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'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 (Δ *lmxgt1-3*) 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 Δ *lmxgt1-3* mutants contained a linear extrachromosomal 40 kb amplification of a region on chromosome 29 not amplified in WT 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 ORFs covering the 29–40k amplicon. The data mapped the suppressor activity to *PIFTC3*, encoding a component of the intraflagellar transport pathway. We discuss possible models by which *PIFTC3* might act to facilitate loss of *GTs* specifically. Surprisingly, by plasmid segregation we showed that continued *PIFTC3* overexpression was not required for Δ *lmxgt1-3* 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 sand fly 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 sand fly 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 hemolymph (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

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thought that this environment is relatively carbohydrate poor (Burchmore & Barrett, 2001, McConville *et al.*, 2007, Naderer & 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 & Coombs, 1982, Mottram & 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 *L. 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 (TMDs) 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 $\Delta lmg1$ (Burchmore *et al.*, 2003) but referred to hereafter for clarity as $\Delta lmxgt1-3$, 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 1) experience an ~8-fold reduction in the storage carbohydrate and virulence factor mannogen that is critical for amastigote viability (Ralton *et al.*, 2003), 2) become highly susceptible to oxidative stress, which they experience inside macrophages (Stafford *et al.*, 2002), and 3) 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 & Landfear, 2006, Rodriguez-Contreras *et al.*, 2007).

In contrast to amastigotes, the $\Delta lmxgt1-3$ 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 $\Delta lmxgt1-3$ 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 $\Delta lmxgt1-3$ DNA was compared to that of wild type *L. mexicana* (Feng *et al.*, 2008) using a DNA microarray probing most known *Leishmania* ORFs. Notably, in the two independent $\Delta lmxgt1-3$ 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 Δ *lmxgt2* knockouts was quantified in the presence of an episomal multicopy cosmid bearing a recombinant version of the 29–40k amplicon, or individual open reading frames (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 Δ *lmxgt1-3* and Δ *lmxgt2* 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 Δ *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. Lastly, In this manner, we were able to generate ‘clean’ or defined single gene deletion mutants for Δ *lmxgt1*, Δ *lmxgt2*, and Δ *lmxgt3* (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 Δ *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 Δ *lmxgt1* (4 out of 6 transformed colonies examined or 67%, Fig. 1B, scheme 1) and Δ *lmxgt3* (6 out of 7 colonies examined or 86%, Fig. 1B, scheme 3) in individual gene replacement experiments (Fig. 2A, B; these numbers refer to the second step of targeted gene replacement in which the null mutants are generated). In contrast, the same strategy readily generated a heterozygous knockout in which the first allele of *LmxGT2* was replaced, but it failed to produce the Δ *lmxgt2* null mutant in 305 colonies (0%) that resulted from the second targeted gene disruption (Fig. 1B, scheme 2, left hand arrow). All these transformants were doubly resistant to the two drugs (hygromycin and puromycin) employed to select for integration of the two gene replacement constructs, and a subset were tested by PCR to confirm that each construct had indeed integrated as the planned replacement in the correct locus. Nonetheless, either PCR or Southern blots of genomic DNA revealed that they all retained an intact *LmxGT2* gene. The observation of consistent retention of a targeted gene simultaneously with generation of the planned replacement, accompanied by tetraploidization or chromosome duplication/aneuploidy, is often considered circumstantial evidence that a gene may be essential (Cruz *et al.*, 1993) or that it at least plays a critical role that makes its deletion difficult to achieve. Hence, deletion of the *LmxGT2* gene in promastigotes was strongly disfavored compared to elimination of the flanking *LmxGT1* or *LmxGT3* genes.

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, 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 radiolabeled probe from the amplicon (Fig. 2D). Indeed Southern blot analysis showed that this same amplicon 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 analog 2-deoxy-D-glucose (R.J.H. Burchmore, unpublished 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 amplicon.

We speculated in the earlier study (Feng *et al.*, 2008) that this amplicon 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). Since the studies above had underscored the difficulty in deleting the *GT2* gene, we transfected cosmid AC5 into heterozygous null mutants at the *LmxGT2* locus ($+/ \Delta lmxgt2$, 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 supplementation by this multicopy cosmid could promote selection of a homozygous $\Delta lmxgt2$ null mutant. Indeed, prior complementation with cosmid AC5 resulted in two $\Delta lmxgt2$ null mutants among 27 colonies examined (Fig. 2A,B), vs 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 $\Delta lmxgt2$ and $\Delta lmxgt1-3$ null mutants, thereby acting as a ‘suppressor’ facilitating the recovery of each null mutant.

