Differential expression analysis of TOR3 knockouts in arginine-starved *Leishmania major*

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Abstract

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The Arginine Deprivation Response (ADR) pathway is essential for *Leishmania* parasites to overcome host macrophage defense mechanisms and establish infection. The activation of this pathway appears to rely in part on regulation by target of rapamycin kinase 3 (TOR3), since TOR3 knockouts do not respond to arginine starvation consistent with ADR activation. Here, we investigate the role of TOR3 in response to arginine starvation via TOR3 knockout in *Leishmania major*. We analyzed mRNA abundance changes in *L. major* by differential gene expression analysis across four conditions: wild type (WT) in the presence of arginine, WT starved of arginine, knockout (KO) in arginine, and KO starved. As expected, we found a small number of changes between starved and unstarved WT samples, with relatively low magnitude (22 up-regulated, 1 down-regulated; fold change ≤ 2.5). Conversely, comparison of starved and

unstarved knockout samples yielded man more differentially expressed genes (1263 up-regulated, 1158 down-regulated) with overall higher fold changes. Comparison of WT and KO samples under starved conditions showed similar results (1533 up-regulated, 1383 down-regulated) while comparison of WT and KO under unstarved showed fewer changes, even in the presence of arginine (463 up-regulated, 261 down-regulated). Together, our results support the hypothesis that TOR3 is involved in the ADR pathway and suggest that it also plays a wider regulatory role in cell homeostasis.

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INTRODUCTION

Leishmania are a parasitic protozoan genus of more than 20 species of single celled eukaryotes that cause a wide spectrum of mammalian diseases. Although the specific outcome an infection will depend on species and host immune response, leishmaniasis has a range of clinical manifestations, from skin lesions and mucous membranes to lethal infection of the spleen and liver[1,2]. Leishmaniasis is currently endemic in 98 countries, with approximately 350 million people in 88 countries at risk for its lethal form. 500,000 new cases diagnosed every year, 10% of which are fatal[3–5].

Leishmania parasites have a digenetic lifecycle and are transmitted between mammalian hosts via female sand flies[6,7]. They proliferate in the fly's midgut, from where a small sub-population will differentiate and migrate to the foregut. These infective promastigotes are injected into hosts during the fly's blood meal where they attach to host macrophages and are ingested via phagocytosis. Upon delivery to the macrophage's phagolysosome, specific environmental conditions trigger a second stage of differentiation from promastigote to amastigote[4,5]. These amastigotes are the acute disease-causing agents[7].

Crucial to the infectivity and survival of *Leishmania* parasites within the host macrophage is their ability to sense, monitor, and respond to changes in intracellular arginine concentrations during infection[2,3,8]. Arginine is the sole precursor trypanothione biosynthesis in the polyamine pathway, the production of which is essential for parasite replication, infectivity, growth, and differentiation[2,5,8–12]. *Leishmania* are auxotrophic for arginine and thus rely on mammalian hosts for the resource[13]. However, arginine is also crucial for host defense mechanisms. Sensing the presence of arginine, macrophages activate the mammalian target of rapamycin complex 1

(mTORC1) and induce a Th1 response[14]. By these means macrophages kill invading pathogens, increasing their uptake of arginine to release reactive oxygen species and synthesize cytotoxic nitric oxide (NO)[3–5].

To counteract the Th1 host response, *Leishmania* activate a Th2 response that redirects arginine towards polyamine biosynthesis instead of NO production[15,16]. To do this, the parasite converts arginine to ornithine via macrophage arginase 1, limiting its availability for NO synthase[5,17]. This dual function of reducing arginine availability for host macrophages and synthesizing the first step in the polyamine pathway altogether depletes intracellular arginine concentrations, creating a narrowing bottleneck in which parasite and host are locked in tight competition[2]. It's the outcome of this struggle for limited resources that largely determines *Leishmania*'s intracellular survival. Thus, they require a highly sensitive mechanism for sensing and responding to the arginine depletion in order to successfully compete with the host.

