin). The black box represents the 29-40k linear amplicon derived from chromosome 29.

B. Generation of the single gene null mutants Δlmxgt1 (scheme 1, circled number 1), Δlmxgt2 (scheme 2) and Δlmxgt3 (scheme 3). The Δlmxgt1 and Δlmxgt3 knockouts could be obtained easily, but the Δlmxgt2 null mutant was not obtained (quantifications in Fig. 2B) by conventional targeted gene replacement (left-hand arrow in scheme 2). Subsequently, prior complementation with an episome encompassing the LmxPIFTC3 ORF (pPIFTC3, right-hand arrow in scheme 2) allowed generation of Δlmxgt2 null mutants that contained this episome (black rectangle-marked pPIFTC3).

C. Assay for identification of the ORF within amplicon 29-40k that facilitates knockout of the LmxGT2 gene. Individual or clustered ORFs (Fig. 2C, p1–p9) from the 29-40k amplicon were subcloned into the pX63NEO-Ri plasmid and transfected into the +/-Δlmxgt2 heterozygote (top). This resulted in nine lines, P1–P9, each bearing a plasmid with a different single or cluster of ORFs (designated pX63NEO-p1-p9). Each line was then transfected with a GT2 targeting construct containing sequence upstream and downstream of the GT2 ORF plus the PAC gene. Puromycin-resistant colonies that emerged from each transfection were tested by genomic Southern blots to determine which ones represented bona fide Δlmxgt2 homozygous null mutants (bottom).
probing most known *Leishmania* open reading frames (ORFs). Notably, in the two independent Δlmxgt1-3 null mutants obtained in our original studies, we observed the presence of an amplified region of chromosome 29 encompassing ~40 kb of DNA and 14 ORFs (referred to here as the 29-40k amplicon).

Here we show that the 29-40k amplicon provides the suppressor function promoting the ability of the hexose transporter null mutants to be recovered. Because we were unable to generate null mutants, by conventional targeted gene replacement strategies, of the *LmxGT2* gene that encodes the most abundantly expressed of the three glucose transporter mRNAs, we also included this single gene deletion in further studies of the suppressor function of the 29-40k amplicon. We developed an assay in which the frequency of recovery of Δlmxgt2 knockouts was quantified in the presence of an episomal multicopy cosmid bearing a recombinant version of the 29-40k amplicon, or individual ORFs overexpressed from multicopy episomes. We present evidence that a single gene within the 29-40k amplicon, *PIFCT3* (encoding a protein acting within the intraflagellar transport pathway), promotes retrieval of the Δlmxgt1-3 and Δlmxgt2 null mutants. However, in contrast to most genetic suppressors, *PIFCT3* or amplicon overexpression was required only transiently during generation of the null mutants.
Unexpectedly, we found by using plasmid segregation methods that the ‘suppressing’ PIFCT3-containing episomes could be rapidly lost from the $\Delta lmxgt1$-3 null mutants without any detectable functional consequences. The suppressing properties of PIFCT3 appeared specific to the GT locus, as no alterations were seen in a wide array of other genetic knockouts. Last, in this manner, we were able to generate ‘clean’ or defined single gene deletion mutants for $Dlmxgt1$, $Dlmxgt2$ and $Dlmxgt3$ (Fig. 1B), allowing us to revisit the roles of the individual GT transporters.

Importantly, the discovery of a ‘transient’ or ‘facilitating’ suppressor suggests the possibility that this is a more general mechanism of genetic suppression, one that had been overlooked. This is likely to have important consequences to genetic analysis of Leishmania and other organisms.

Results

Null mutants are readily obtained for the single transporters GT1 and GT3 but not GT2

The $\Delta lmxgt1$-3 null mutant (Fig. 1A) is impaired in growth of promastigotes and especially of amastigotes (Burchmore et al., 2003), demonstrating the importance of this family of transporters in both life cycle stages. However, to define potentially distinct biological functions of GT1, GT2 and GT3 permeases, it was necessary to generate single gene null mutants by targeted gene replacement (Cruz and Beverley, 1990) in which the two alleles of each gene were replaced by drug selectable markers (Fig. 1B), flanked by targeting segments of DNA upstream and downstream of the individual ORF. This approach readily generated null mutants $\Delta lmxgt1$ (four out of six transformed colonies examined or 67%, Fig. 1B, scheme...
Fig. 2. A. Southern blot of genomic DNA digested with BglII/EcoRI from wild type (WT), \(\Delta\)lmxgt1-3, \(\Delta\)lmxgt1, \(\Delta\)lmxgt2 and \(\Delta\)lmxgt3 null mutants. The panel on the left shows the ethidium bromide stained gel, and the panel on the right is the blot hybridized with a 676-nucleotide fragment representing the 3’ half of the LmxGT2 ORF. The 8.3 kb band represents LmxGT1, the 3.6 kb band is LmxGT2, and the 1.8 kb band is LmxGT3.

B. Table showing the number and percentages of colonies representing confirmed null mutants (positive colonies) for various targeted gene replacement experiments. The null mutants showed correct integration, as assessed by PCR amplification employing primers on either side of the 5’ and 3’ insertion sites, and the absence of the appropriate hybridizing bands or bands on genomic Southern blots, as shown in (A). Knockout experiments either employed parasite lines without any prior complementation (top three rows) or lines that had been complemented following the first round of integration (bottom five rows) with cosmid AC5 or the pX63NEO-RI or pXNG4 episomal expression vectors carrying Knockout experiments either employed parasite lines without any prior complementation (top three rows) or lines that had been complemented following the first round of integration (bottom five rows) with cosmid AC5 or the pX63NEO-RI or pXNG4 episomal expression vectors carrying one or several inserted ORFs from this cosmid. [PIFTC3-int] represents a line in which the LmxPIFTC3 gene was integrated into rDNA repeats to promote overexpression from this highly transcribed locus.

