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# Transcriptomic analysis reveals metabolic switches and surface remodeling as key processes for stage transition in *Trypanosoma cruzi*

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American trypanosomiasis is a chronic and endemic disease, which affects millions of people. *Trypanosoma cruzi*, its causative agent, has a life cycle that involves complex morphological and functional transitions, as well as a variety of environmental conditions. This requires a tight regulation of gene expression, which is achieved mainly by post-transcriptional regulation. In this work we conducted an RNAseq analysis of the three major life cycle stages of *T. cruzi*, amastigotes, epimastigotes and trypomastigotes. This analysis allowed us to delineate specific transcriptomic profiling for each stage, and also to identify those biological processes of major relevance in each state. Stage specific expression profiling evidenced the plasticity of *T. cruzi* to adapt quickly to the different conditions, with particular focus on membrane remodeling and metabolic shifts along the life cycle. Epimastigotes, which replicate in the gut of insect vector, showed higher expression on genes related to energy metabolism, mainly Krebs cycle, respiratory chain and oxidative phosphorylation related genes, and anabolism related genes associated to nucleotide and steroid biosynthesis; also a general down regulation of surface glycoproteins was seen at this stage. Trypomastigotes, living extracellularly in the bloodstream of mammals, express a plethora of surface proteins and signaling genes involved in invasion and evasion of immune response. Amastigotes mostly express membrane transporters and genes involved in regulation of cell cycle, and also express a specific subset of surface glycoproteins coding genes. In addition, these results allowed to improve the annotation of Dm28c genome, identifying new ORFs and set the stage for construction of networks of co-expression, which can give clues about coded proteins of unknown functions.

1 **Transcriptomic analysis reveals metabolic switches and**  
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13

14 **Abstract**

15 American trypanosomiasis is a chronic and endemic disease, which affects millions of people.  
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17 functional transitions, as well as a variety of environmental conditions. This requires a tight regulation of  
18 gene expression, which is achieved mainly by post-transcriptional regulation. In this work we conducted  
19 an RNAseq analysis of the three major life cycle stages of *T. cruzi*, amastigotes, epimastigotes and  
20 trypomastigotes. This analysis allowed us to delineate specific transcriptomic profiling for each stage, and  
21 also to identify those biological processes of major relevance in each state. Stage specific expression  
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23 focus on membrane remodeling and metabolic shifts along the life cycle. Epimastigotes, which replicate in  
24 the gut of insect vector, showed higher expression on genes related to energy metabolism, mainly Krebs  
25 cycle, respiratory chain and oxidative phosphorylation related genes, and anabolism related genes  
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27 was seen at this stage. Trypomastigotes, living extracellularly in the bloodstream of mammals, express a  
28 plethora of surface proteins and signaling genes involved in invasion and evasion of immune response.  
29 Amastigotes mostly express membrane transporters and genes involved in regulation of cell cycle, an  
30 also express a specific subset of surface glycoproteins coding genes. In addition, these results allowed to

31 improve the annotation of Dm28c genome, identifying new ORFs and set the stage for construction of  
32 networks of co-expression, which can give clues about coded proteins of unknown functions.

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## 36 1. Introduction

37 *Trypanosoma cruzi* is the causative agent of Chagas disease, a chronic and endemic disease affecting  
38 millions of people mainly in America (<http://www.who.int/mediacentre/factsheets/fs340/en/>). This  
39 protozoan parasite has a complex life cycle involving both vertebrate and invertebrate hosts, and  
40 extracellular and intracellular stages (Brenner 1973). These environmental switches involve dramatic  
41 changes in the physiology of these parasites. In fact, *T. cruzi* has three main stages during its life cycle:  
42 trypomastigotes (infective and non-replicative), amastigotes (replicative and intracellular in the vertebrate  
43 host), and epimastigotes (replicative and insect-specific) (Brenner 1973; Vickerman 1976).

44 These stages have been defined initially by morphological characteristics (Chagas 1909) and, as  
45 expected, they imply changes at the cellular level, including surface composition and energy metabolism.  
46 Specifically, while epimastigotes are highly active in catabolism and anabolism related pathways, and  
47 potentially use nutrients from different origins (lipids, proteins, sugars) (Cazzulo 1984; Cazzulo 1992),  
48 trypomastigotes have low levels of transcription and translation, being specialized in attachment and  
49 infection of cells. In turn, amastigotes, although metabolically more active than trypomastigotes, do not  
50 have the versatility of epimastigotes in response to different nutritional situations (Engel et al. 1987), even  
51 though very little information is available about amastigote metabolism.

52 Regarding the cellular surface, *T. cruzi* has a dense glycocalyx formed by a large number of GPI-  
53 anchored proteins that to a certain extent constitutes an identity hallmark of these parasites (Acosta-  
54 Serrano et al. 2001). These surface proteins belong to several multigene families, product of gene  
55 expansion phenomena, which represents a characteristic feature of *T. cruzi*. Their biological relevance  
56 relies on the interaction with the immune system, resistance to low pH, and antibody clearance among  
57 others (Buscaglia et al. 2006). These parasites are potentially able to remodel their surface, although  
58 large-scale studies of all the surface genes at the transcriptomic level were not performed up to date.

59 The above mentioned changes obviously require a fine regulation of gene expression. However, unlike  
60 most eukaryotes, trypanosomes have peculiarities in the genome organization and transcription. The  
61 genome of trypanosomatids is organized in clusters of protein-coding genes located on the same DNA

62 strand, separated by relatively short intergenic regions (Daniels et al. 2010). With a few exceptions,  
63 genes do not contain introns, and the clusters are transcribed as long nuclear polycistronic units. This  
64 particular organization probably explains why only a few promoters have been found in trypanosomes.  
65 mRNAs maturation in trypanosomes involves *trans*-splicing and polyadenylation. *Trans* splicing, by a  
66 similar mechanism to that involved in *cis*-splicing, is responsible for the addition of a capped spliced  
67 leader sequence (SL RNA) in the 5'UTR of each gene. This process is coupled to the polyadenylation of  
68 the 3' end of the gene located upstream on the same polycistronic RNA molecule. As a consequence, a  
69 molecule of mature mRNA (capped, polyA<sup>+</sup>, transpliced) is released from the polycistron and exported to  
70 the cytoplasm, where it can be translated. Therefore, in trypanosomes the 5'UTR is the sequence  
71 segment located between the SL and the first translated codon, whereas the 3' UTR is defined in the  
72 same way as in other eukaryotes. In contrast to bacterial operons, trypanosomatid polycistronic units do  
73 not contain genes that are functionally related. Moreover, despite their contiguity in the primary transcript,  
74 individual genes from the same transcription unit can show markedly different expression patterns  
75 (Vanhamme & Pays 1995). This observation indicates that in trypanosomes regulation of gene  
76 expression operates mainly at the post-transcriptional level. The final outcome of protein production of  
77 trypanosomatids is indeed regulated at different levels with complex mechanisms. Recently, it has been  
78 demonstrated by ribosome profiling the relevance of mRNA translation efficiency in the abundance of  
79 specific proteins in *Trypanosoma cruzi* (Smircich et al. 2015) and other trypanosomes (Jensen et al.  
80 2014; Parsons et al. 2015; Vasquez et al. 2014). However, in trypanosomatids only about 10 percent of  
81 the reads obtained by this technique are mappable due to the shortness of the sequence covered by the  
82 ribosome and the high amount of repetitive sequences and multigene families in these species.  
83 Therefore, some precaution is needed with the interpretation of the overall results. Regulation of gene  
84 expression in trypanosomes operates mainly at the transcriptional level, and numerous studies have  
85 demonstrated that 3' UTR regions affect mRNA stability, and hence differential expression (Clayton 2016;  
86 Kramer & Carrington 2011). Although the exact mechanisms allowing specificity are still unknown, some  
87 evidences have indicated that different domains in the 3' UTRs could explain, at least in part, changes in  
88 expression (da Silva et al. 2006; Di Noia et al. 2000; Jager et al. 2008). In spite of the importance of post-  
89 transcriptional changes, standard RNAseq analysis has proved to be a very informative tool for assessing

90 expression profiles in trypanosomatids (Dillon et al. 2015; Fernandes et al. 2016; Greif et al. 2013; Kolev  
91 et al. 2010; Li et al. 2016; Siegel et al. 2010). Moreover, recently a RNAseq analysis of the *T. cruzi* Y  
92 strain infecting fibroblasts was published (Li et al. 2016), reinforcing this concept by analyzing the  
93 expression of the infection in a time course simultaneously in the parasite and the host cell.

94 In this work we aimed to know which were the main transcriptomic changes during the life cycle of *T.*  
95 *cruzi*, with special emphasis on surface and energy metabolism remodeling. RNAseq of the three main  
96 stages of the parasite was performed, and allowed us to identify genes with important variation in their  
97 expression patterns (statically significant and with large size effects) at the RNA level. A systemic view  
98 about the features of gene reprogramming along the life cycle of *Trypanosoma cruzi* can be significant for  
99 future identification of key molecules to be used in the control of Chagas disease.

100

## 101 **2. Material & Methods**

### 102 **2.1. Parasites**

103 Epimastigotes were grown in liver infusion tryptose medium (LIT) supplemented with 10 % heat  
104 inactivated fetal bovine serum (FBS) at 28°C (Robello et al. 1997). Trypomastigotes were collected from  
105 supernatants of infected monolayers of Vero cells (ATCC® CCL-81)  
106 in DMEM medium at 37°C under 5% CO<sub>2</sub>. Extracellular amastigotes were obtained by incubating  
107 trypomastigotes recently released from the cells in DMEM medium at 37°C under 5% CO<sub>2</sub> for 24 h as  
108 previously described (Chiribao et al. 2012).

109

### 110 **2.2. RNA purification and quality control**

111 Parasites were washed three times with PBS, and pellets directly lysed with Tri Reagent (Sigma-Aldrich,  
112 USA). In order to obtain high quality samples the extracted RNA was further purified with IllustraRNAspin  
113 Mini Kit (GE Healthcare). Quantification was performed in a Qubit (Invitrogen), exhibiting a high content of  
114 total RNA, and quality was tested in a BioAnalyzer 2100 (Agilent Technologies), obtaining RNA integrity  
115 number (RIN) values above 8 in all the samples.