Within minutes of encountering arginine-starved conditions, *Leishmania* parasites launch a coordinated mitogen-activated protein kinase 2 (MAPK2)-mediated arginine deprivation response (ADR). In response to the absence of the necessary arginine ligand, parasites increase arginine transport within the macrophage via up-regulation of amino acid permease 3 (AAP3), a highly specific arginine transporter[8,18]. Observed in both Old and New World species, it's through this mechanism that the parasite monitors metabolic deprivation and can induce a response at the gene level. The ADR is crucial not only for overcoming the arginine bottleneck, but for both successful intracellular development within the host and, ultimately, pathogenesis[4,17,19].

Recent findings suggest the ADR may be regulated by a target of rapamycin (TOR) kinase. TOR kinases are a highly conserved and versatile family of proteins with key regulatory roles in eukaryotes, particularly amino acid sensing. Usually forming complexes with several other

proteins, TOR kinases play important roles in cellular responses to stimuli and stresses, especially nutrient availability, during growth and proliferation. These processes are highly coordinated during infection cycles of eukaryotic pathogens, such as in mTORC1, which activates its pro-inflammatory response to increase arginine availability[20]. While most organisms encode one or two TOR kinases, trypanosomatid genomes encode for three (*Leishmania*) or four (*T. brucei*). TOR1 and TOR2 in *Leishmania* are similar in structure and necessity to eukaryotes. TOR3, however, does not complex with the TORC1/2 proteins and contains a unique PDZ domain. Generally speaking, these domains are often found in signaling complexes located at cell membranes. Proteins containing these domains also often play key roles in anchoring membrane receptor proteins to cytoskeletal components — together suggesting that TOR3 may interact with *Leishmania* arginine sensing to help regulate the ADR pathway in a distinct mechanism from other TOR-mediated processes[5,21].

Further, TOR3 gene expression has shown to be crucial for parasite establishment and survival in macrophages. While *L. major* strains lacking TOR3 genes show normal morphology, rapamycin sensitivity, and differentiation, they also exhibit slower growth and are unable to replicate in macrophages *in vitro* or induce pathology *in vivo*. They do not upregulate AAP3 expression in the absence of arginine and are generally sensitive to starvation of glucose but not amino acids[20]. Preliminary investigations have also suggested that TOR3 associates with arginine sensors under high concentrations, regulating AAP3 to homeostatic levels[5]. In concert, these findings give strong evidence that TOR3 may be a negative regulator of the ADR.

This investigation is thus an initial exploration into the role of TOR3 in *Leishmania major* gene expression and mRNA abundance under conditions of arginine starvation. We used transcriptional profiling (RNA-sequencing) to identify differentially expressed genes in *L. major*

wild-type (WT) versus *L. major* TOR3 knockout (KO) in variable arginine concentrations. Cell lines were grown in both arginine-rich and starved environments, sequenced, and analyzed. Expression changes in the wild-type after starvation were consistent with previous findings. In conditions of starvation, mutants showed dramatic dysregulation against both unstarved mutants and starved wild-types. Mutants also showed significant differential expression even in the presence of arginine. Together, these results suggest TOR3 has a significant involvement in downstream responses to arginine starvation, which may include both the ADR pathway and other regulatory mechanisms

RESULTS

Two distinct *L. major* cell lines were grown in three replicates: the wild-type (WT) with a functioning TOR3 gene and the knockout (KO), where TOR3 function was removed via homologous recombination. Each cell line culture was initially grown in an arginine-rich environment, RNA isolated, moved to an arginine-deprived environment, and RNA isolated again. The resulting data set is twelve unique samples, three replicates each of the following conditions: WT unstarved, WT starved, KO unstarved, and KO starved. Reads were aligned against the *L. major* strain Friedlin genomic data obtained from TriTrypDB version 44 using Bowtie2[22–24]. Mapped percentages ranged between 37.5-80.5% and were counted with featureCounts[25]. These raw counts were then imported into RStudio for analysis.