C. Schematic representation of the amplified segment of chromosome 29 identified in two \(\Delta\)lmxgt1-3 null mutants. This figure is modified from fig. S1B in Feng et al. (2008). The data are from a comparative genomic hybridization experiment in which genomic DNA from WT and \(\Delta\)lmxgt1-3 null mutants were hybridized to oligonucleotides (represented by diamonds) spanning the \(L\). major genome. The \(y\)-axis represents the log2 value of the hybridization signal for \(\Delta\)lmxgt1-3 WT. The positions of the 14 ORFs (from genes LmxM08_29.2730-29.2860) are indicated by the bars below the plot. (The ampiclon was originally thought to contain 15 ORFs based on the microarray data (Feng et al., 2008), but genomic Southern blots have subsequently demonstrated that ORF designated LmxM08_29.2870 is not amplified in the \(\Delta\)lmxgt1-3 mutant). The designations p1-p9 below the ORFs indicate the single or multiple ORFs that were inserted into the pX63NEO-RI plasmid expression vector and employed to transfect the heterozygous null mutant of LmxGT2. The one plasmid construct/ORF (p3/ORF4) that facilitated knockout of the LmxGT2 ORF, the right panel was hybridized with the LmxPIFTC3 ORF, and the panel below was hybridized with a probe from the LmxGT4 ORF as a loading control.

D. Southern blot of undigested genomic DNA from WT and \(\Delta\)lmxgt1-3 null mutant. Intact chromosomes were separated using a clamped homogeneous electric field gel. The left panel represents the ethidium bromide stained gel, and the right panel represents the blotted filter hybridized with a probe from the LmxPIFTC3 ORF. Chr 29 represents the hybridization signal for LmxPIFTC3 on chromosome 29, and Ampliclon represents the amplified linear 40 kb segment of chromosome 29 present in the \(\Delta\)lmxgt1-3 null mutant but not in WT parasites. E. Southern blots of genomic DNA, digested with BglII/EcoRI, from WT parasites, the \(\Delta\)lmxgt1-3 null mutant, this null mutant made by previous complementation with the LmxPIFTC3 ORF ([pPIFTC3]), and four of the null mutants that have been cured of the LmxPIFTC3 episome (C1–C4) and isolated by fluorescence-activated cell sorting as described in the text. The blot on the left was hybridized with a probe representing the LmxGT2 ORF, the right panel was hybridized with the LmxPIFTC3 ORF, and the panel below was hybridized with a probe from the LmxGT4 ORF as a loading control.

Amplification of an extragenic suppressor facilitates retrieval of null mutants of the entire GT1-3 gene cluster or of GT2

Previous comparative genomic hybridization studies (Feng et al., 2008) comparing wild type and \(\Delta\)lmxgt1-3 null mutants revealed an unanticipated amplification of an ~40 kb segment of chromosome 29 in \(\Delta\)lmxgt1-3 cells (Figs 1A and 2C). This amplification (termed 29-40k) appears in the \(\Delta\)lmxgt1-3 null mutant that was obtained by double targeted gene replacement, but not in wild type parasites or the heterozygous null mutant (Feng et al., 2008), and is represented by a linear extrachromosomal segment of ~40 kb in Southern blots of undigested genomic DNA hybridized with a radioactively labelled probe from the ampiclon (Fig. 2D). Indeed, Southern blot analysis showed that this same ampiclon was also present in a second independently generated \(\Delta\)lmxgt1-3 null mutant that had been made by subjecting the heterozygous +/~\(\Delta\)lmxgt1-3 line (Fig. 1A), to a ‘loss of heterozygosity protocol’ involving selection with the cytotoxic glucose analogue 2-deoxy-D-glucose (R.J.H. Burchmore, unpubl. data) (Gueiros-Filho and Beverley, 1996). Hence, two different strategies used to recover \(\Delta\)lmxgt1-3 homozygous null mutants resulted in lines bearing the 29-40k ampiclon.

We speculated in the earlier study (Feng et al., 2008) that this ampiclon might function as a suppressor that facilitated the generation of the null mutant. To test this
model, we first generated a cosmid library of wild type *L. mexicana* DNA using the shuttle vector cLNEO (Ryan et al., 1993), and recovered a cosmid encompassing ORFs 1–11 (cosmid AC5) of the 14 ORFs present in the chromosome 29-40k amplicon (Fig. 2C). As the studies above had underscored the difficulty in deleting the *GT2* gene, we transfected cosmid AC5 into heterozygous null mutants at the *LmxGT2* locus (+/∆*lmxgt2*, Fig. 1B), generated from a single round of targeted gene replacement employing a construct containing a hygromycin resistance marker, and lacking the 29-40k amplification. We then introduced a second *GT2* targeting fragment encompassing a puromycin resistance marker to determine whether introduction of a second marker, and lacking the 29-40k amplification. We then introduced a second *GT2* targeting fragment encompassing a puromycin resistance marker to determine whether supplementation by this multicopy cosmid could promote selection of a homozygous ∆*lmxgt2* null mutant. Indeed, prior complementation with cosmid AC5 resulted in two ∆*lmxgt2* null mutants among 27 colonies examined (Fig. 2A and B), versus 0/305 in the studies above lacking AC5. This observation supports the notion that amplification of the 29-40k amplicon sequences included in cosmid AC5 promotes the ability to retrieve viable ∆*lmxgt2* and ∆*lmxgt1-3* null mutants, thereby acting as a ‘suppressor’ facilitating the recovery of each null mutant.

It is notable that the frequency of ∆*lmxgt2* null mutants transfected with the AC5 cosmid (Fig. 2B, 7%) is still ~10-fold less than that for untransfected ∆*lmxgt1* or ∆*lmxgt3* null mutants (67% and 86% respectively). Thus, the suppressor function of the AC5 cosmid is not sufficient to completely reverse the barrier to generating the ∆*lmxgt2* null mutant. Nonetheless, it did greatly facilitate the process of deleting a gene that we have not been able to knock out in the absence of a suppressing amplicon.