116

**117 2.3. RNA-seq library construction, quality control and sequencing**

118 Directional libraries were constructed for each *T. cruzi* stage, by using oligo dT primers and reverse  
119 transcription. Quality control of the length of the library was done with BioAnalyzer DNA 1000 kit (Agilent  
120 Technologies), and quantification was performed with Kappa Library Quantification Kit (Kapa,  
121 Biosystems). 15 pM of the libraries (mean length=350nt), were clustered on an Illumina Single Read Flow  
122 Cell in cBot (Illumina). Single read 72 and 36 cycles of sequencing was performed on GAIIX instrument  
123 with Illumina Sequencing kits (TruSeq SBS v5-GA kit, Illumina). Raw data were deposited in the NCBI  
124 database under SRA accession number SRP072022.

**125 2.4 Bioinformatics and data analysis**

126 For each stage, two libraries were generated of 36 and 72 bp each. Reads were filtered for ribosomal  
127 RNA, and a minimum of quality phred score of 20. After filtering a total of  $43.98 \times 10^6$ ,  $40.52 \times 10^6$  and  
128  $44.28 \times 10^6$  reads of amastigotes, trypomastigotes and epimastigotes were obtained, respectively. Reads  
129 were aligned to the reference genome of *T. cruzi* Dm28c (24, 30-Mar-2015) using Bowtie (allowing  
130 two/three base pair mismatches for 36/72bp reads respectively).

131 To estimate transcript levels we used ERANGE software that considers the unique regions of the genes  
132 to re-normalize the assignment of multimatching reads. The CDS plus an extension of 200bp at both  
133 sides (to the normalization process) were processed. The raw counts are presented in Table S9.

134 Differential expression analyses were performed using the R/Bioconductor package DESeq2.

135 Normalized counts were obtained from DESeq2 with the function `count(dds, normalized=T)`, and are  
136 presented in Table S9. Genes were considered as differentially expressed (DEGs) when the following  
137 conditions were met: they were statistically significant as indicated by a FDR value lower than 0.05 (FDR  
138 is the False Discovery Rate, a correction of the p-value to account for multiple simultaneous tests) and  
139 had a fold change in transcript abundance of at least two (in either direction).

140

141 Gene Ontology enrichment analyses were performed using Tritypdb tools (<http://tritypdb.org>) with

142 Fisher exact test filtering for false discovery rate (FDR) lower than 0,05.



143 Visual inspection of the alignment was performed using The Integrative Genomics Viewer (IGV)  
144 (Robinson et al. 2011).

145 In order to find possible new transcripts, libraries were pooled and aligned to reference genome using  
146 Bowtie (seed length 15, maximum mismatches in seed 1). The de novo RNA-Seq transcript assembly  
147 was performed using Cufflinks including the bias correction, the 'rescue method' for multi-reads and the 3'  
148 overhang-tolerance set to 200 (Trapnell et al. 2010). TransDecoder was used to identify candidate coding  
149 regions within transcript sequences and open reading features (ORFs) smaller than 300pb were  
150 discarded. Functional annotation of the translated ORF was done by HMM search (HMMER 3.1 (Mistry et  
151 al. 2013) against pfam database (Finn et al. 2014) and by Blastp search against nr NCBI (all non-  
152 redundant GenBank CDS translations) filtering for e-value lower than  $1 \times 10^{-5}$ . Further filter including  
153 minimum alignment identity of 60%, minimum query alignment length 60% and, minimum subject  
154 alignment length 60% were incorporated to select complete genes.

155 3'UTR sequences of Dm28c genes were obtained from TriTrypDB.org extracting 400pb after the  
156 transcriptional stop codon.

157 Alignments were performed with Clustalw2 (Larkin, Blackshields et al. 2007) and phylogenetic analysis  
158 with PhyML3.1 (Guindon et al. 2010). Modelgenerator (Keane et al. 2006) was used to select adequate  
159 substitution models. JTT was used for protein sequences and HKY85 was selected for nucleotide  
160 sequences. Visualization of phylogenies was performed with Figtree 1.4.2  
161 (<http://tree.bio.ed.ac.uk/software/figtree/>).

162 For GPI identification we used PredGPI predictor (Pierleoni et al. 2008). A total of 782 proteins were  
163 identified to present at least one potential GPI-modification site.

164

### 165 **3. Results and discussion**

166 In the present work we have determined and compared the transcriptome profiling of the three main life  
167 cycle stages of the parasite *Trypanosoma cruzi*. PolyA<sup>+</sup> RNA from parasites was purified, and libraries  
168 were constructed for amastigotes (A3 and A7), trypomastigotes (T3 and T7) and epimastigotes (E3 and  
169 E7), and sequenced by Illumina technology; after filtering for poor quality sequencing scores, we got  
170 around  $4 \times 10^7$  reads for each stage (Table 1). Reads were aligned to the reference genome of *T. cruzi*

171 Dm28c using Bowtie (Langmead et al. 2009), allowing two mismatches for 36nt reads and three  
 172 mismatches for 72nt reads. The amounts of reads that were of good quality and also aligned to reference  
 173 genome for each library are presented in Table 1. As shown in this table around 60% of the reads map  
 174 into the reference genome, which represents a significant proportion taking into account the still  
 175 incomplete state of the Dm28c genome assembly.

**Table 1 RNA-seq mapping statistics.**

Library	Readlength	Total reads	Alignedreads	Alignedreads	% readcounts in CDS*	% readcounts in CDS*
A3	36	37.972.908	21.083.527	55,5	10.150.097	48,1
A7	72	6.011.870	2.644.677	44,0	1.314.640	49,7
T3	36	37.549.496	24.880.138	66,3	10.941.002	44,0
T7	72	2.976.919	1.604.848	53,9	716.431	44,6
E3	36	35.740.671	24.024.322	67,2	9.482.211	39,5
E7	72	8.540.435	5.496.063	64,4	2.192.983	39,9

\* CDS extended 200bp at both sides

A: amastigotes, T: tryomastigotes, E: epimastigotes

176

177 In order to determine and quantify the transcript levels of each gene in the different stages, reads were  
 178 assigned to coding sequences (CDS) using Enhanced Read Analysis of Gene Expression ERANGE  
 179 (Mortazavi et al. 2008). Basically ERANGE assigns reads that map uniquely in the genome. For those  
 180 reads matching equally to two or more sites, this program uses the extended coding sequences (we have  
 181 chosen 200nt at both sides of the CDS, see Methods) assigning them to their most likely site. Total reads  
 182 counts are presented in Table 1. It is noteworthy that 50 % of the mapped reads do not map in CDS but in  
 183 other regions of the genome (Table 1). Visual inspection of the density (sequencing depth) of mapped  
 184 reads suggests that several reads mapped on UTRs regions, predominantly on 3'UTR. It should be  
 185 mentioned that *T. cruzi* UTR lengths vary according to the gene size, and have been estimated from  
 186 experimentally mapped genes to range from 10-400 bp for 5'UTR and 17-2800 bp for 3'UTR (Brandao &  
 187 Jiang 2009), being 3'UTR 2-3 times longer than its corresponding 5'UTR (Ziccardi & Brandao 2011). This  
 188 result reinforces previous findings showing the relevance of 3' UTRs in the regulation of gene expression  
 189 (Coughlin et al. 2000; da Silva et al. 2006; Di Noia et al. 2000; Nozaki & Cross 1995; Weston et al. 1999).

190 A second point that needs to be considered here is that most of the sequenced *T. cruzi* genomes are still  
191 in a "draft-like" form, mainly due to the high number of repetitive sequences. Indeed, *T. cruzi* genome  
192 consists of more than 50% repeats that include surface molecule genes and several other gene families,  
193 as well as the poorly characterized retroelements (Arner et al. 2007; El-Sayed et al. 2005). Additionally,  
194 we cannot discard that many assembled regions are not completely annotated. In this regard the RNA-  
195 seq data can be used to help detect novel transcripts and new genes. In the following sections we will  
196 focus our analysis on surface genes, metabolic pathways and the annotation of potentially new genes.

197

### 198 **3.1. Highest expressed genes**

199 Analysis of 500 most expressed genes revealed that 277 genes are common to epimastigotes,  
200 amastigotes and trypomastigotes. Gene ontology enrichment showed that these genes are related to  
201 microtubule movement, chromosome organization, DNA packaging and conformation change, response  
202 to stress, cell cycle progress, chromatin assembly among others (Table 2 and Table S1). These results  
203 suggest the relevance of epigenetic regulation in *T. cruzi* life cycle. Also amino acid activation (synthesis  
204 of aminoacyl-tRNAs) and other proteins related to translation machinery appear to be relevant in driving  
205 changes through the cycle (Table S1). Metabolic pathway analysis of shared highly expressed genes  
206 showed that aminoacyl-tRNA biosynthesis, purine metabolism, glycolysis and porphyrin metabolism are  
207 the most important. Protein synthesis, folding and degradation pathways were also very represented in  
208 this group with many ubiquitin-proteasome system genes, translation factors and chaperones being  
209 greatly expressed. It is noteworthy that some aminoacyl-tRNA synthetases show high expression profiles:  
210 glutamyl, isoleucyl, prolyl and valyl-tRNA synthetases. We cannot discard that these enzymes might have  
211 additional domains and roles (Table S7). It is known that leucyl-tRNA synthetase participates as a sensor  
212 that mediates amino acid dependent mTORC1 activation (Han et al. 2012) and glutamyl-tRNA  
213 synthetase participates in the antiapoptotic activity of glutamine by its interaction with ASK1 (Ko et al.  
214 2001). Due to the relevance of proline in parasite differentiation (Contreras et al. 1985; Tonelli et al. 2004)  
215 it is tempting to speculate that prolyl-tRNA synthetase might also be involved in sensing and/or regulation  
216 roles. Activation of translation, expression of aminoacyl-tRNA synthetases, folding and ubiquitin

217 proteasome expression suggest that besides epigenetic control, protein remodeling also plays a relevant  
 218 role in parasite stage transition.