Following filtering and normalization, we performed principal component (PCA) and clustering analyses on the raw count data. The wild type (WT) conditions clustered as expected:

replicates of the same condition were close to each other, and all WT samples grouped tightly together (Fig. 1A). While we expected the same results for the knockout (KO) condition, we found instead that replicate 1 of the arginine-starved KO samples clustered more tightly with replicates 2 and 3 of the unstarved samples than it did within its own treatment group (Fig. 1A, circled). Upon closer inspection via clustering analysis, we found that, similarly, replicate 1 of the WT starved samples clustered between replicates 2 and 3 of the unstarved samples (Fig. 1C).

We suspect these observations may be results of an error in methodology. The starved samples were moved from an arginine-rich environment to an arginine-starved to trigger any deprivation response. However, what we have observed instead is that replicates 1 of both starved genotypes (WT and KO) cluster more tightly with replicates from their respective unstarved groups. To prevent any confounding results in our subsequent analyses, we removed replicate 1 from all samples, reran the PCA, and re-clustered (Fig. 1B and D). We determined this removal of replicates to be the safest course of action, as there may be artifacts of any error in downstream analyses. With the first replicate in each group removed, we proceeded to differential expression analysis, performed on the initial raw reads set with DEseq2[26].

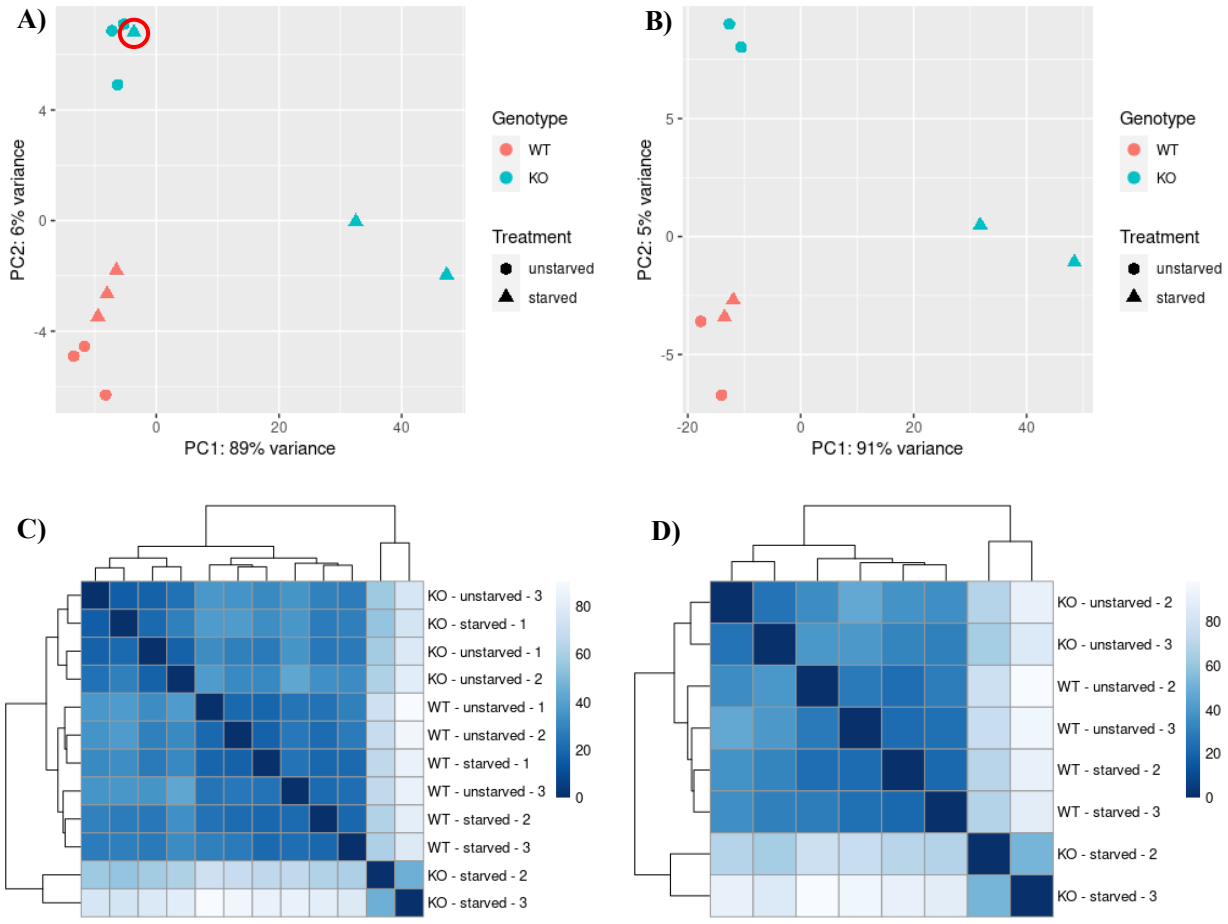

Figure 1. Principal component and clustering analyses of Wild-type and TOR3 null mutants in arginine rich and starved environments. A) Principal component analysis of all cell line samples, separated by genotype and treatment condition. Replicate 1 of the starved knockout lines circled in red. B) Principal component analysis repeated without replicate 1 in any of the conditions, twelve samples reduced to eight total. C) Cluster analysis of sample distances on all replicates. D) Repeated cluster analysis of sample distances without replicates 1.