### Identification of a single ORF on the chromosome 29 amplicon as the suppressor

To determine which gene or genes within the 29-40k amplicon provide the suppressor function, we developed the assay outlined in Fig. 1C. Single ORFs or segments containing several ORFs from this amplicon [Fig. 2C, the 14 ORFs are *Lmx.M08* 29.2730-29.2860 in the *L. mexicana* genome (Aslett et al., 2010)] were amplified by the polymerase chain reaction employing the primers in Table S1 and then subcloned into the pX63NEO-RI (Valdes et al., 2004) expression vector. The inserts in these expression vectors are indicated by the bars and brackets marked p1–p9 in Fig. 2C, and they span the entire 29-40k amplicon. Each construct was then transfected into the amplicon-free +/∆*lmxgt2* heterozygous deletion to generate nine cell lines called P1–P9. Each of these lines was subsequently tested for the ability to delete *GT2* by using a targeted replacement construct containing a puromycin resistance marker (PAC; Fig. 1C). Colonies that arose from each of the P1–P9 lines were tested by genomic Southern blots, as in Fig. 2A, to determine how many of them represented bona fide knockouts of the *GT2* gene. Notably, only for line P3, bearing an episomal copy of ORF4 or *Lmx.M08* 29.2760 (subsequently named *LmxPIFTC3*), were ∆*lmxgt2* null mutants obtained. Furthermore, these were retrieved at a frequency comparable to that seen for lines carrying cosmid AC5 (7/114 or 6.1% for line P3 vs 2/27 or 7.4% for AC5; Fig. 2B). Similarly, integration of ORF4 into the rDNA locus using the plR1SAT expression vector (Capul et al., 2007) allowed retrieval (4/22 or 18%) of ∆*lmxgt2* null mutants (∆*lmxgt2*[PIFTC3*int]), Fig. 2B). In contrast, no ∆*lmxgt2* null mutants were obtained out of 632 transfectants (Fig. 2B, ∆*lmxgt2*[other ORFs]) representing the other eight lines tested (P1, P2 and P4–P9; between 58 and 100 colonies tested for each line). Furthermore, introduction of the p3 plasmid into an amplicon-free heterozygous deletion of the entire LmxGT1-3 locus (+/∆*lmxgt1-3*) enabled the recovery of three ∆*lmxgt1-3* null mutants among 19 transformant colonies (16%). These results support the conclusion that overexpression of ORF4/ *Lmx.M08* 29.2760, present in both cosmid AC5 and the 29-40k amplicon, enabled recovery of ∆*lmxgt2* and ∆*lmxgt1-3* null mutants that were not obtained in its absence.

The suppressing ORF4/Lmx.M08.29.2760 encodes an intraflagellar transport protein designated LmxPIFTC3

The *L. mexicana* gene Lmx.M08.29.2760 (Aslett et al., 2010) is an orthologue of gene Tb927.3.3000 from the related parasite *Trypanosoma brucei* (68% amino acid identity). Tb927.3.3000 was previously shown to be required for flagellum formation in *T. brucei* (Absalon et al., 2008) and the protein it encoded was named PFTC3 (putative intraflagellar transport protein C3). *T. brucei PIFTC3* (Franklin and Ullu, 2010) was subsequently shown to physically associate with proteins from intraflagellar transport (IFT) particles A and B (Rosenbaum, 2002). Hence, we designate the *L. mexicana* orthologue of Tb927.3.3000 as *LmxPIFTC3*. Models for how alterations in the IFT pathway involving *LmxPIFTC3* might promote the ability to recover ∆*lmxgt1-3* and ∆*lmxgt2* null mutants are considered in Discussion.

Amplification of the *LmxPIFTC3* gene facilitates retrieval of ∆*lmxgt1-3* null mutants but is not essential for their long-term survival

In principle, amplification of the *LmxPIFTC3* gene could either (i) be essential for normal growth or viability of ∆*lmxgt2* and ∆*lmxgt1-3* null mutants, or (ii) facilitate obtaining such mutants by promoting their viability during or after integration of the targeted gene replacement construct but not be essential for long-term survival once the null mutant has become established. The first possibility is of course...
the typical mode of genetic suppression. To address this question, we employed the ‘plasmid shuffling’ strategy that has been applied recently for *Leishmania* parasites (Murta et al., 2009). In this approach (see Fig. S1 for schematic explanation of plasmid shuffling), *Δlmxgt1-3* parasites were first generated by transfection of the ampiclon-free +/+*lmxgt1-3* heterozygote with the pXNG4-*LmxPIFTC3* plasmid, followed by the second round of targeted gene replacement (Fig. 1B, scheme 2, right-hand arrow). As the pXNG4-*LmxPIFTC3* plasmid also encompasses an expressed *GFP* gene, GFP fluorescence can be used as a measure of relative plasmid copy number in single cells. This culture was grown for ~2.5 passages (~15 cell doublings) in the absence of nourseothricin (SAT, the selectable marker on the pXNG4 plasmid) to allow a subpopulation of parasites to arise that had either lost or retained a very low copy number of the pXNG4-*LmxPIFTC3* plasmid. Then, the entire population was sorted by flow cytometry for cells that had lost or retained pXNG4-*LmxPIFTC3* (GFP dim or GFP bright; Figs S1 and S2). The clonal lines were allowed to grow further in the absence of nourseothricin, and each line was then tested to determine whether it had retained or lost the pXNG4-*LmxPIFTC3* plasmid. If overexpression of *LmxPIFTC3* is essential for long-term viability of the null mutants, all viable lines from both the ‘GFP bright’ and ‘GFP dim’ populations will retain the pXNG4-*LmxPIFTC3* plasmid and will be nourseothricin-resistant. In contrast, if overexpression of the *LmxPIFTC3* gene is not essential for long-term viability, the GFP dim population (most of which have lost the pXNG4-*LmxPIFTC3* plasmid) should produce a high percentage of viable clonal lines in the absence of selection for the pXNG4 marker, and it should be possible to retrieve viable null mutants that have lost the plasmid and thus are neither nourseothricin-resistant nor contain an amplified *LmxPIFTC3* gene. Hence, plasmid shuffling provides a quantitative test of whether a supplementing gene is essential for parasite viability.