It is notable that the frequency of $\Delta lmxgt2$ null mutants transfected with the AC5 cosmid (Fig. 2B, 7%) is still ~10-fold less than that for untransfected $\Delta lmxgt1$ or $\Delta lmxgt3$ 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 $\Delta lmxgt2$ 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 $+/ \Delta lmxgt2$ heterozygous deletion to generate 9 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 Δ *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 pIR1SAT expression vector (Capul *et al.*, 2007) allowed retrieval (4/22 or 18%) of Δ *lmxgt2* null mutants (Δ *lmxgt2* [*PIFTC3int*], Fig. 2B). In contrast, no Δ *lmxgt2* null mutants were obtained out of 632 transfectants (Fig. 2B, Δ *lmxgt2* [other ORFs]) representing the other 8 lines tested (P1, P2, and P4–P9; between 58–100 colonies tested for each line). Furthermore, introduction of the p3 plasmid into an amplicon-free heterozygous deletion of the entire *LmxGT1-3* locus (+/ Δ *lmxgt1-3*) enabled the recovery of three Δ *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 in the 29–40k amplicon, enabled recovery of Δ *lmxgt2* and Δ *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 ortholog 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 PIFTC3 (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* ortholog of Tb927.3.3000 as *LmxPIFTC3*. Models for how alterations in the IFT pathway involving *LmxPIFTC3* might promote the ability to recover Δ *lmxgt1-3* and Δ *lmxgt2* null mutants are considered in the Discussion.

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

In principle, amplification of the *LmxPIFTC3* gene could either 1) be essential for normal growth or viability of Δ *lmxgt2* and Δ *lmxgt1-3* null mutants, or 2) 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 Supporting Information Fig. S1 for schematic explanation of plasmid shuffling), Δ *lmxgt1-3* parasites were first generated by transfection of the amplicon-free +/ Δ *lmxgt1-3* heterozygote with the pXNG4-*LmxPIFTC3* plasmid, followed by the second round of targeted gene replacement (Fig. 1B, scheme 2, right hand arrow). Since 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; Supporting Information, Figs. S1, 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 Supporting Information Fig. S2 for fluorescence activated cell sorting 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 Δ *lmg1-3* 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 1 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) Δ *lmg1-3* 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 Δ *lmg1-3* 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 Δ *lmg1-3* 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 from the original Δ *lmg1-3* 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 Δ *lmg1-3* null mutant. Thus, expulsion of the pXNG4-*PIFTC3* 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 Δ *lmg1-3* 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 Δ *lmg1-3* and Δ *lmg2* null mutants, but is not essential for viability once the null mutants have been obtained.

Functional characterization of Δ *lmg1*, Δ *lmg2*, and Δ *lmg3* 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 promastigotes 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 *LmxGT1* or *LmxGT3* had no phenotype compared to wild type parasites. In contrast, deletion of *LmxGT2* ($\Delta lmxgt2$) 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 $\Delta lmxgt2$ null mutant with an episomal copy of *LmxGT2*.

Other phenotypic differences between wild type and $\Delta lmxgt1-3$ promastigotes that are thought to diminish viability of $\Delta lmxgt1-3$ 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 H_2O_2 , compared to wild type parasites (Fig. 4C). While the $\Delta lmxgt1-3$ 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 $\Delta lmxgt1-3$ null mutant (ratio of mannogen in $\Delta lmxgt1-3$ /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 $\Delta lmxgt2$ mutant ($\Delta lmxgt1$ /WT = 0.69 ± 0.09 ; $\Delta lmxgt2$ /WT = 0.32 ± 0.03 ; $\Delta lmxgt3$ /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 $\Delta lmxgt1-3$ null mutants.