Differential expression analysis of KO and WT samples shows that TOR3 deletion has significant effect on mRNA abundance following arginine starvation. As expected, and in line with previous findings[17], we observed few changes in gene expression after starvation in WT cell lines (22 up-regulated, 1 down-regulated; fig. 1A) with relatively low magnitude of change (log fold change

<2.5 in either direction). Among the 22 significantly up-regulated genes is AAP3, as found in previous RNA-seq investigations of arginine starvation and giving further support of its role in the deprivation response pathway. Conversely, arginine starvation in the KO cell lines showed much higher changes in gene expression (1158 up-regulated, 1263 down-regulated; fig. 1B) at high magnitudes, up to 32 times under- or over-expressed from levels in an arginine-rich environment. Observing the differences in both quantity and magnitude of changes in mRNA abundance between wild type and TOR3 knockout cell lines suggest some downstream effect of TOR3, given the distinct response to arginine starvation.

Some changes also occur in knockouts regardless of arginine starvation. Differential expression between WT and KO cell lines in the presence of arginine showed changes in several hundred genes, bidirectionally (463 up-regulated, 261 down-regulated; fig. 2C). This suggests that TOR3 may have additional roles outside of arginine starvation, and in addition to the ADR pathway may contribute some noise to our observations across other conditions. However, by far the largest magnitude of change occurs in KO cell lines starved of arginine compared to starved WT cell lines (Fig. 2D). We observe 1533 genes up-regulated and 1383 down-regulated, suggesting that if TOR3 is even somewhat important under 'normal' conditions, it appears to be much more important in the context of arginine starvation.

Brief investigation into the significantly dysregulated genes in each condition revealed that in addition to the starved WT samples, AAP3 is up-regulated in KO samples compared to WT samples in the presence of arginine (Fig. 2C). AAP3 is not significantly regulated, however, in KO samples following starvation (Fig. 2B), suggesting the ADR is not activated in the absence of functioning TOR3.