The results of plasmid shuffling are shown in Table 1. First, both GFP bright and GFP dim populations [column A, see Fig. S2 for fluorescence-activated cell sorting (FACS) profiles] had high plating efficiencies (73% and 86% respectively, column B) in the absence of drug selection, consistent with amplification of the *LmxPIFTC3* gene not being essential for long-term viability of the *Δlmxgt1-3* null mutant. Second, all single cells obtained from a GFP bright population contained the pXNG4-*LmxPIFTC3* plasmid (70/70 or 100%, column C), as revealed by the resistance of the clonal lines to the drug nourseothricin. Third, in contrast, only one out of the 83 or 1% of cells (column C) obtained from the GFP dim population retained the pXNG4-*LmxPIFTC3* plasmid, as determined by resistance to nourseothricin. Most significantly, from the GFP dim population it was possible to generate many (82/83 or 99%, column D) *Δlmxgt1-3* viable lines that had lost the pXNG4-*LmxPIFTC3* plasmid and that were no longer resistant to nourseothricin. Southern blot analysis of four of the cured lines (C1–C4) showed that they were indeed *Δlmxgt1-3* null mutants (Fig. 2E), and that they had neither retained the amplified *LmxPIFTC3* gene encompassed in the pXNG4 plasmid, nor had they generated the chromosome 29 amplicon that is apparent from the increased intensity of the *LmxPIFTC3* band in the original *Δlmxgt1-3* null mutant but not in the C1–C4 lines. These results indicate that amplification of the *LmxPIFTC3* gene promotes retrieval of the null mutant (Fig. 2B), but is not essential for its long-term survival, once the null mutant has become established (Table 1 and Fig. 2E).

Phenotypic characterization of several of these cured lines revealed that they had properties indistinguishable

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<td><strong>GFP fluorescence (FACS)</strong></td>
<td>Number of wells showing parasite growth without drug (plating efficiency)</td>
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<td>Number of wells showing absence of growth in presence of nourseothricin (loss of plasmid)</td>
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<td><strong>GFP bright</strong></td>
<td>70/96 = 73%</td>
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<td><strong>GFP dim</strong></td>
<td>83/96 = 86%</td>
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Table 1. Glucose transporter knockouts do not require an amplified *LmxPIFTC3* gene for long-term viability.
from the original Δlmxgt1-3 null mutant. Specifically, promastigotes from the cured lines grew at the same rate as the original null mutant at 26°C (Fig. 3A), but they did not grow at 33°C (qualitative observation not quantified); they were strongly impaired in growth inside murine bone marrow-derived macrophages (Fig. 3B), and they did not take up glucose (Fig. 3C), all characteristics of the original Δlmxgt1-3 null mutant. Thus, expulsion of the pXNG4-LmxPIFTC3 plasmid was not accompanied by a secondary genetic alteration that restored uptake of hexoses, nor were the cured lines impaired in growth compared to the Δlmxgt1-3 lines that contained the amplified PIFTC3 gene. These results support the notion that amplification of the LmxPIFTC3 gene acts by ‘facilitating suppression’, in that it promotes recovery of the Δlmxgt1-3 and Δlmxgt2 null mutants, but is not essential for viability once the null mutants have been obtained.

Functional characterization of Δlmxgt1, Δlmxgt2 and Δlmxgt3 null mutants

The studies above provided clean, defined null mutants lacking the individual GT genes, enabling us to revisit the role(s) of the individual genes in Leishmania metabolism and infectivity, the second objective of this investigation. Initial characterization of each null mutant in promastig-
otes involved assaying the rate of uptake of the substrate [14C]D-glucose (Fig. 4A) and growth of promastigotes in glucose-containing medium (RPMI containing 11 mM D-glucose, Fig. 4B). In each case, deletion of \( \text{LmxGT1} \) or \( \text{LmxGT3} \) had no phenotype compared to wild type parasites. In contrast, deletion of \( \text{LmxGT2} \) resulted in reduction of glucose uptake by ~5-fold compared to wild type parasites (Fig. 4A) and a significant reduction in the rate of growth and stationary phase cell density (Fig. 4B), while both phenotypes were restored to wild type levels by complementation of the \( \Delta \text{lmxgt2} \) null mutant with an episomal copy of \( \text{LmxGT2} \).

Other phenotypic differences between wild type and \( \Delta \text{lmxgt1-3} \) promastigotes that are thought to diminish viability of \( \Delta \text{lmxgt1-3} \) parasites in the environment they encounter inside macrophages are their increased...
sensitivity to oxidative stress and elevated temperature (Rodriguez-Contreras et al., 2007) and the strong reduction in the level of the storage carbohydrate and virulence factor mannogen (Rodriguez-Contreras and Landfear, 2006), previously called β-mannan (Ralton et al., 2003). In contrast, none of the individual null mutants experienced increased sensitivity to treatment with H2O2, compared to wild type parasites (Fig. 4C). While the Δlmxgt1-3 null mutant is temperature-sensitive because it grows at 26°C but not 33°C (Fig. 4B versus Fig. 4D), each of the individual null mutants is viable at both temperatures. Finally, while mannogen levels were greatly reduced in the Δlmxgt1-3 null mutant (ratio of mannogen in Δlmxgt1-3/WT = 0.12 ± 0.01, n = 2), mannogen was not as strongly reduced in any of the individual null mutants, but the greatest degree of reduction was in the Δlmxgt2 mutant (Δlmxgt2/WT = 0.32 ± 0.03; Δlmxgt3/WT = 0.52 ± 0.05; n = 2). Overall, these observations indicate that even in the absence of the major hexose transporter LmxGT2, the other two hexose permeases can provide sufficient glucose uptake to avoid many, but not all, of the phenotypic impairments seen in the Δlmxgt1-3 null mutants.