In several experiments described below, we employed the $\Delta lmxgt1-4$ null mutant in which the *LmxGT4* gene (Feng *et al.*, 2008) has been knocked out in addition to the *LmxGT1-3* genes (Fig. 1A, 29–40k 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 $\Delta lmxgt1-3$ 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. Since $\Delta lmxgt1-4$ has the same phenotypes as $\Delta lmxgt1-3$ in macrophage infections (loss of viability in macrophages), hexose uptake assays (no measurable uptake of hexoses), and sensitivity to killing by H_2O_2 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, $\Delta lmxgt1-4$, $\Delta lmxgt1$, $\Delta lmxgt2$, and $\Delta lmxgt3$ null mutants were used to infect murine bone marrow derived macrophages, and the number 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 $\Delta lmxgt1-4$ null mutants were strongly impaired in growth (as were $\Delta lmxgt1-3$ 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 $\Delta lmxgt1-4$ 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, expression 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 Δ *Lmxgt4* null mutant, as the Δ *Lmxgt1-3* 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. Since 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, Δ *Lmxgt1-4* null mutants, 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 Δ *Lmxgt1-4* 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 μ 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). Fig. 6B shows dose response curves for GlcN inhibition of uptake of 100 μ M [3 H]D-glucose by each of the three hexose transporters. The calculated (Cheng and Prusoff, 1973) K_i values for GlcN (Fig. 6B legend) range from 0.89–2.3 mM, indicating that this amino sugar is a somewhat lower affinity substrate than D-glucose (K_m values of 0.11–0.34 mM). Hence, another metabolically important role that the LmxGT1-3 permeases play within amastigotes is the uptake of amino sugars, and the inability of the Δ *Lmxgt1-3* mutant to take up amino sugars is likely one reason why this null mutant does not survive within macrophages.

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 intraflagellar transport pathway acted as the suppressing locus, especially since the key target GT2 is not localized specifically to the flagellum (Burchmore *et al.*, 2003; Piper *et al.*, 1995).

Furthermore, the properties of the *PIFTC3* suppressor did not follow the classic suppressor scenario outlined above. Instead, once the Δ *Lmxgt1-3* 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 (TK) gene enabling selection for its loss by flow cytometry or ganciclovir selection (Murta *et al.*, 2009). In such tests the Δ *Lmxgt1-3* 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 Δ *Lmxgt1-3* 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 inside primary murine macrophages, and inability to take up glucose (Fig. 3).

Thus rather remarkably, our data suggest that *PIFTC3* overexpression facilitates recovery of the $\Delta lmxgt1-3$ and $\Delta lmxgt2$ 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 *PIFTC3* 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 has 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, unpublished data). In *Leishmania* a number of genetic alterations whose roles in parasite biology remain enigmatic are known – T region amplifications in *L. tarentolae* (Petrillo-Peixoto and Beverley, 1989), unselected amplifications in *L. major* (Beverley and Coburn, 1990), and extra-chromosomal amplifications in *L. 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 $\Delta lmxgt1-3$ or $\Delta lmxgt2$ 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 (Suppl. Fig. S3), none of these mutants exhibited amplification of *PIFTC3*. These mutants included two that were also difficult to obtain ($\Delta ldcps$ and $\Delta ldhprt/\Delta ldxppt$), and one that represented a flagellar membrane protein ($\Delta lmxgt1$). Hence, amplification of *PIFTC3* 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 $\Delta lmxgt1-3$ 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 *PIFTC3* act as a transient/facilitating suppressor for GT deletion?

The absence of an obvious connection between the *PIFTC3* 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 the 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). Since 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. Since 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. Metabolomic 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 $\Delta lmxgt1-3$ and $\Delta lmxgt2$ 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 *Trypanosoma brucei* to become resistant to the cytotoxic adenosine analog 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 lose viability in macrophages regardless of whether they have ($\Delta lmxgt1-3$ 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. 4 A,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 (Suppl. 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 Δ *lmxgt1* and Δ *lmxgt3* null mutants exhibited no phenotypic differences in uptake of glucose or growth and viability measurements in promastigotes, the Δ *lmxgt2* null mutant showed greatly reduced glucose uptake and impaired growth at both 26° 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 Δ *lmxgt1-3* 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 hexose sensing (Landfear and Ignatushchenko, 2001), since 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 Δ *lmxgt1* 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 G418 or 50 µg/ml phleomycin or nourseothricin. Growth curves for promastigotes were determined by fixing parasites in 1% formaldehyde followed by counting triplicate samples on a hemacytometer grid. Infections of primary macrophages from BALB/c mice with stationary phase promastigotes were performed as described (Rodríguez-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 d and 5 d post-infection using HEMA 3 Stain following the manufacture'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 2.8×10^4 independent recombinants (~25x coverage). *E. coli* bearing Lmex-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 the polymerase chain reaction (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 2×10^7 *L. mexicana* promastigotes were prepared as described (Hanson *et al.*, 1992, Wilson *et al.*, 1992). Chromosomes of wild type and Δ *lmxgt1-3* cells were fractionated by pulsed field gel electrophoresis (PFGE) on a contour-clamped homogeneous electric field (CHEF) (Chu *et al.*, 1987) gel apparatus (Bio-Rad). Chromosomes were separated on a 0.8% agarose gel at 14°C for 20 h in 0.5X 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 [32 P]-labeled *LmxPIFTC3* 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 Δ *lmxgt1-3*[p*PIFTC3*] parasites were passed in the absence of nourseothricin selection for two passages (3.5 d 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 5×10^6 cells were harvested, resuspended in 1 ml Dulbecco's Phosphate Buffered Saline (PBS) (GIBCO Invitrogen), and filtered through CellTrics 50 μ m filters (Partec) to remove clumps. Single cells were then