**Table 2** Gene Ontology enrichment of commonly highest expressed genes

GO term	% genes present	Fold enrichment	p value
microtubule-based movement	21.0	5.4	2.73e-6
cellular component movement	20.3	5.2	3.72e-6
microtubule-based process	18.3	4.7	1.01e-5
Chromosome organization	35.3	9.1	1.46e-4
DNA conformation change	31.3	8.0	8.54e-4
Organelle organization	19.4	5.0	9.16e-4
DNA packaging	44.4	11.4	1.03e-3
Cellular component organization	12.3	3.2	5.39e-3
cellular component organization at cellular level	12.7	3.27	7.75e-3
cellular component organization or biogenesis	10.6	2.7	7.86e-3
Chromosome condensation	100.0	25.7	7.94e-3
Sister chromatid cohesion	100.0	25.7	7.94e-3
Chromosome segregation	100.0	25.7	7.94e-3
response to stress	12.1	3.1	9.99e-3

219

### 220 3.2. Differential expression of *T. cruzi* surface genes

221 The surface of *T. cruzi* is covered by a dense glycocalyx and its composition is characteristic of each  
 222 differentiation stage, being most of these glycoproteins attached to the plasma membrane by a  
 223 glycosylphosphatidyl inositol (GPI) anchor (de Lederkremer & Agusti 2009). Most of the surface proteins  
 224 belong to multigene families and are involved in the interaction with their hosts (De Pablos & Osuna  
 225 2012). Several studies have been performed in the different protein groups aimed to shed light on their  
 226 structure, post-translational modifications, their role in the infection and prevalence and the importance as  
 227 markers or possible drug targets (Acosta-Serrano et al. 2000; Buscaglia et al. 2006; De Pablos & Osuna  
 228 2012; Freitas et al. 2011; Kawashita et al. 2009). The first *T. cruzi* genome studies have given a more  
 229 integrative view of the complexity of these expanded families, and have even allowed the identification of  
 230 a new protein family named Mucin-associated surface proteins (El-Sayed et al. 2005; Franzén et al. 2011;  
 231 Franzén et al. 2012; Grisard et al. 2014). The main multigene families correspond to trans-sialidases  
 232 (TS), mucins (MUC), mucin-associated surface proteins (MASP), dispersed gene family-1 (DGF-1) and  
 233 metalloproteases (GP63). In order to further analyze these families and their expression, we first

234 performed a genomic analysis using public data available in the Tritryp database that includes four *T.*  
 235 *cruzi* genomes. Table 3 shows the number of members of these multigene families (including  
 236 pseudogenes) in different strains analyzed. As it can be observed, the strains exhibit substantial  
 237 differences in the content of the multigene families. This variation in the membrane composition can  
 238 constitute a characteristic of the different phylogenetic groups. We further investigated the transcript  
 239 levels of these gene families during the life cycle of the parasite, and we found that 560 genes encoding  
 240 surface proteins are differentially expressed along the life cycle, most of them (499) up regulated in  
 241 trypomastigotes (Table 3 and Figure S2). This result is in line with previous reports indicating that most of  
 242 the members of these families are relevant in the infective stages of the parasite (De Pablos & Osuna  
 243 2012) and also with the enhanced expression of specific enzymes for O-glycosylation in this stage  
 244 (Chiribao et al. 2012). However, a more in depth analysis of surface multigene families shows that all of  
 245 them have stage specific genes and some genes are not expressed at all (Figure 1).

246

**Table 3** Membrane components in *T. cruzi* and those differentially expressed in Dm28c

	TS	MASP	Mucin	DGF-1	GP63	CRP
CL BrenerGenome:						
Brener	419	345	230	205	93	1
Non-Esmeraldo	579	501	321	186	160	2
Esmeraldo-like	526	531	339	174	172	0
<i>T. marinkellei</i>	841	337	69	709	129	15
Sylvio	1112	249	76	984	126	4
Dm28c	659	311	116	69	60	13
<b>DEG</b>	295 (45%)	205 (66%)	25 (22%)	1 (1%)	26 (43%)	9 (69%)
Upregulated in trypomastigote	268 (92%)	198 (97%)	25 (100%)	0 (0%)	10 (38%)	9 (100%)

247

248 **3.2.1. Trans-sialidases (TSs).** TSs were described as the largest gene expansion phenomena in the CL  
 249 Brener strain of *T. cruzi* (El-Sayed et al. 2005), and as we show in Table 3, the analysis of all currently  
 250 available genomes confirms that it constitutes a general phenomena in *T. cruzi*. An interesting  
 251 observation from the analysis of TSs expression is that all of them are regulated during the life cycle of

252 the parasite (Figure 1): 268 genes (80%) are up regulated in trypomastigotes, but 33 genes are almost  
253 exclusively expressed in amastigotes, whereas 31 genes are up regulated in epimastigotes (Figure 1).  
254 Moreover the figure evidenced two groups of TS genes that are upregulated in trypomastigotes but  
255 clearly appear switched off in epimastigotes (\* in Figure 1), or switched off in amastigotes (\*\* in Figure 1).  
256 It has been well established the relevance of TSs in the infective stages of *T. cruzi*, due to their  
257 participation in adhesion and invasion of host cells through different functions. In the first place, *T. cruzi* is  
258 unable to synthesize sialic acid, and relies for its acquisition on the surface TSs. TSs are capable of  
259 transferring sialic acid residues from host sialoglycoconjugates to parasite mucins (Ferrero-García et al.  
260 1993; Frasch 2000; Schenkman et al. 1991). Second, these proteins participate in the recognition  
261 process through binding to specific receptors (laminin, Trk, among others), and it has been demonstrated  
262 that the different variants could explain at least in part the organ tropism of these parasites (Magdesian et  
263 al. 2001; Tonelli et al. 2010). Finally, non-infective epimastigotes also express functional trans-sialidases,  
264 and, despite their role is unclear, it is accepted that they can participate in insect-parasite interactions and  
265 metacyclogenesis (Chaves et al. 1993).

266 The described functions of TSs are clearly related to invasion, escape from parasitophorous vacuole,  
267 modulation of immune response and apoptosis and hence the predominance of TSs in trypomastigotes.  
268 Nevertheless, some TS members are specifically expressed in amastigotes and epimastigotes. To  
269 analyze the characteristics of these groups, we conducted comparisons among the stage-specific up  
270 regulated TSs, namely those exclusively expressed in epimastigotes (eTS), in trypomastigotes (tTS) or in  
271 amastigotes (aTS). We compared among these groups of proteins basic features such as predicted GPI  
272 anchor addition signal and estimated molecular weight. Our results show that eTS and aTS are smaller  
273 than tTS, the mean predicted molecular weight were 27,7KDa for eTS, 55,3 kDa for aTS and 67,6 kDa for  
274 tTS (Figure S1). Protein characterization showed that eTS are around 60 kDa and tTS molecular weights  
275 ranging from 120 to 240 kDa (Briones et al. 1995; Schenkman et al. 1991). These differences may be  
276 attributed to oligomer formation but also to glycosylation, which are not included in our prediction. In  
277 addition, analysis of GPI anchor prediction revealed that none of eTS contain predicted GPI anchor sites  
278 (100%), 43% of aTS contain predicted GPI sites whereas 60.6% of tTS are probably GPI anchored.  
279 These results support previous reports showing that only 70% of GPI anchored TSs are released after

280 phospholipase C treatment in trypomastigotes (Rosenberg et al. 1991), and also the fact that eTS are not  
281 released after addition of phospholipase C (Rosenberg et al. 1991).

282 Complement regulatory proteins (CRP) constitute a subgroup belonging to the TS superfamily that were  
283 analyzed separately for two reasons: first, their relevant role restricting the activation of the complement  
284 pathway and the lysis of the parasite (Norris 1998) (a resistance function that in *Leishmania* is mediated  
285 by one group of GP63 proteases (Grandgenett et al. 2000) and second because we found that, like strain  
286 Sylvio X10/1, Dm28c has 13 CRP genes whereas in other strains they are single (or few) copy genes  
287 (Table 3). Our analysis showed that all CRP genes were significantly over expressed in trypomastigotes,  
288 and even more, 70% were almost exclusively expressed in trypomastigotes (Figure 1 and Table S2). It  
289 was previously reported that CRPs are expressed in metacyclic and cell derived trypomastigotes surface,  
290 but they are undetectable in epimastigotes and amastigotes (Norris 1998). Our results show a correlation  
291 between protein and mRNA levels for this gene family, and also confirm their specificity for  
292 trypomastigotes as expected considering their function. Further studies comparing complement-mediated  
293 lysis between other *T. cruzi* strains can give clues about correlation between this group of genes,  
294 complement resistance and infectivity.

295 **3.2.2. Mucin-associated surface proteins (MASPs).** MASP genes were up-regulated in trypomastigotes  
296 (97%) which confirm previous studies obtained using 3'UTR as a probe in Northern blot experiments in  
297 the CLBrenner strain (Bartholomeu et al. 2009). However, a discrete number of genes were found as  
298 differentially up-regulated specifically in amastigotes or epimastigotes (Figure 1 and Table S2). Being the  
299 second largest multigene family in *T. cruzi*, MASPs were described for the first time after sequencing the  
300 *T. cruzi* genome (El-Sayed et al. 2005), and their precise function and expression remains unclear  
301 Differences in genes up-regulated in specific stages of the parasite deserve further investigation, which  
302 can help to unravel the precise function of MASP family components.

303 **3.2.3. Mucins (MUCs).** MUCs, also named mucin-like proteins, are the major component of the *T. cruzi*  
304 surface. They play different roles according to the stage and environment; in epimastigotes they are  
305 smaller, more conserved and participate in the adhesion to the perimicrovillar membrane in the insect

306 intestine. They also confer protection against proteolysis (Buscaglia et al. 2006). Mucins expressed in  
307 trypomastigotes have high glycan content and diversity, higher molecular weights, and their roles are  
308 related to attachment and penetration as well as immune evasion (Buscaglia et al. 2006). Although our  
309 analysis reveals that only 25 differentially expressed mucin genes are upregulated in trypomastigotes in  
310 comparison with both amastigotes and epimastigotes (fold change greater than two and FDR < 0.05,  
311 Figure 1), the rest of the them, are expressed at higher levels in trypomastigotes: all TcMUCI (19 genes)  
312 and 52 out of 72 TcMUCII (Table S9). On one hand, the fact that most of the TcMUCII are highly  
313 expressed in trypomastigotes is in agreement with previous experiments showing that this subgroup is  
314 the preferably mucin at this stage (Buscaglia et al. 2006). On the other hand, the highest expressed  
315 mucins in amastigotes do not belong to TcMUCI group but TcMUCII, in disagreement with previous  
316 immunofluorescence experiments showing TcMUCI as the predominant group at this stage (Buscaglia et  
317 al. 2006). Two facts are worth stressing: first, only 19 genes have been identified in Dm28c so far (a  
318 reduced number in comparison with other available genomes), so we cannot rule out the possibility of not  
319 being appreciating the full picture. Second, all TcMUCI genes have a moderate expression, lower in  
320 average than that of TcMUCII for all stages. Finally, there is also a group of 23 genes that drew our  
321 attention because they present very low or almost no expression in all three stages. The reason why  
322 these genes are turned off in the three stages analyzed escapes our knowledge. Additional studies on the  
323 expression of these and other membrane proteins are essential to shed light on these topics.