In several experiments described below, we employed the Δlmxgt1-4 null mutant in which the LmxGT4 gene (Feng et al., 2008) has been knocked out in addition to the LmxGT1-3 genes (Fig. 1A, amplicon present). The LmxGT4 protein is expressed at a lower level than LmxGT1-3, has very low affinity for hexoses and may be a high-affinity permease for some unknown substrate. Nonetheless, extrachromosomal amplification of the LmxGT4 gene can occur over time in the Δlmxgt1-3 null mutant and can partially suppress its mutant phenotypes, e.g. impaired growth inside macrophages, due to its ability to mediate significant uptake of hexoses when strongly overexpressed. As Δlmxgt1-4 has the same phenotypes as Δlmxgt1-3 in macrophage infections (loss of viability in macrophages), hexose uptake assays (no measurable uptake of hexoses), and sensitivity to killing by H2O2 or elevated temperature, and it cannot undergo spontaneous amplification of the deleted LmxGT4 gene, it was employed as a more stable hexose transporter null mutant for experiments in Figs 5 and 6.

To examine the effects of individual knockouts in amastigotes, wild type, Δlmxgt1-4, Δlmxgt1, Δlmxgt2 and Δlmxgt3 null mutants were used to infect murine bone marrow-derived macrophages, and the numbers of intracellular parasites were quantified at 2 h, 1 day and 5 days post infection (Fig. 5A). There were no significant differences in the numbers of intracellular parasites at 2 h for any of parasite lines, indicating that none of them was compromised in entry into macrophages. At 5 days post infection, the Δlmxgt1-4 null mutants were strongly impaired in growth [as were Δlmxgt1-3 mutants (Burchmore et al., 2003)], but none of the individual null mutants was defective in survival and replication within macrophages. These results indicate that in amastigotes, where LmxGT1, LmxGT2 and LmxGT3 mRNAs are present at similar levels (Burchmore and Landfear, 1998), none of the permeases plays a uniquely critical role. However, complementation of the Δlmxgt1-4 null mutant with any one of the LmxGT1-3 genes expressed from the episomal pX63NEO vector (LeBowitz et al., 1991) was able to only modestly promote viability of the null mutants inside macrophages (Fig. 5B). Hence, while none of the LmxGT1-3 permeases is critical for survival in amastigotes,

Fig. 5. Growth of (A) individual null mutants and (B) the Δlmxgt1-4 null mutant complemented with individual LmxGT genes inside primary murine bone marrow macrophages at 37°C. Macrophages were infected at a multiplicity of 5 with stationary phase promastigotes, and the number of intracellular parasites per 100 macrophages was determined at 2 h, 1 day and 5 days post infection. Data represent the mean ± SD of three replicate measurements.

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sion of only one permease from an episome still results in quantitatively impaired growth in macrophages. We emphasize that deletion of the \(LmxGT4\) gene is not responsible for compromised viability in the \(Dlmxgt1-4\) null mutant, as the \(Dlmxgt1-3\) null mutant is equally impaired in growth inside macrophages (Fig. 3B).

**Transport of amino sugars by LmxGT permeases**

Recent studies (Naderer et al., 2010) suggest that amino sugars such as glucosamine (GlcN) and N-acetylglucosamine (GlcNAc) serve as central carbon sources for amastigotes residing inside macrophage phagolysosomes and are critical for amastigote viability. As these amino sugars appear to be crucial nutrients for the infective stage of the life cycle, it is important to determine which permeases mediate their uptake. To this end, uptake of GlcN was quantified in wild type, \(Dlmxgt1-4\) null mutant, and in this null mutant individually complemented with each of the \(LmxGT1-3\) genes. These assays (Fig. 6A) indicate that GlcN uptake is reduced to background levels in the \(Dlmxgt1-4\) null mutant but is significantly restored by complementation with any of the \(LmxGT1-3\) genes. Thus, all three permeases mediate uptake of GlcN. Similar assays employing 250 \(\mu\)M GlcNAc confirm that it is also taken up by these three permeases, but the velocity of uptake for each parasite line was ~10-fold lower than that of GlcN (data not shown). Figure 6B shows dose–response curves for GlcN inhibition of uptake of 100 \(\mu\)M \[^{[3H]}\)D-glucose by each of the three hexose transporters. The calculated (Cheng and Prusoff, 1973) \(K_i\) values for glucosamine for two such experiments were 2.3 \(\pm\) 1.2, 0.89 \(\pm\) 0.01 and 1.2 \(\pm\) 0.36 mM for LmxGT1, LmxGT2 and LmxGT3 respectively.

**Discussion**

**Transient suppression by PIFTC3**

In this work we have shown that amplification of a region bearing the \(LmxPIFTC3\) gene, or overexpression of the
PIFTC3 ORF alone, enables deletions of the hexose transporter genes of *L. mexicana* to be recovered. These deletions appear to be otherwise difficult to obtain. Thus, *PIFTC3* amplification/overexpression in most respects meets the definition of a classic genetic suppressor, e.g. a second site mutation enabling mutations of the primary target locus. In the case of GT transporters, candidates for the usual genetic suppressors might be expected to be ones acting to ameliorate the metabolic consequences to loss of the uptake of glucose or other sugars, even in the presence of compensating levels of the amino acid proline. As such, their activity would be required continuously to compensate for loss of GT. Thus, it was rather surprising to find that a component of the IFT pathway acted as the suppressing locus, especially as the key target GT2 is not localized specifically to the flagellum (Piper et al., 1995; Burchmore et al., 2003).