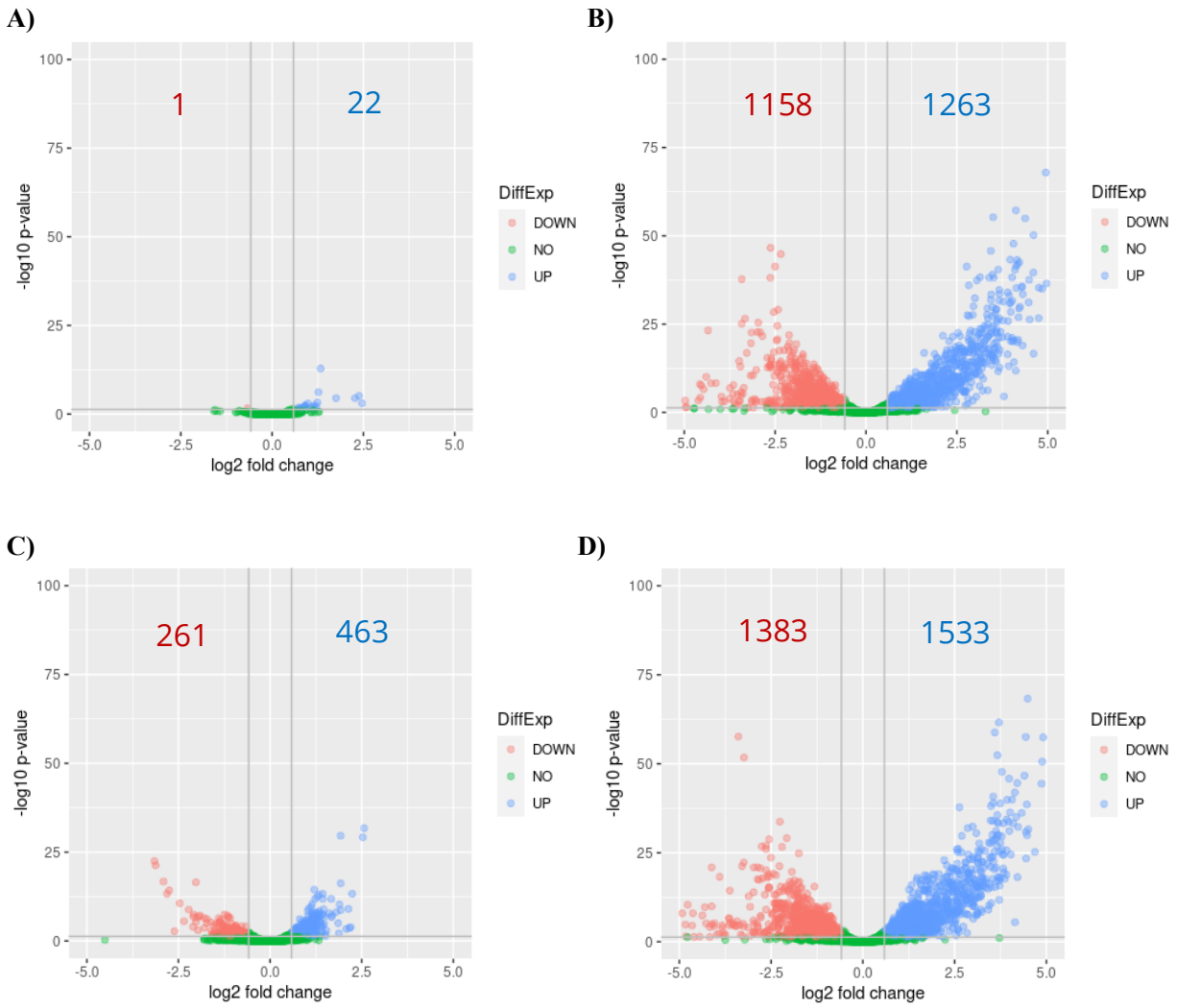

Figure 2. Differential expression analysis of mRNA abundance in wild type and TOR3 null mutants subjected to arginine starvation conditions. Significant change in regulation marked at adjusted p-value > 0.05 and a log fold-change greater or less than 0.585 (equivalent to 1.5-fold change in mRNA abundance). A) expression changes in WT cell lines after starvation compared to unstarved WT samples. B) expression changes in TOR3 cell lines after starvation compared to unstarved mutants. C) Unstarved mutants compared to unstarved WT cells. D) Starved mutants compared to starved WT cells.

DISCUSSION

In this investigation we show that TOR3 loss-of-function has a significant downstream effect on RNA abundance in the presence, but especially the absence of arginine. We find that, consistent with previous literature, AAP3 is up-regulated in WT cells following arginine starvation. Compared with WT cells in arginine, AAP3 is additionally up-regulated in arginine-rich KO samples. Massive dysregulation in arginine-starved KO samples and lack of significant AAP3 regulation further supports the hypothesis that TOR3 is an upstream regulator of the ADR pathway. Significant changes in the KO samples regardless of arginine starvation also suggest at additional regulatory roles.