324 **3.2.4. GP63.** GP63 proteins are surface GPI anchored metalloproteases present in *Leishmania spp.*,  
325 African trypanosomes and *T. cruzi* (Cuevas et al. 2003; LaCount et al. 2003). In *Leishmania* species  
326 GP63 proteins and their coding genes have been extensively studied (evolution, organization of  
327 multigene family and its role in invasion,) (Yao et al. 2003). However in *T. cruzi* little is known about these  
328 genes. As it is clear from Table 3, most available genomes of *T. cruzi* contain about 170 genes per  
329 haploid genome, but in Dm28c there are only 60 annotated GP63 genes. The expression analysis of  
330 these genes allowed us to divide them into two groups: those that were found to be barely expressed in  
331 all stages of the parasite (50%), and a second group of genes that are differentially expressed (Figure 1  
332 and Figure S3). This latter, in turn, can be divided into two sub-groups: a first one significantly up-  
333 regulated in trypomastigotes, and a second subgroup up-regulated in amastigotes (containing 17 and 9



334 genes respectively). This suggests a fine regulation of the steady state levels of their mRNAs.  
335 Grandgenett and collaborators have suggested dividing the family in two categories according their  
336 expression in different stages and the length of their 3'UTRs (Grandgenett et al. 2000). Subsequently,  
337 Cuevas and co-workers defined again two subgroups: Tcgp63-I that is widely expressed and Tcgp63-II  
338 that is scarcely detected in Northern blot analyses (Cuevas et al. 2003). Our results, which are in line with  
339 these previous works, give now a more complete view of the expression pattern of all the GP63 family,  
340 indicating a relevant role in the mammal stages of the parasite.

341 Due to the probable relevance of UTR regions in stage specific expression regulation (Grandgenett et al.  
342 2000), and taking into account the differences in sequence and length of the 3'UTR, we further  
343 investigated the GP63 genes and their 3'UTRs. We first conducted a phylogenetic analysis of the amino  
344 acid sequences. By doing this we could observe that all GP63 genes that are DEGs in amastigotes  
345 clustered together and the same was true for GP63 genes that were up regulated in trypomastigotes with  
346 the exception of 3 sequences (Figure S3). More interesting though, are the results from the analysis of  
347 the 3'UTR, which show three groups of sequences clearly differentiated: those that belong to isoforms  
348 highly expressed in amastigotes, those associated to genes highly expressed in trypomastigotes and  
349 those associated to the group of genes with almost no expression in any stage of life cycle (Figure 2).  
350 The fact that each main group of 3'UTR is associated to a specific stage of the life cycle is a strong  
351 indication of the relevance of 3'UTR in post transcriptional regulation. Whether this is due to the presence  
352 of sequences or motives that either stabilize or degrade GP63 mRNA differentially during life cycle is  
353 uncertain. Further work will be necessary to identify mRNA-conserved motives in these genes and RNA  
354 binding proteins or small RNAs responsible for this tight regulation.

355

356 **3.2.5. Dispersed gene family 1.** Located in sub-telomeric regions, this group of proteins is greatly  
357 expanded in CLBrenner strain where they are divided in at least 3 groups (Kawashita et al. 2009).  
358 However, the first aspect that attracted our attention of this family is its reduction in the Dm28c strain,  
359 which contains only 69 annotated gene copies (Table 3). Taking into consideration that several genes  
360 appear to be incomplete (since they exhibit a reduction in length to less than 3000 bp, when the

361 estimated size is around 10 kb) the low number of DGF-1 genes in Dm28c most likely is the consequence  
362 of inaccurate genome assembly. Previous studies detected DGF-1 gene expression in different stages  
363 (Kawashita et al. 2009). Moreover, proteomic analyses find DGF-1 proteins in the parasite surface  
364 (Atwood et al. 2006). Our results do not show differential expression of these genes among the different  
365 three stages analyzed, but it should be pointed out that there is a group of DGF-1 genes that are almost  
366 not expressed. Nevertheless these results deserve further investigation because the incomplete  
367 annotation of this group could lead to erroneous conclusions.

368 The results obtained in this study, concerning the stage specific membrane composition variation and the  
369 surface remodeling during stage transitions, are depicted in Figure S2. This figure summarizes the  
370 expression levels of each gene belonging to surface multigene families in the different stages and  
371 highlights the stage-specific genes (Figure S2.A); The general picture of expression of these families (i.e  
372 total read counts of each family in the three stages) is also represented (Figure S2.B).

### 373 **3.3. Metabolic switch through *T. cruzi* life cycle.**

374 **3.3.1. Oxidative metabolism.** We have found that genes belonging to Krebs cycle, respiratory chain and  
375 oxidative phosphorylation, were significantly up regulated in epimastigotes, suggesting an enhanced  
376 respiratory activity at this stage (Figure 3A and Table S3). However expression of genes related to  
377 respiration was detected in all stages. Analysis of respiratory chain genes showed very low expression of  
378 complex I associated components (NADH dehydrogenase subunit, NADH-ubiquinone oxidoreductase) in  
379 all samples (Table S3). This result supports the notion that complex I is not very active in *T. cruzi* as was  
380 suggested previously (Carranza et al. 2009). On the other hand, the relevance of succinate-dependent  
381 respiration was evidenced here by the up regulation of NADH dependent fumarate reductase in  
382 epimastigotes, which generates succinate as the main source of electrons in the respiratory chain  
383 (Denicola-Seoane et al. 1992). The increase in oxidative metabolism in epimastigotes is in concordance  
384 with its high anabolic profile, which suggests that in this stage synthesis of macromolecules and  
385 particularly of steroids is favored.

386 Regarding the increase in respiration related genes, we wondered if the expression of antioxidant  
387 enzymes and NADPH production coding genes were also upregulated, as a strategy of avoiding oxidative  
388 damage. Analysis of antioxidant genes also showed a general up regulation of many genes that  
389 participates in antioxidant defenses in epimastigotes (trypanothione reductase, GPX, and trypanredoxin  
390 peroxidase) that could play a role against reactive oxygen species produced by high respiratory chain  
391 activity (Figure 3B and Table S4)). In contrast, ascorbate dependent peroxidase (APX) is upregulated in  
392 mammalian stages (trypomastigotes and amastigotes). APX uses ascorbate as electron donor (Logan et  
393 al. 2007), and it has been proposed that in both stages *T. cruzi* is able to synthesize this vitamin (Logan  
394 et al. 2007). In this context, the down regulation of APX in epimastigotes might reflect the inefficiency in  
395 ascorbate synthesis and/or reduction in this stage. The reduced levels of APX mRNA genes is also in line  
396 with the empirical observation that ascorbic acid content in epimastigotes is between 1,6 and 3,6 times  
397 lower than trypomastigotes (Clark et al. 1994).

398 Concerning the expression of antioxidant genes, pentose phosphate pathway genes were also  
399 upregulated in epimastigotes (Figure 3C and Table S6), suggesting a greater production of NADPH,  
400 which acts as an electron donor in detoxifying reactions but also a production of ribose phosphate for  
401 nucleotide synthesis.

402 Another remarkable point is that enzymes for fermentation (acetaldehyde dehydrogenase and alcohol  
403 dehydrogenase) were highly upregulated in epimastigotes. These results show that during this stage of  
404 life cycle the parasites can be adapted to different metabolic conditions, particularly to different oxygen  
405 conditions for ATP production.

406

407 **3.3.2. Lipid Metabolism.** Analysis of genes related to lipid metabolism in *T. cruzi* main stages showed  
408 significant differences. Both epimastigotes and amastigotes (compared to non-dividing trypomastigotes)  
409 up regulate key genes involved in phospholipid and sterol biosynthesis (Figure 4 and Table S5).  
410 Comparison of genes related to lipid synthesis in amastigotes and epimastigotes revealed that the former  
411 not only have higher mRNA levels of genes involved in phospholipid synthesis (phosphatidic acid  
412 phosphatase, choline ethanolamine kinase) but also of desaturases which allow the generation of  
413 polyunsaturated fatty acids that maintain membrane fluidity under variable environment (Table S5).

414 Concerning sterol biosynthesis pathways it was demonstrated that in amastigotes it is similar to  
415 epimastigotes but simpler, being cholesterol (probably derived from the host) up to 80% in weight of total  
416 sterols (Liendo et al. 1999). Analysis of sterol synthesis genes showed an up regulation in most genes of  
417 this pathway in epimastigotes (Figure 4). This may constitute an advantage due to the variable  
418 temperature in the insect host and the subsequent requirement to regulate membrane fluidity. In this  
419 sense, it was shown that epimastigotes have the ability to rapidly adapt and remodel their lipid content in  
420 response to temperature changes (Florin-Christensen et al. 1997).

421

422 We have also analyzed the expression of genes related to catabolism of fatty acids and found that all  
423 stages have similar levels of mRNAs encoding beta oxidation enzymes, although some genes relevant for  
424 activation and transport of fatty acids exhibited increased mRNA levels in amastigotes and epimastigotes  
425 (fatty acyl CoA synthase, carnitine *O*- acyl transferase). Down regulation of fatty acid oxidation related  
426 genes in trypomastigotes was also observed by Li and collaborators (Li et al. 2016) during the transition  
427 from trypomastigotes to intracellular amastigotes. Atwood and co-workers (Atwood et al. 2006) suggested  
428 a shift from carbohydrate to fatty acid catabolism in the transition of trypomastigotes to amastigotes, on  
429 the basis of proteomic data that showed the presence of  $\beta$  oxidation enzymes as well as Krebs cycle  
430 intermediates. In contrast, metabolic studies confirm that amastigotes can use glucose as a carbon  
431 source generating acetate, glycerol and pyruvate (Sanchez-Moreno et al. 1995). It is important to note  
432 that metabolic, transcriptomic and proteomic studies had been carried out using *in vitro* approaches,  
433 sometimes differing from real physiological and environmental conditions. Our data shows that  
434 epimastigotes, amastigotes and trypomastigotes express high levels of  $\beta$  oxidation related genes, but  
435 some of them, like acyl CoA dehydrogenase and enoil CoA isomerase are up regulated in epimastigotes  
436 (Table S5). Comparison of amastigotes and trypomastigotes revealed that fatty acid oxidation genes  
437 (ketoacyl-CoA thiolase, enoyl-CoA hydratase) were overexpressed in the former, as in amastigotes of the  
438 Y strain (Li et al. 2016), suggesting that it constitutes a general feature of amastigotes, independently of  
439 the lineage.