Furthermore, the properties of the *PIFTC3* suppressor did not follow the classic suppressor scenario outlined above. Instead, once the Δlmxgt1-3 null mutants were obtained in the presence of overexpressed *PIFTC3*, maintenance of the amplified *PIFTC3* gene was no longer required. This was shown clearly by plasmid segregation tests, in which *PIFTC3* overexpression was mediated by expression from the pXNG4 plasmid, which encodes both a GFP and herpesvirus thymidine kinase gene enabling selection for its loss by flow cytometry or ganciclovir selection (Murta et al., 2009). In such tests the Δlmxgt1-3 line yielded clonal lines at frequencies similar to controls retaining plasmid (83/96 vs 70/96; Table 1), quantitatively establishing a lack of requirement for continued presence of *PIFTC3* overexpression. Moreover, the Δlmxgt1-3 segregants lacking pXNG4-LmxPIFTC3 showed properties identical to the parent line, including comparable growth rates as promastigotes at 26°C, severe impairment of growth at 33°C or as amastigotes in primary murine macrophages, and inability to take up glucose (Fig. 3).

Thus, rather remarkably, our data suggest that *PIFTC3* overexpression facilitates recovery of Δlmxgt1-3 and Δlmxgt2 null mutants, but it is not required for their continued growth. In this respect, *PIFTC3* can be seen to act as a ‘transient’ or ‘facilitating’ suppressor, enabling recovery of a mutant but inessential for its propagation thereafter. Thus, amplification of *PIFTC3* might be loosely analogous to the role of a catalyst in a chemical reaction that decreases but does not eliminate the barrier between two chemical states.

**Transient or facilitating suppression, a new mode of genetic suppression**

The novel form of genetic suppression we have described for PIFCT3 has not been described previously in other organisms. Potentially, it may have been overlooked for technical reasons, or a lack of follow-up study given its unexpected nature. This raises the possibility that in many selections, amplifications or other genetic alterations may play enabling roles in acquisition of other genetic modifications, but then are dispensable. Indeed, recent studies of a locus implicated in the synthesis of *Leishmania* surface phosphoglycans have yielded results similar to those described here, in that knockouts could only be obtained in the presence of an ectopic suppressing gene; but once knockouts were obtained, the suppressor was now fully dispensable (H. Guo and S. Beverley, unpubl. data). In *Leishmania* a number of genetic alterations whose roles in parasite biology remain enigmatic are known – T region amplifications in *Leishmania tarentolae* (Petrillo-Peixoto and Beverley, 1989), unselected amplifications in *Leishmania major* (Beverley and Coburn, 1990), and extrachromosomal amplifications in *Leishmania donovani* (Ghosh et al., 1999) and *Endotrypanum* (Lopes et al., 1990). With the advent of rapid whole-genome sequencing it is clear that myriad other gene amplifications abound in different *Leishmania* isolates (Downing et al., 2011). Potentially, those amplifications of cryptic or unexplained function may have arisen indirectly, as ‘facilitating’ suppressors enabling other genetic modifications to arise. It seems likely that similar phenomena may occur in other organisms.

We emphasize that amplification of the *PIFTC3* gene appears to be specific for facilitating the recovery of Δlmxgt1-3 or Δlmxgt2 null mutants, and does not arise commonly to support viability of a broad range of null mutants. Thus, when 16 additional null mutants in three species of *Leishmania* were examined on genomic Southern blots (Fig. S3), none of these mutants exhibited amplification of *PIFTC3*. These mutants included two that were also difficult to obtain (Δidcps and Δldhprt/Δldtpr), and one that represented a flagellar membrane protein (Δlmxgt1). Hence, amplification of PIFCT3 is not a general suppressor that arises frequently when parasites experience stresses due to loss of various genes.

Notably, even though amplification of the *PIFTC3* gene is not required once the Δlmxgt1-3 null mutant has become established, the 29-40k amplicon is retained in these mutants over extensive passage. This observation may indicate an inherent stability for this linear amplicon that does not apply to the pXNG4 plasmid bearing the LmxPIFTC3 gene, separate from any functional involvement ameliorating the loss of GT2.

**How might PIFCT3 act as a transient/facilitating suppressor for GT deletion?**

The absence of an obvious connection between the PIFCT3 protein, a presumed component of IFT-A and IFT-B particles (Franklin and Ullu, 2010) that mediate
anterograde and retrograde movement of cargo within the flagellum (Rosenbaum, 2002), and parasite metabolism makes the LmxPIFTC3 gene a curious candidate for suppression of glucose transporter null mutant phenotypes. One possibility is that overexpression of LmxPIFTC3 protein could have an indirect effect on cellular metabolism in some way that promotes increased viability of the hexose transporter null mutants (metabolism model). As PIFTC3 proteins are assembled into multi-protein particles, overexpression of PIFTC3 would likely cause the excess protein to emerge as a monomer in the absence of sufficient partner IFT particle proteins to accommodate all the PIFTC3 into complexes. As PIFTC3 proteins contain multiple protein–protein interaction domains, such as TPR (Andrade et al., 2001) and WD-40 repeats (Xu and Min, 2011), the excess PIFTC3 may assemble with other proteins with which it does not normally interact when present at stoichiometric levels with its IFT partners, and such abnormal interactions might affect sugar metabolism or compensatory metabolic pathways. Global approaches such as quantification of a broad range of metabolites (Creek et al., 2012) may be required to dissect the mechanism of PIFTC3 suppression. Additionally, development of an anti-LmxPIFTC3 antibody and examination of potential novel protein complexes in overexpressors of the cognate gene will be necessary to test the scenario advanced above.