separated by fluorescence-activated cell sorting (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 μ l of RPMI medium. One plate of 'GFP bright' cells and 6 plates of 'GFP dim' cells (cells with fluorescent intensity equivalent to that of non-GFP expressing parasites) were collected as described (Murta *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 μ g/ml nourseothricin and the number 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 H₂O₂ 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 radiolabeled 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 (BioRad, CA). Time courses were performed at 100 μ M of [¹⁴C(U)]D-glucose (Moravek Biochemicals, CA), and 250 μ M [1-¹⁴C]D-glucosamine hydrochloride. For substrate saturation curves of transport by LmxGT1 (Tran *et al.*, In press), incubations with [6-³H]D-glucose (Moravek Biochemicals, CA) were performed for 30 seconds, 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. ($K_m = 352 \pm 112 \mu$ M). Glucosamine inhibition curves for parasites expressing individual LmxGT transporters were carried out using 100 μ M of [6-³H]D-glucose as substrate and 0.05–50 mM D-glucosamine. K_i values for D-glucosamine were estimated by fitting in the data to one site competition equation employing Prism 4.0b GraphPad Software, Inc., employing a K_m value for D-glucose of 352 μ M by LmxGT1 (this paper), 109 μ M by LmxGT2 and 208 μ M by LmxGT3 (Burchmore *et al.*, 2003).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1A

1

Figure 1C

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 gray 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 (p*PIFTC3*, right hand arrow in scheme 2) allowed generation of Δ *lmxgt2* null mutants that contained this episome (black rectangle marked p*PIFTC3*). (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 pX64NEO-RI plasmid and transfected into the $+/ \Delta$ *lmxgt2* heterozygote (top). This resulted in 9 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 Δ *lmxgt2* homozygous null mutants (bottom).

Fig. 2. (A) Southern blot of genomic DNA digested with BglII/EcoRI from wild type (WT), $\Delta lm xgt1-3$, $\Delta lm xgt1$, $\Delta lm xgt2$, and $\Delta lm xgt3$ 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 band or bands on genomic Southern blots, as shown in part (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 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 Δ *lmxgt1-3* null mutants. This figure is modified from Supplementary Fig. S1B in (Feng *et al.*, 2008). The data are from a Comparative Genomic Hybridization experiment in which genomic DNA from wild type and Δ *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 ratio of the hybridization signal for Δ *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 amplicon 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 Δ *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* or *LmxGT1-3* loci is emboldened. The region spanned by the insert of cosmid AC5 (which encompasses ORFs 1–11) is also indicated. (D) Southern blot of undigested genomic DNA from WT and Δ *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 Amplicon represents the amplified linear 40 kb segment of chromosome 29 present in the Δ *lmxgt1-3* null mutant but not in WT parasites. (E) Southern blots of genomic DNA, digested with BglII/EcoRI, from WT parasites, the Δ *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.

Fig. 3.

Phenotypic characterization of $\Delta lmg1-3$ null mutants that were generated by transfection with the pXNG4-*LmxPIFTC3* plasmid followed by curing of the plasmid as explained in the text (cured lines C1–C4). The lines examined in each experiment are indicated at the right. $\Delta lmg1-3$ refers to the original null mutant with the 29–40k amplicon, while $\Delta lmg1-3[pPIFTC3]$ is the null mutant generated from the heterozygous knockout that was transfected with the pXNG4-*LmxPIFTC3* plasmid. The cured C1–C4 lines were generated from this latter line. (A) Growth of promastigotes at 26°C. (B) Enumeration of intracellular parasites in murine primary bone marrow derived macrophages at 2 hours, 1 day, and 5 days post infection. (C) Uptake of 100 μ M [14 C]D-glucose from 0–60 seconds. The $\Delta lmg1-3[pPIFTC3]$ and C2 lines show no measurable uptake, and the data points overlap. Graphed values represent the mean $\pm$ standard deviation (SD) of three replicates.