440

441 **3.3.3. Glucose catabolism.** Trypomastigotes are present in the blood of their mammalian host, where  
442 glucose is abundant; amastigotes reside in the cytoplasm of mammalian cells where free glucose is  
443 scarce; and epimastigotes live in the digestive tract of the insect (an amino acid rich medium), which has  
444 sources of free glucose during or immediately after bloodmeals. The transcriptomic profiling of genes  
445 encoding glycolytic enzymes showed that all of them are expressed, but epimastigotes present higher  
446 mRNA levels of hexokinase, phosphofruktokinase, glyceraldehyde-3-phosphate dehydrogenase and  
447 enolase than trypomastigotes and amastigotes (Figure 5 and Table S6). Comparison of normalized  
448 mRNA levels (ncounts/Kb) showed significant differences between the genes in the same pathway  
449 (Figure 5). An overview of Figure 5 shows that the most highly expressed glycolytic genes are in the  
450 extremes of the graphics, that is, in the initial and final steps of glycolysis. These are the most relevant  
451 enzymes since they catalyze either the points of regulation of the pathway and/or reactions related to  
452 ATP production. It is well known that intermediate reactions depend on the availability of substrates, and  
453 they do not need to have high levels of expression, this is the case of genes 4, 6, 7 and 9 (Figure 5).  
454 Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) constitute an exception since their level of  
455 transcription is higher. However, it should be noted that this gene encode cytosolic, instead of glycosomal  
456 enzyme and probably high concentrations are required due to the lack of compartmentalization. On the  
457 other hand, the GAPDH reaction is responsible for the first "high energy" intermediate formation and then,  
458 it is a hub for ensuring metabolic flux of the pathway. In summary, although epimastigotes present higher  
459 level of some key glycolytic genes, all the stages are prepared for glucose degradation. Additionally we  
460 cannot discard that these differences in mRNA levels could also be a strategy for glycolysis regulation  
461 under different stimuli like hypoxia or glucose availability. This kind of regulation has been observed in  
462 yeast (Daran-Lapujade et al. 2007), where post-transcriptional regulation play major roles in modulating  
463 metabolism.

464

465 A particular highlight of our results is that amastigotes present a drastic reduction of hexokinase (HK)  
466 mRNA levels (almost 7 and 3 times respect to epimastigotes and tryptomastigotes respectively, see  
467 Figure 5). It has been well established that amastigotes use mainly amino acids as primary source of  
468 energy (Silber et al. 2005), and the drastic down regulation of HK expression could imply a reduction of

469 glycolysis. In fact, glucose transporters are not expressed in this stage (Silber et al. 2009) in agreement  
470 with the low intracellular glucose concentration of around 20  $\mu$ M (Malliopoulou et al. 2006). However, the  
471 rest of the glycolytic enzymes do not decrease their expression, suggesting that the pathway could be  
472 active in the presence of hexose phosphates, but specific transporters have not been described in *T.*  
473 *cruzi*. Therefore, the HK decrease can reflect a switch to gluconeogenesis at this stage. It should be  
474 noted that, as mentioned above, amastigotes express high levels of genes related to pentose pathway,  
475 including those coding for the non-oxidative phase of the pathway (Figure 3C). These enzymes are  
476 responsible for the interconversion of monosaccharide-phosphates from 3 to 7 C, then generating hexose  
477 phosphates as substrates for glycolysis.

478 Finally we have found that genes encoding enzymes necessary for fermentation (acetaldehyde  
479 dehydrogenase and alcohol dehydrogenase) were highly up regulated in epimastigotes. These results  
480 show that they are adapted to different metabolic conditions, as well as to different oxygen conditions for  
481 ATP production. It is known that trypanosomatids produce and excrete reduced fuels, not only in  
482 anaerobiosis, but also in the presence of oxygen (Cazzulo 1992). Interestingly, Sanchez-Moreno and  
483 coworkers have showed that epimastigotes but not amastigotes, release ethanol to the media using  
484 glucose as a carbon source (Sanchez-Moreno et al. 1995), which is in line with our findings.

485

486 **3.3.4. Protein and amino acid metabolism.** Protein synthesis and degradation plays important roles in  
487 parasites, which suffer morphological changes and nutritional stresses through their life cycle. Synthesis  
488 of new specialized proteins and glycoproteins is necessary for adaptation and survival in each stage.  
489 Furthermore, proteins and amino acids can be used as major carbon sources for ATP production  
490 depending on the stage and/or the environment (Cazzulo 1984).

491 In *T. cruzi*, amino acid catabolism is very relevant in proliferative stages, amastigotes use amino acids for  
492 energy production and epimastigotes also use amino acids when glucose is not available (Bringaud et al.  
493 2006). In addition, some amino acids like arginine and proline play additional roles like energy store and  
494 differentiation respectively (Silber et al. 2005). Among amino acids, proline has an outstanding role

495 promoting the differentiation of intracellular forms, from epimastigotes-like to trypomastigotes (Tonelli et  
496 al. 2004) as well as in metacyclogenesis (Homsy et al. 1989).

497 Our results emphasize the significance of amino acids in *T. cruzi* biology and metabolism as indicated by  
498 the high expression of several amino acid permeases and transporters in epimastigotes, trypomastigotes  
499 and amastigotes. Our transcriptomic analysis revealed that each stage presents specific highly expressed  
500 amino acid transporters, even trypomastigotes. Nevertheless most transporters were up regulated in  
501 amastigotes and epimastigotes, suggesting that amino acid metabolism is more relevant in proliferative  
502 stages than in trypomastigotes (Figure 6 and Table S7).

503

504 It has been demonstrated that epimastigotes can metabolize asparagine, aspartate, glutamine, glutamate  
505 and branched amino acids like valine, leucine and proline, and their oxidation converges in aspartate and  
506 glutamate. Glutamate can participate as substrate of transaminases or deaminases and enter the Krebs  
507 cycle (Silber et al. 2005). To check these aspects we looked for genes involved in valine, leucine and  
508 isoleucine degradation and their expression through the life cycle. Our data agree with the idea that  
509 catabolism of these amino acids is increased in epimastigotes since most genes of this pathway were up  
510 regulated in this stage. It should be pointed out that one of the most important enzymes involved in  
511 branched amino acid degradation, the oxoisovalerate dehydrogenase complex, is also upregulated in  
512 amastigotes (Figure 6, Table S7).

513 The amino group of glutamate can be transferred to pyruvate by transaminases (alanine  
514 aminotransferase or tyrosine aminotransferase) or alternatively transferred to water by glutamate  
515 dehydrogenases, releasing  $\text{NH}_3$ . In this context we looked for glutamate dehydrogenases coding genes  
516 (NADP<sup>+</sup> and NAD<sup>+</sup> dependent) and tyrosine and alanine aminotransferases and it was found that both  
517 glutamate dehydrogenases were upregulated in epimastigotes, in particular NADP<sup>+</sup> dependent glutamate  
518 dehydrogenase mRNA levels increased 10 fold in this stage (Figure 6, Table S7). All these results  
519 strongly support the idea that amino acid and nitrogen metabolism is enhanced in epimastigotes due to  
520 the scarcity of carbohydrates and the abundance of proline in the terminal portion of the digestive tube of

521 the triatomine (Manchola et al. 2016). In this sense, a general down regulation of processes related to  
522 amino acid metabolism and transport was observed in trypomastigotes, supporting the idea that amino  
523 acids are not the preferred fuel when they parasite the mammal host (Bringaud et al. 2006).

524 As mentioned above, arginine participates in energy storage through a reaction catalyzed by arginine  
525 kinase. This reaction generates phosphoarginine, which serves as an ATP, and phosphate reservoir and  
526 supports burst of cellular activity during the life cycle (Alonso et al. 2001). Our results show that this gene  
527 is significantly up regulated in epimastigotes and trypomastigotes; and this might reflect the fact that  
528 amastigotes have a constant supply of glucose and amino acids and therefore do not need such energy  
529 storage. Another explanation is that arginine kinase activity was not directly correlated with mRNA levels,  
530 a result already shown for epimastigotes (Alonso et al. 2001).

531 Finally, expression analysis of genes related to proteasomal degradation during the cycle showed that 24  
532 genes coding proteasome subunits are highly expressed during the cycle (Table S7), this is not surprising  
533 since parasites undergo radical morphological changes which are carefully controlled by proteasome  
534 mediated proteolysis (Munoz et al. 2015).

### 535 **3.4. Annotation of new genes**

536 During the analysis we found that some genes were not annotated in the Dm28C strain. Additionally the  
537 visual inspection of mapped reads evidences transcriptional activity in regions that were annotated as  
538 intergenic and also encompass relatively long (>300 nt) open reading frames (ORFs). These three facts  
539 led us to look for possible non annotated genes. For this purpose we assembled the transcriptome  
540 including all reads from the different libraries. From this assembly we identified 9521 novel transcripts (not  
541 coincident with the known annotation) containing 1400 ORFs regions with a minimum length of 300 pb.  
542 These ORFs were subsequently validated by HMM and Blast searches against public databases giving a  
543 total of 858 new possible coding sequences. These predictions correspond to genes or gene segments  
544 that were not annotated in the Dm28c strain. To identify ORFs with higher chances of corresponding to  
545 complete CDSs (within this population of non annotated genes) we decided to use a more stringent  
546 criterion in this annotation step and kept only those ORFs that align with an annotated protein entry with



547 the following requirements: minimum alignment identity: 60%, minimum query alignment length 60% and,  
548 minimum subject alignment length 60%. With this procedure we identified 247 putative proteins that were  
549 not previously annotated in Dm28c. They mostly correspond to hypothetical proteins, but also we  
550 identified several surface components (TS, mucins, MASP, etc), retrotransposon hot spot proteins,  
551 among other genes (see Table S8). In particular we found the tryparedoxin 1 (TXN I) gene almost  
552 identical (99.3% identity) to the *T. cruzi* tryparedoxin CAC85916.1. Overall these results illustrate the  
553 importance of continuing to progress in the annotation process combining different sources of data and  
554 manual curation (Table S8).