The mechanism whereby LmxPIFTC3 facilitates generation of glucose transporter null mutants should also explain why the null mutants can be cured of the amplification once they have become established. One possibility is that overexpression of LmxPIFTC3 promotes an alteration in parasite metabolism that supports promastigote survival in the absence of glucose uptake, e.g. by inducing gluconeogenesis or catabolism of alternate fuel sources such as amino acids or fatty acids. Once the parasites have shifted to this altered metabolic strategy, they may be able to survive without overexpressed LmxPIFTC3. Metabolic analysis of both steady state levels of metabolic intermediates and flux through various metabolic pathways in wild type parasites and those overexpressing LmxPIFTC3 may help elucidate such a mechanism. This hypothesis also raises the possibility that selection under some appropriate physiological condition might promote retrieval of \(\Delta lmxt1-3\) and \(\Delta lmxt2\) null mutants in the absence of PIFTC3 amplification or that other genetic variations could also promote retrieval of these mutants.

It is noteworthy that such a transient shift in glucose utilization has been advanced previously to explain the ability of procyclic form T. brucei to become resistant to the cytotoxic adenosine analogue tubercidin (Drew et al., 2003). Specifically, induction of RNAi against either the procyclic form hexose transporter THT2 or hexokinase promoted resistance to tubercidin, a compound that was found to disrupt glycolysis by inhibiting the glycolytic enzyme phosphoglycerate kinase. The gradual silencing of glycolysis by RNAi was proposed to allow the procyclic parasites to adapt metabolically so that they could survive subsequent acute tubercidin inhibition of glycolysis. Such a metabolic adaptation might be promoted by overexpression of LmxPIFTC3. In this case, the metabolic shift induced by overexpression of LmxPIFTC3 would account for the transient suppressor phenotype, because it might allow parasites to survive an abrupt loss of glucose uptake and metabolism by pre-adapting them to use of alternate energy sources. However, it is important to note that such a putative metabolic adaptation in L. mexicana promastigotes, induced by amplification of the LmxPIFTC3 gene, does not promote survival of mammalian stage amastigotes that have undergone deletion of the glucose transporters. Such parasites loose viability in macrophages regardless of whether they have (\(\Delta lmxt1-3\) null mutant) or do not have (the null mutant cured of the LmxPIFTC3 amplification) an amplified LmxPIFTC3 gene (Fig. 3B).

An alternate model for suppression (cell biology model) would postulate that overexpression of PIFTC3 affects some cell biological process, possibly IFT, and that altered IFT in some way transiently promotes viability of the glucose transporter null mutants. For instance, this type of model might invoke a connection between potentially altered flagellar trafficking of LmxGT1 in the PIFTC3 overexpressors and global glucose transport or metabolism, possibly by an as yet uncharacterized sensory function for this flagellar permease (Berbari et al., 2009; Bloodgood, 2010). How such a mechanism would operate is currently obscure, as a complete knockout of the LmxGT1 gene does not affect overall glucose transport or cell growth (Fig. 4A and B), at least once this null mutant has become stably established. Furthermore, LmxGT1 (flagellar), LmxGT2 (cell body) and LmxGT3 (cell body) have unaltered trafficking, compared to wild type promastigotes, in parasites overexpressing the PIFTC3 gene via its integration into the rDNA locus (Fig. S4). These results indicate that suppression by PIFTC3 is unlikely to work by altering trafficking of the glucose transporters.

**Insights gained from null mutants of individual GT genes**

One significant objective of the current study was to interrogate the potentially distinct roles of each of the three transporter isoforms, GT1, GT2 and GT3, by generating single gene null mutants. While the \(\Delta lmxt1\) and \(\Delta lmxt3\) null mutants exhibited no phenotypic differences in uptake of glucose or growth and viability measurements in promastigotes, the \(\Delta lmxt2\) null mutant showed greatly reduced glucose uptake and impaired growth at both 26°C and 33°C (Fig. 4). These results are consistent with
the higher abundance of LmxGT2 mRNA compared to LmxGT1 and LmxGT3 (Burchmore and Landfear, 1998) and establish that the LmxGT2 permease is the principal transporter for hexoses in promastigotes. In contrast, none of the individual null mutants was compromised for growth as amastigotes inside murine macrophages (Fig. 5A), compared to the pronounced loss of viability apparent for the Δlmxgt1-3 null mutant that is deficient in all three permeases. This observation may reflect the fact that the mRNAs for all three transporters are expressed at similar levels in amastigotes (Burchmore and Landfear, 1998) and that loss of a single transporter therefore has a limited impact on uptake of hexoses.

The individual null mutants generated here may be useful for continued studies on potentially distinct biological functions for the three glucose transporter isoforms. Thus, we have speculated that LmxGT1 may play a role in hexasose sensing (Landfear and Ignatashchenko, 2001), as many membrane proteins that are selectively localized to cilia or flagella in other eukaryotes have functions in environmental sensing (Berbari et al., 2009; Bloodgood, 2010). Hence, searching for phenotypic differences between wild type and Δlmxgt1 null mutants under conditions where hexose sensing might become apparent is one way of testing this hypothesis.

Experimental procedures

Parasite culture and macrophage infection

Promastigotes of L. mexicana wild type (strain MNYZ/BZ/62/ M379) or various glucose transporter null mutants derived from this wild type strain were cultured at 26°C in RPMI 1640 medium containing 11 mM glucose and 10% heat-inactivated fetal bovine serum. Transfections employed to generate null mutants or episomally complemented cell lines were performed as reported (Robinson and Beverley, 2003) and complemented cell lines were grown in the RPMI containing 100 μg ml⁻¹ G418 or 50 μg ml⁻¹ phleomycin or nourseothricin. Growth curves for promastigotes were determined by fixing parasites in 1% formaldehyde followed by counting triplicate samples on a haemacytometer grid. Infections of primary macrophages from BALB/c mice with stationary phase promastigotes were performed as described (Rodriguez-Contreras et al., 2007). Residual extracellular promastigotes were removed by gently washing the chambers with PBS at 2 h post infection. Macrophages were stained 2 h, 1 day and 5 days post infection using HEMA 3 Stain following the manufacturer’s instructions (Fisher Scientific). Stained slides were examined microscopically, and intracellular parasites per 100 macrophages were determined by counting ~300 macrophages per sample.