Fig. 4.

Phenotypic characterization of null mutants in single *LmxGT* genes. Cell lines studied are indicated in the legend within each panel. Values represent the mean $\pm$ SD for three replicate measurements for all data in this figure. (A) Uptake of 100 μM [^{14}C]D-glucose by wild type (WT) and null mutant cell lines. The background uptake at $t=0$ was subtracted from all data points. (B) Growth at 26°C of WT and null mutants in RPMI medium containing 11 mM D-glucose and supplemented with 10% fetal bovine serum. (C) Dose-response curves for treatment of WT and null mutant parasites with H_2O_2 . Parasites were incubated for 1h with various concentrations of H_2O_2 as described previously (Rodriguez-Contreras *et al.*, 2007) and then returned to RPMI for 24h before determining cell density. The calculated EC_{50} values for $\Delta lmxgt1$, $\Delta lmxgt2$, and $\Delta lmxgt3$ were 120 ± 43 , 135 ± 23 , and $122 \pm 15 \mu\text{M}$, respectively from three independent experiments. (D) Growth at 33°C of WT and null mutants. Comparison of the data to those in part (B) demonstrate that the $\Delta lmxgt1-3$ null mutant grows at 26°C but not at 33°C. All other null mutants continue to grow at 33°C.

Fig. 5.

Growth of (A) individual null mutants and (B) the $\Delta lmg1-4$ 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 2h, 1 day, and 5 days post-infection. Data represent the mean $\pm$ SD of three replicate measurements.

Fig. 6. Uptake of 250 μ M [¹⁴C]D-glucosamine by $\Delta lmgT1-4$ parasites complemented with individual *LmxGT* genes. Data represent the mean $\pm$ SD for three replicate measurements. (A) Uptake was measured over a 90 s time course, and the uptake at t=0 was subtracted from all data points. (B) Uptake of 100 μ M [³H]D-glucose over 30 s was measured in the presence of various concentrations of unlabeled D-glucosamine. Calculated K_i values for glucosamine for two such experiments were 2.3 ± 1.2 , 0.89 ± 0.01 , and 1.2 ± 0.36 mM for *LmxGT1*, *LmxGT2*, and *LmxGT3* respectively.

Table 1

Glucose transporter knockouts do not require an amplified *LmxPIFTC3* gene for long-term viability. Plasmid shuffling employing FACS sorting demonstrates that Δ *lmg1-3* mutants, generated by prior complementation with the pXNG4-*LmxPIFTC3* plasmid, can expel this plasmid and remain viable once the null mutant has become established. Δ *lmg1-3* parasites were first grown for ~2.5 passages in the absence of the selective drug nourseothricin to generate a sub-population of parasites with few or no copies of the pXNG4-*LmxPIFTC3* plasmid. These parasites were then sorted by flow cytometry into 96-well plates. One plate received single cells that expressed high levels (GFP bright) of GFP (i.e. retained the pXNG4-*LmxPIFTC3* plasmid), and another plate received single cells that exhibited only background (GFP dim) GFP fluorescence (i.e. either no plasmid or a low copy number) (column A). The number of wells in each 96-well plate that grew in RPMI medium without nourseothricin (column B) was determined, and the number of these wells that also survived subsequent inoculation into RPMI containing 50 μ g/ml nourseothricin (column C) was determined. The non-resistant lines (99% of GFP Dim wells, column D) had lost the pXNG4-*LmxPIFTC3* plasmid (Fig. 2E) and represented viable Δ *lmg1-3* null mutants that no longer retained an amplified *LmxPIFTC3* gene.

The single colony from GFP Dim cells that grew in the presence of nourseothricin (column C) likely arose from a parasite that had a low copy number of the pXNG4-*LmxPIFTC3* plasmid at the time of sorting, giving rise to low fluorescence but the ability to grow in the presence of the drug (Murta *et al.*, 2009).

A GFP Fluorescence (FACS)	B Number of wells showing parasite growth without drug (Plating Efficiency)	C Number of wells showing growth in presence of nourseothricin (retained plasmid)	D Number of wells showing absence of growth in presence of nourseothricin (loss of plasmid)
GFP Bright	70/96 = 73%	70/70 = 100%	0 = 0%
GFP Dim	83/96 = 86%	1/83 = 1%	82/83 = 99%