555 The expression analyses presented here were further compared to known and particular protein  
556 expression profiles (Figure S4). These group of genes are: i) flagellum associated genes down-regulated  
557 in amastigotes which present a small non-emergent flagellum; ii) genes related to conversion of histidine  
558 to glutamate up-regulated in epimastigotes allowing to this stage to take advantage of the abundance of  
559 histidine in the gut of its insect vector (Parodi-Talice et al. 2004); iii) mucins up regulated in  
560 trypomastigotes, being TcMUC II predominant (70%) over TcMUC I (Figure S4) (Buscaglia et al. 2006); In  
561 all the above examples we found a correlation between mRNA expression and protein levels, indicating  
562 that regulation of gene expression in trypanosomes is multifactorial, and both translation (Smircich et al.  
563 2015) and transcription profiles this work and (Li et al. 2016) are relevant for specific biological functions.  
564

#### 565 4. Conclusions

566 In this work we conducted a RNA-seq analysis in *Trypanosoma cruzi*, a species of great medical  
567 importance since it is the causative agent of Chagas disease. We have sequenced RNA populations from  
568 the three stages of the life cycle of the parasite using the Illumina technology. This technology in  
569 combination with computational tools was used to perform a comparative analysis of gene expression  
570 along the life cycle of *T. cruzi*.

571 A correlation between patterns of gene expression and previously described metabolic features of each  
572 stage was found. Metabolic pathway analysis of highly expressed genes in epimastigotes revealed that  
573 they are related to ATP production pathways such as Krebs cycle, pyruvate metabolism, respiratory  
574 chain, oxidative phosphorylation and nitrogen metabolism. Biosynthetic pathways related genes are also  
575 upregulated at this stage, being the most important steroid biosynthesis. Gene ontology analysis confirms  
576 the pathway enrichment analysis, since the Biological process related to ATP biosynthesis like  
577 carbohydrate and amino acid metabolic catabolism are the most important in epimastigotes, whereas  
578 most of the surface genes are down regulated at this stage (Figure 7).

579 .

580

581 In amastigotes (intracellular replicative forms) the highest expressed genes are related to regulation of  
582 cell cycle, protein and amino acid catabolic processes, adhesion and signaling. Adhesion includes the  
583 expression of many surface proteins involved in invasion like stage specific trans-sialidases, GP63, and  
584 MASPs. The high expression level of transporters, particularly amino acid transporters, shows that the  
585 parasite uses host cytoplasmic metabolites at this stage (Figure 8). Some of these characteristics were  
586 recently reported for amastigotes also in the Y strain suggesting that the metabolic hallmarks of this  
587 stage are independent of the *T. cruzi* lineage considered.

588 Trypomastigotes (non-replicative and infective form) exhibit a predominance of surface protein genes,  
589 those encoding trans-sialidases, MASPs, GP63, mucins and complement regulatory proteins represent

590 more than 50% of the transcripts. Enrichment analysis (GO Biological processes) of these genes  
591 revealed that cell adhesion, microtubule-based flagellum, peptidase, signal transducer activity and  
592 calmodulin binding are the most relevant ones, in agreement with the specialization of trypomastigotes in  
593 movement, adhesion, invasion and signaling (Figure 8).

594 1400 ORF regions with a minimum length of 300pb were identified, that eventually correspond to 858 new  
595 coding sequences. By using stringent matching conditions, 247 proteins were identified as non-  
596 annotated in the Dm28c genome.

597 In summary, transcriptome profiling of the three main developmental stages of *Trypanosoma cruzi* has  
598 shown which genes and processes are related to each stage, and allows to conclude that surface  
599 remodeling and metabolic switches are at the basis of differentiation

600

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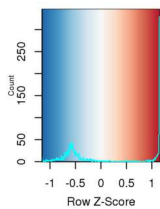


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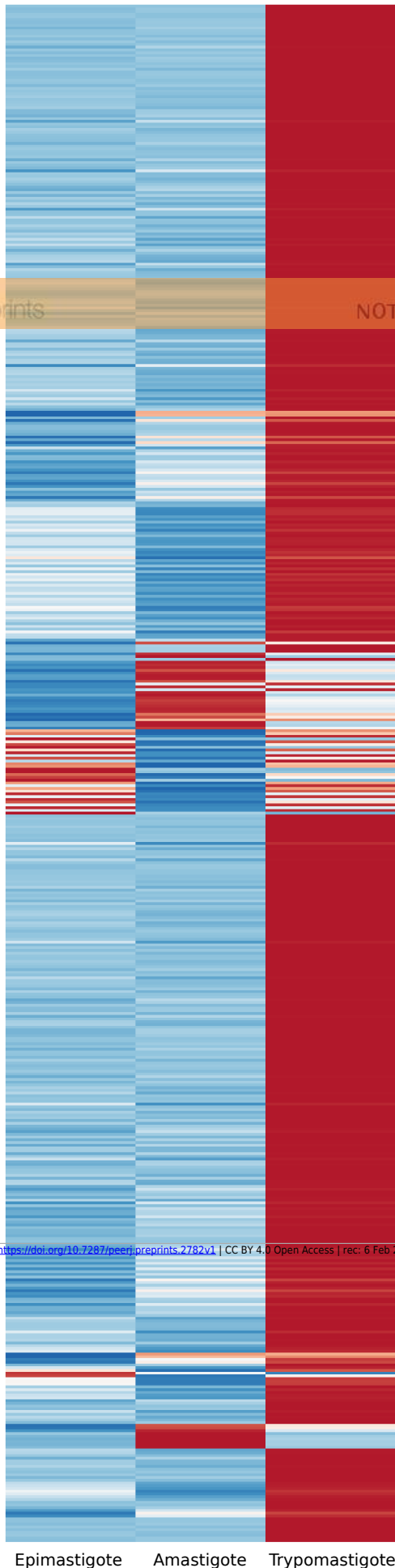
**Figure 1**(on next page)

Differential expression of the surface components

Heatmap of glycoproteins genes significantly regulated during the life-cycle of *Trypanosoma cruzi*. The z-score is plotted, red bars represent up regulation and blue bars represent down regulation. Numbers correspond to number of genes. (\*) remarks TS genes barely expressed in epimastigotes.(\*\*) remarks TS genes barely expressed in amastigotes. A total of 31 and 33 TS genes were up regulated in epimastigotes and amastigotes, respectively. Of them, 19 and 18 genes were also upregulated in trypomastigotes. The total number of Trans-sialidases upregulated in trypomastigotes is 268.