Recombinant DNA constructs and null mutants

Details of recombinant DNA constructs and generation of glucose transporter null mutants are presented in Supporting information associated with the online version of this article.

Preparation of cosmid library

A cosmid library was prepared in the shuttle vector cLNEO (B3592) from partially Sau3A-digested L. mexicana M379 genomic DNA as described previously (Ryan et al., 1993), yielding 2.8 × 10⁴ independent recombinants (~25× coverage). Escherichia coli bearing Lmx-cLNEO cosmids were screened for inserts overlapping the chromosome 29 amplicon using standard protocols (Sambrook et al., 1989).

Isolation and hybridization of nucleic acids

Genomic DNA was isolated using DNAZol reagent (Invitrogen) and Southern blots were performed using standard protocols (Sambrook et al., 1989). Cosmid and plasmid DNA were isolated using the Wizard plus purification system (Promega). Automated DNA sequencing was performed by the Core Facility of the Vollum Institute at the Oregon Health & Science University using an Applied Biosystems 16-capillary 3130xl automated sequence analysis system.

DNA probes

The 676-nucleotide fragment representing the 3’ terminal half of the LmxGT2 ORF, the 1.7 kb full-length LmxPIFTC3 ORF and a 1.8 kb region of the LmxGT4 ORF were amplified from L. mexicana genomic DNA using PCR. PCRs were performed using high-fidelity MasterAmp™ Extra-long Polymerase Mix (Epicentre Biotechnologies) following manufacture’s instruction.

Pulsed field gel electrophoresis

InCert agarose plugs (0.5% agarose) containing 2 × 10⁷ L. mexicana promastigotes were prepared as described (Hanson et al., 1992; Wilson et al., 1992). Chromosomes of wild type and Δlmxgt1-3 cells were fractioned by pulsed field gel electrophoresis on a contour-clamped homogeneous electric field (Chu et al., 1987) gel apparatus (Bio-Rad). Chromosomes were separated on a 0.8% agarose gel at 14°C for 20 h in 0.5× Tris–borate–EDTA buffer with 60/120 s pulse times, essentially as described (Hanson et al., 1992). The gels were stained with ethidium bromide and subjected to Southern blot analysis, using [32P]-labelled Lmx-PIFTC3 ORF as the probe.

Plasmid shuffling

The LmxPIFTC3 ORF was subcloned into the BglII site of the pXNG4 plasmid (Murta et al., 2009) to generate the pXNG4-LmxPIFTC3 expression vector that also expressed GFP. This plasmid was transfected into a heterozygous knockout of the LmxGT1-3 locus (Burchmore et al., 2003) followed by a second round of targeted gene replacement to generate the homozygous null mutant complemented with pXNG4-LmxPIFTC3. These Δlmxgt1-3[pPIFTC3] parasites were passed in the absence of nourseothricin selection for two passages (3.5 days per passage, ~20-fold dilution per passage), diluted again and grown to early log phase to
generate a subpopulation of parasites that had lost the episome and thus exhibited only background levels of GFP fluorescence. About $5 \times 10^6$ cells were harvested, resuspended in 1 ml of Dulbecco’s PBS (Gibco Invitrogen), and filtered through CellTrics 50 µm filters (Partec) to remove clumps. Single cells were then separated by FACS, using a FACS Vantage SE with Diva Option (Becton Dickinson, CA) high-speed cell sorter (488 nm), into individual wells of 96-well plates, each containing 150 µl of RPMI medium. One plate of ‘GFP bright’ cells and six plates of ‘GFP dim’ cells (cells with fluorescent intensity equivalent to that of non-GFP-expressing parasites) were collected as described (Murga et al., 2009). Plates were incubated at 26°C for 1 week and the number of wells of a 96-well plate that grew in RPMI was determined. Subsequently, cells were replicated into RPMI containing no drug or 50 µg ml$^{-1}$ nourseothricin and the numbers of wells that grew in RPMI with or without nourseothricin were determined. Four clones (C1–C4) that grew in RPMI without nourseothricin but died in RPMI with nourseothricin were selected as cured strains for further molecular and phenotypic characterization.

Phenotypic characterization

Measurement of parasite killing by H$_2$O$_2$ and growth at 33°C versus 26°C were performed as described (Rodriguez-Contreras et al., 2007). Quantification of mannogen (β-mannan) levels was performed as detailed in reference (Rodriguez-Contreras and Landfear, 2006).

Uptake assays

Assays for uptake of radiolabelled substrates were performed by an oil-stop method as reported (Seyfang and Landfear, 2000), and protein determination for normalization was performed using the DC Protein Assay Kit (Bio-Rad, CA). Time-courses were performed at 100 µM [6-3H]D-glucose (Moravek Biochemicals, CA), and 250 µM [1-14C]D-glucosamine hydrochloride. For substrate saturation curves of transport by LmxGT1 (Tran et al., 2012), incubations with [6-3H]D-glucose (Moravek Biochemicals, CA) were performed for 30 s, a time period over which uptake is linear. Four independent experiments ($n = 4$) were carried out for LmxGT1 and these data were fitted to the Michaelis–Menten equation by non-linear regression using Prism 4.0b, GraphPad Software, Inc. ($K_m = 352 \pm 112$ µM). Glucosamine inhibition curves for parasites expressing individual LmxGT transporters were carried out using 100 µM [6-3H]D-glucose as substrate and 0.05–50 mM D-glucosamine. $K_v$ values for D-glucosamine were estimated by fitting the data to one site competition equation employing Prism 4.0b GraphPad Software, Inc., employing a $K_m$ value for D-glucosamine of 352 µM by LmxGT1 (this paper), 109 µM by LmxGT2 and 208 µM by LmxGT3 (Burchmore et al., 2003).

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**Supporting information**

Additional supporting information may be found in the online version of this article.