Our analysis of WT samples supports previous investigations into AAP3 and arginine starvation. Here, we show that AAP3 is up-regulated in starved WT samples and unstarved KO samples. This is consistent both with investigations into AAP3 regulation of WT cells in the absence of arginine[4,17] and more recent findings that TOR3 knockout lines have higher levels of AAP3 even in the presence of arginine (results not yet published). AAP3 has a unique quality of two haploid gene copies (AAP3.1 and AAP3.2) where only mRNAs from 3.2 are sensitive to and up-regulated under conditions of arginine starvation[4,8]. We see this confirmed in our findings, where cases of AAP3 up-regulation in our results correspond only to the AAP3.2 gene in the *L. major* Friedlin strain (LmjF.31.0880). Although this investigation is specific to *L. major,* similar findings with respect to AAP3 abundance in arginine-starved *L. donovani* suggest that successful TOR3 knockout will yield similar results[8].

Through differential expression analysis, we also found that TOR3 loss-of-function impacts RNA abundance regardless of starvation but has an even more significant effect compared to starved WT cells. Together these suggest an important role of TOR3 under normal conditions

that is exacerbated in arginine starvation. It may also be possible that these effects are not the result of TOR3 knockout alone. Rather, downstream observations may be a combination of TOR3 non-functionality and nutrient starvation; the cells, under immense stress and without TOR3 to allow adaptation to conditions in a way that is not detrimental to growth and development, may have to dysregulate a large number of genes to compensate. However, it is also important to note that TOR3 deletion is known to slow growth in *L. major* even under normal conditions[5,20]. Slow growth similarly affects RNA abundance, and this investigation examines the correlation between treatment conditions and RNA abundance.

Thus, this investigation is not without limitations. Notably, there is a significant loss of power in only conducting our analyses with two biological replicates for each condition. This was a decision made as a result of unusual clustering of the first replicates and the decision to remove them in the case of downstream artifacts. We suspect a failure to adequately remove these samples from an arginine-rich environment may have occurred. Regardless, it is our recommendation that this experiment be repeated again with three independent biological replicates. Additionally, it is unclear from our findings and experimental design which of the observed changes in mRNA abundance resulted directly from arginine starvation or are a consequence and artifact of slowed growth rates. For clarity, we suggest future time-course analyses of TOR3 knockout in arginine starvation to elucidate the effects of both slowed growth and starvation over time. Finally, future research should include Gene Ontology (GO) analysis of significantly regulated genes and an add-back experiment of TOR3 functionality.

In conclusion, our findings collectively suggest that TOR3 is an important homeostatic regulator for normal growth rate and gene expression in *L. major*. Following arginine starvation, TOR3 mutants show substantial dysregulation of gene expression and growth. These results give

further evidence towards a regulatory role of TOR3 in the ADR pathway and an additionally much larger role in metabolism and homeostasis.

METHODS

Leishmania **culture and targeted gene replacement.** *Leishmania major* Friedlin clones and *L. major* TOR3 knockouts were grown in two subsequent media: arginine-rich followed by arginine-starved. RNA from 3 independent replicates in each group (WT, KO) was isolated after the growth period in each media for a total of 6 WT samples and 6 KO samples, 3 of each starved or unstarved. The TOR3 null lines were generated with gene replacement by homologous recombination following the protocol developed by da Silva and Beverley (2010) for *L. major TOR* kinase knockout[20].

RNA-seq and data analysis. Paired-end reads were aligned against the *L. major* strain Friedlin genomic data obtained from TriTrypDB version 44 (https://tritrypdb.org/) using Bowtie2. Aligned reads were counted with featureCounts[22–25]. These raw counts were then imported into RStudio (http://www.rstudio.com/). Only protein-coding genes with more than one read in at least two samples were kept for analysis. Remaining genes were normalized with a regularized log transformation, the results of which were used for principal component and clustering analysis. Differential gene expression analysis was performed on six comparison pairs (WT arg+ vs. WT arg-; KO arg+ vs. KO arg-; WT arg+ vs. KO arg+; WT arg- vs. KO arg-; arg+ vs. arg-; WT vs. KO) using the R package DEseq2[26]. Significantly differentially expressed genes had adjusted p-values < 0.05 and log2 fold changes > 1.5 or < -1.5.

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