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**TS**

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**MASP**

198

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4

3

25

9

17

9

**Mucin**

**GP63**

**CRP**

Epimastigote

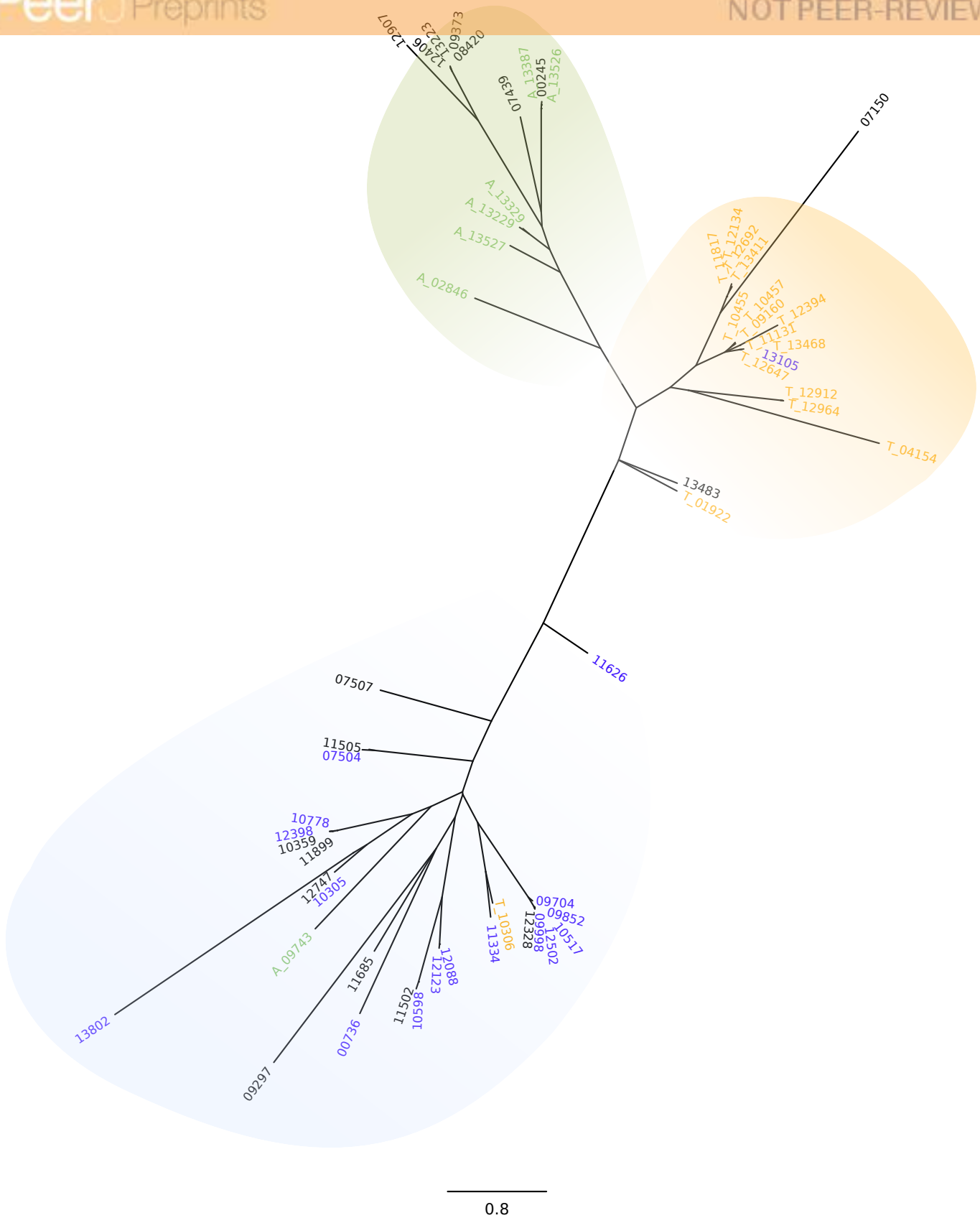
Amastigote

Trypomastigote

**Figure 2** (on next page)

## GP63 3'UTR phylogeny

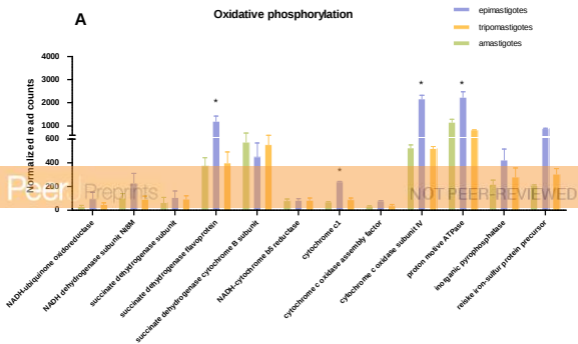
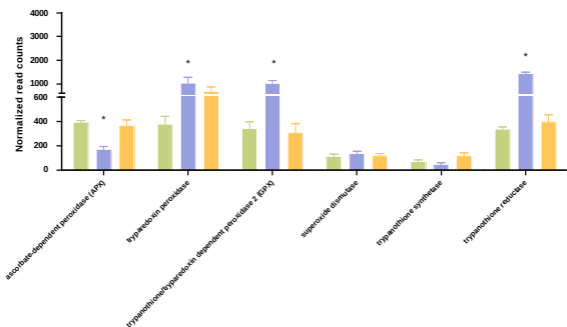
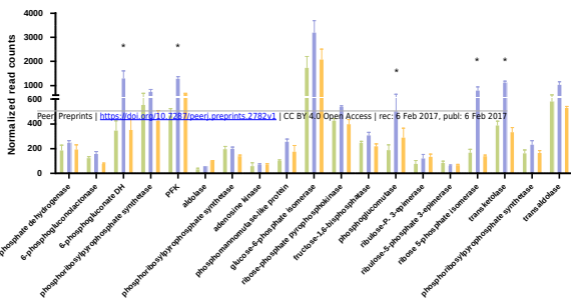
Neighbor-joining tree of the 3'UTR of GP63 genes; numbers correspond to gene ID in Dm28c annotation. Differentially up-regulated in A (green), differentially up-regulated in T (orange), very low or null levels of expression (blue).



**Figure 3**(on next page)

Differential expression in metabolic pathways

Normalized read counts for each stage is shown for A) Oxidative phosphorylation; B) Antioxidant defense; and C) Pentose phosphate pathway. Different cycle stages are represented: amastigotes (green), epimastigotes (blue) and trypomastigotes (orange). (\*) Denotes differentially expressed genes.

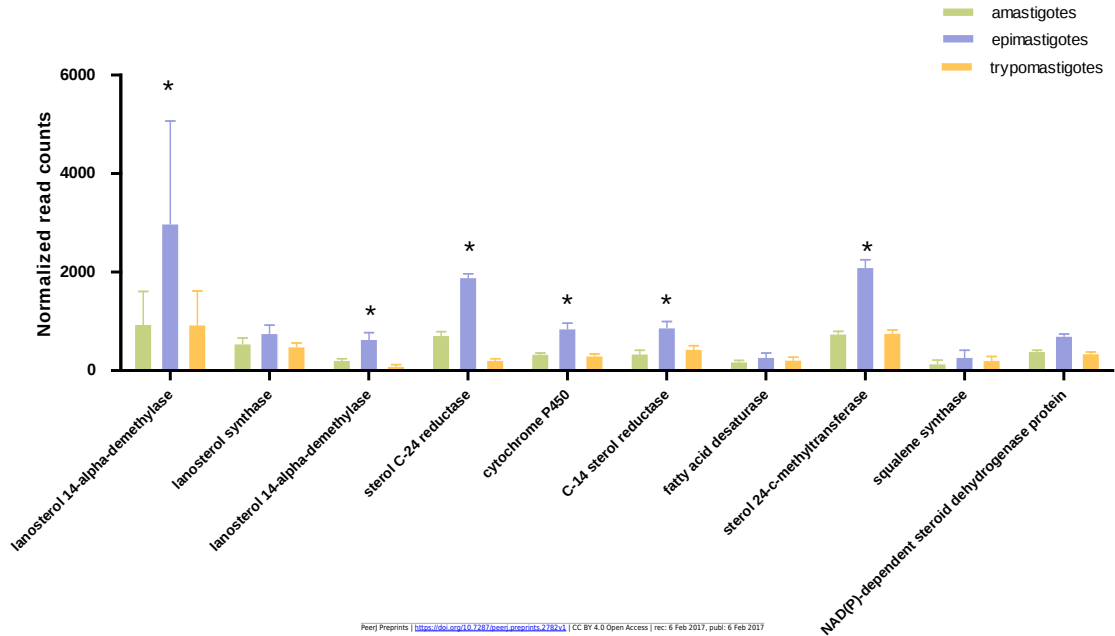
**A****Oxidative phosphorylation****B****Antioxidant defense****C****Pentose Phosphate Pathway**

**Figure 4**(on next page)

Expression of steroid biosynthesis related genes

Total normalized count of reads is shown for Steroid metabolism genes for the three stages: amastigotes (green), epimastigotes (blue) and trypomastigotes (orange). (\*) Denotes differentially expressed genes.

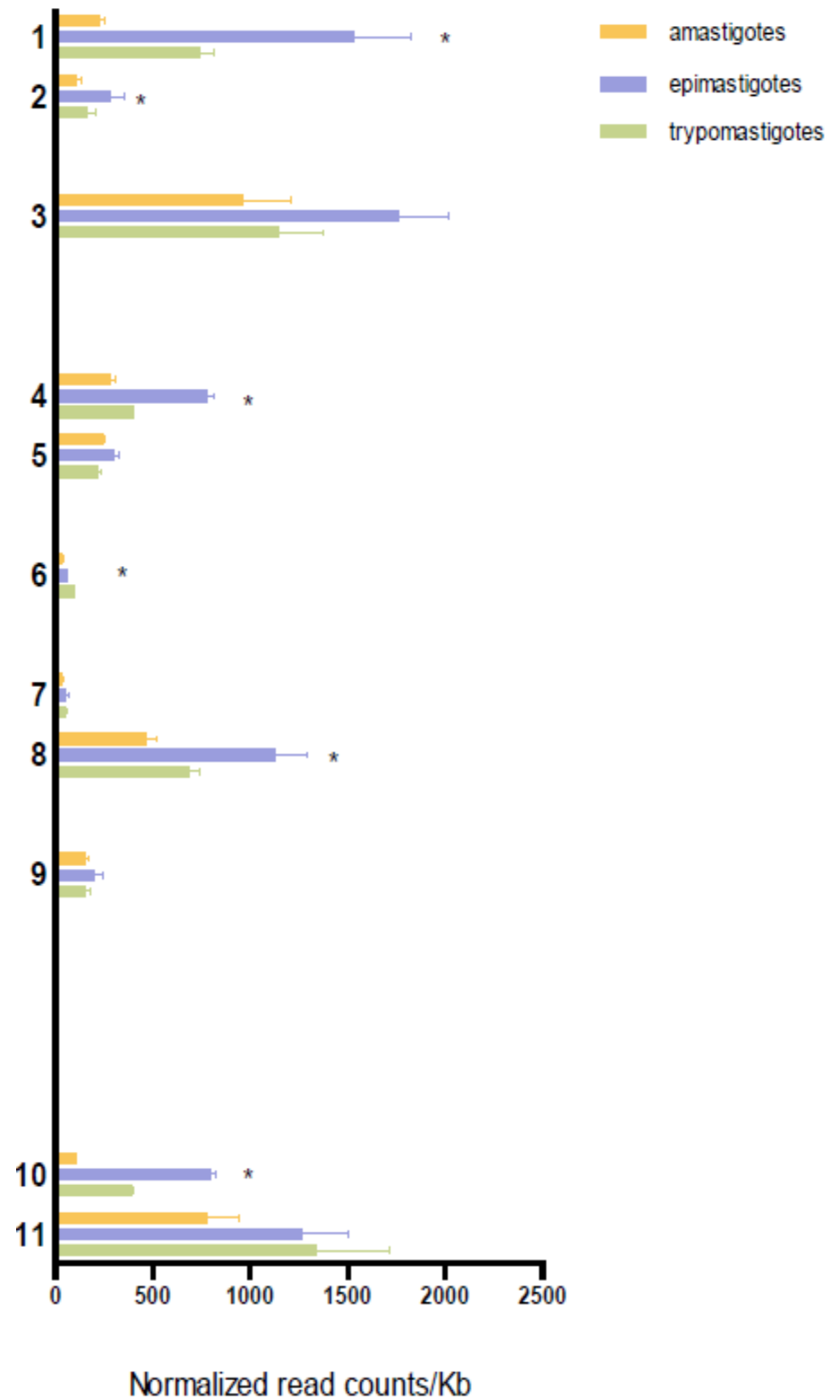
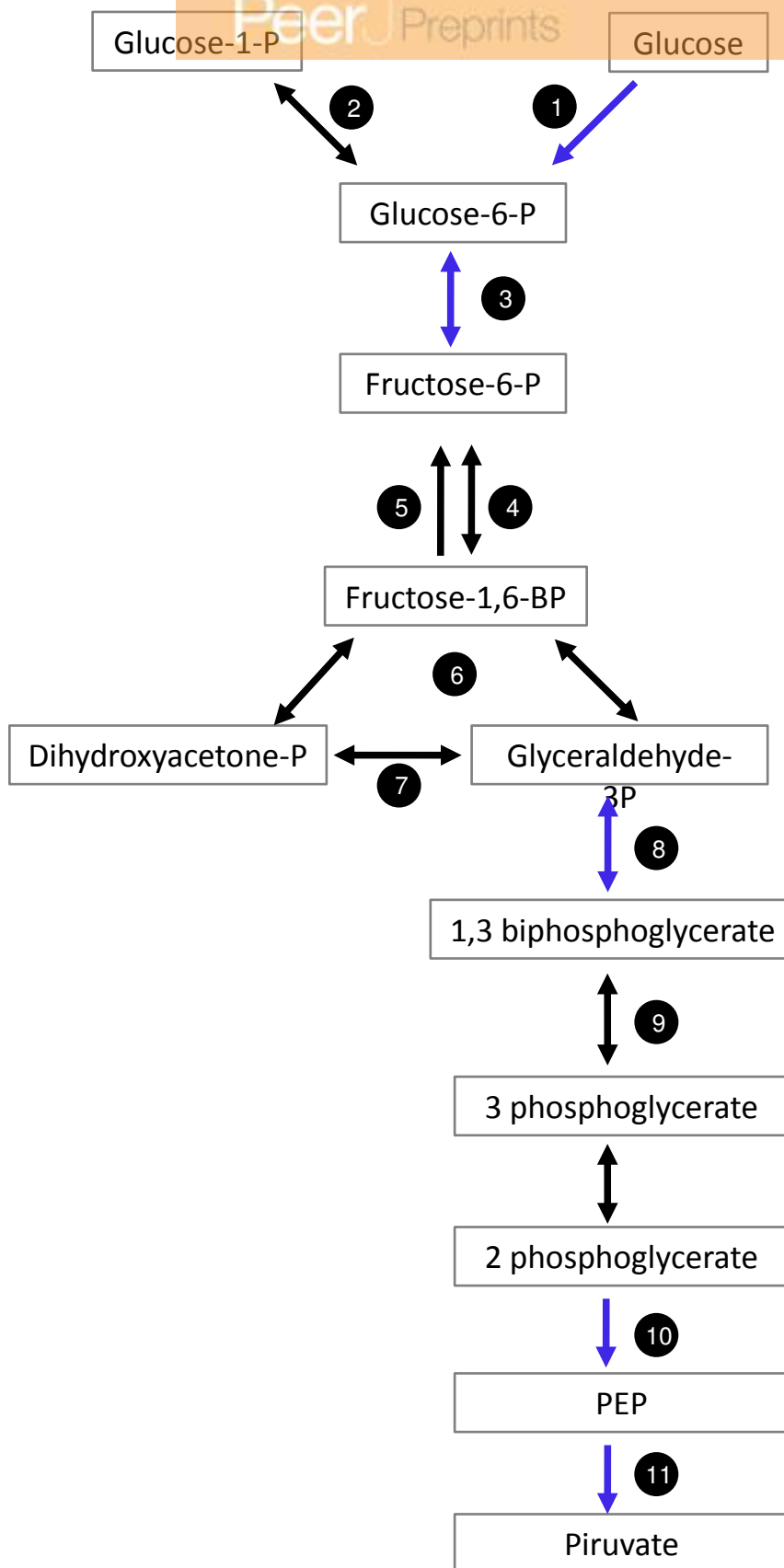




**Figure 5**(on next page)

## Glucose metabolism

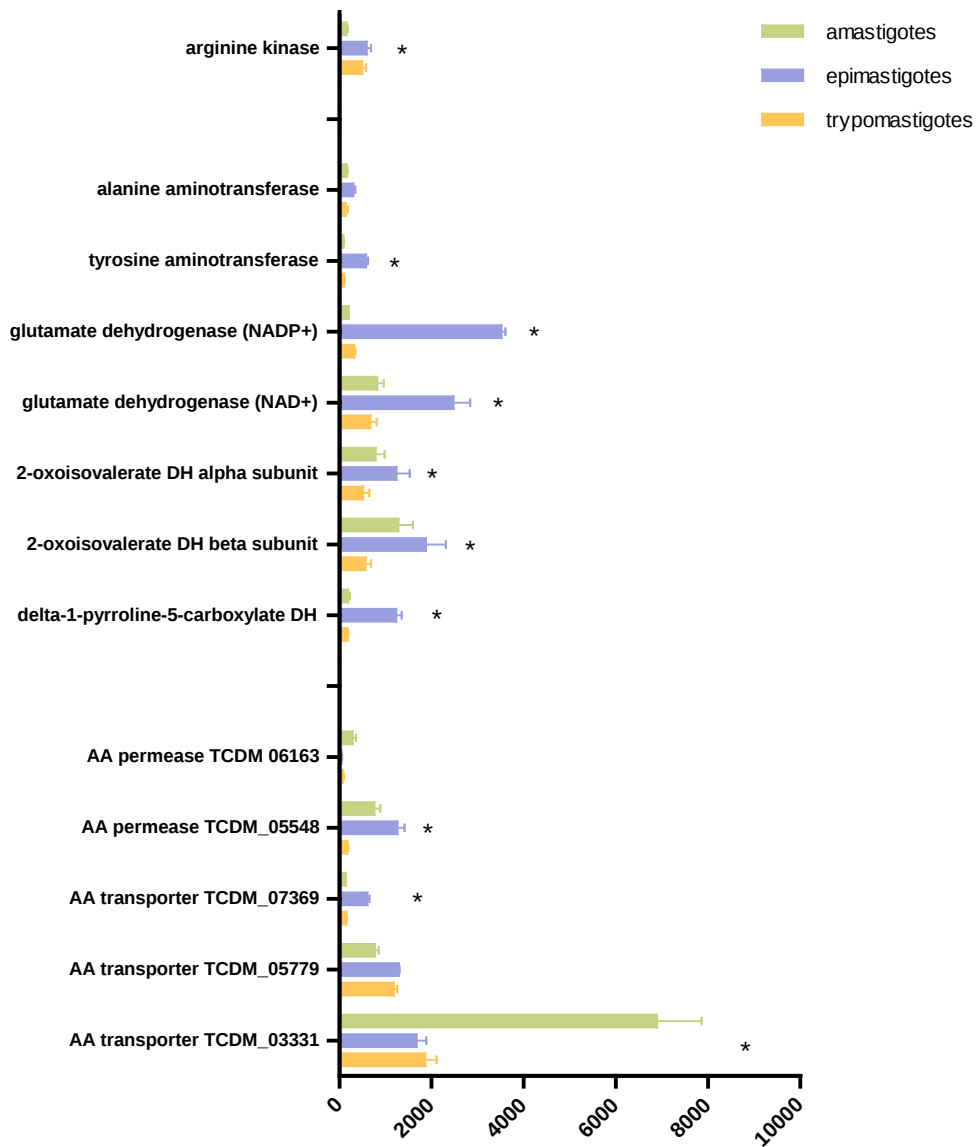
A) Schematic diagram of glucose catabolism. Each reaction is assigned with a number (1: hexokinase, 2: phosphoglucomutase, 3: glucose-6-phosphate isomerase, 4: phosphofructokinase, 5: fructose-1,6-biphosphatase, 6: aldolase, 7: triosephosphate isomerase, 8: glyceraldehyde 3-phosphate dehydrogenase, 9: phosphoglycerate kinase, 10: enolase and 10: pyruvate kinase 2). B) Expression of glucose metabolism genes of each reaction is shown as normalized count per gene size in kilobases. The three cycle stages are represented: amastigotes (green), epimastigotes (blue) and trypomastigotes (orange). (\*) Denotes differentially expressed genes.



**Figure 6** (on next page)

Expression of genes related to amino acid transport and metabolism

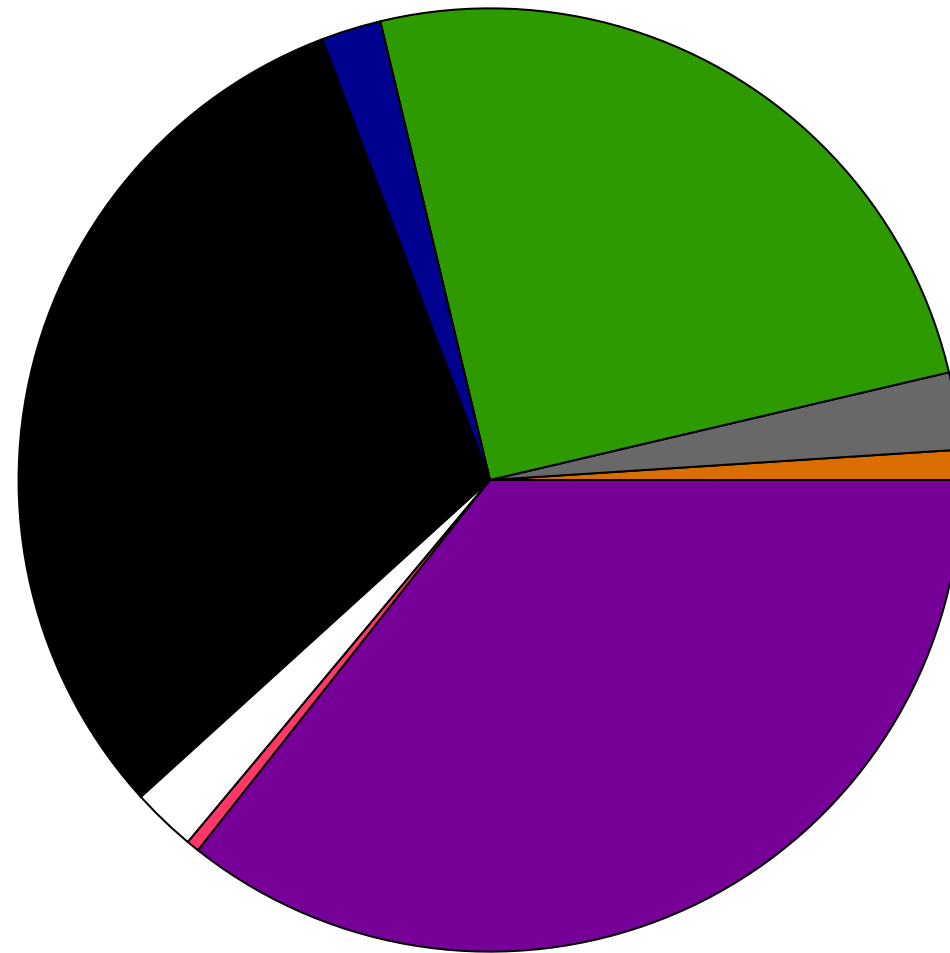
Total normalized Read counts of some genes coding amino acids transporters and nitrogen and amino acid related metabolism. The three cycle stages are represented: amastigotes (green), epimastigotes (blue) and trypomastigotes (orange). (\*) Differentially expressed.



**Figure 7** (on next page)

Membrane down-regulated genes in Epimastigotes

Pie chart representation of the percentage of down-regulated genes in epimastigote of each membrane protein category analyzed.



- CRP
- mucins
- MASP
- GP63
- Trans-sialidases
- Putative surface proteins
- Other surface related proteins
- Non surface related proteins

**Figure 8**(on next page)

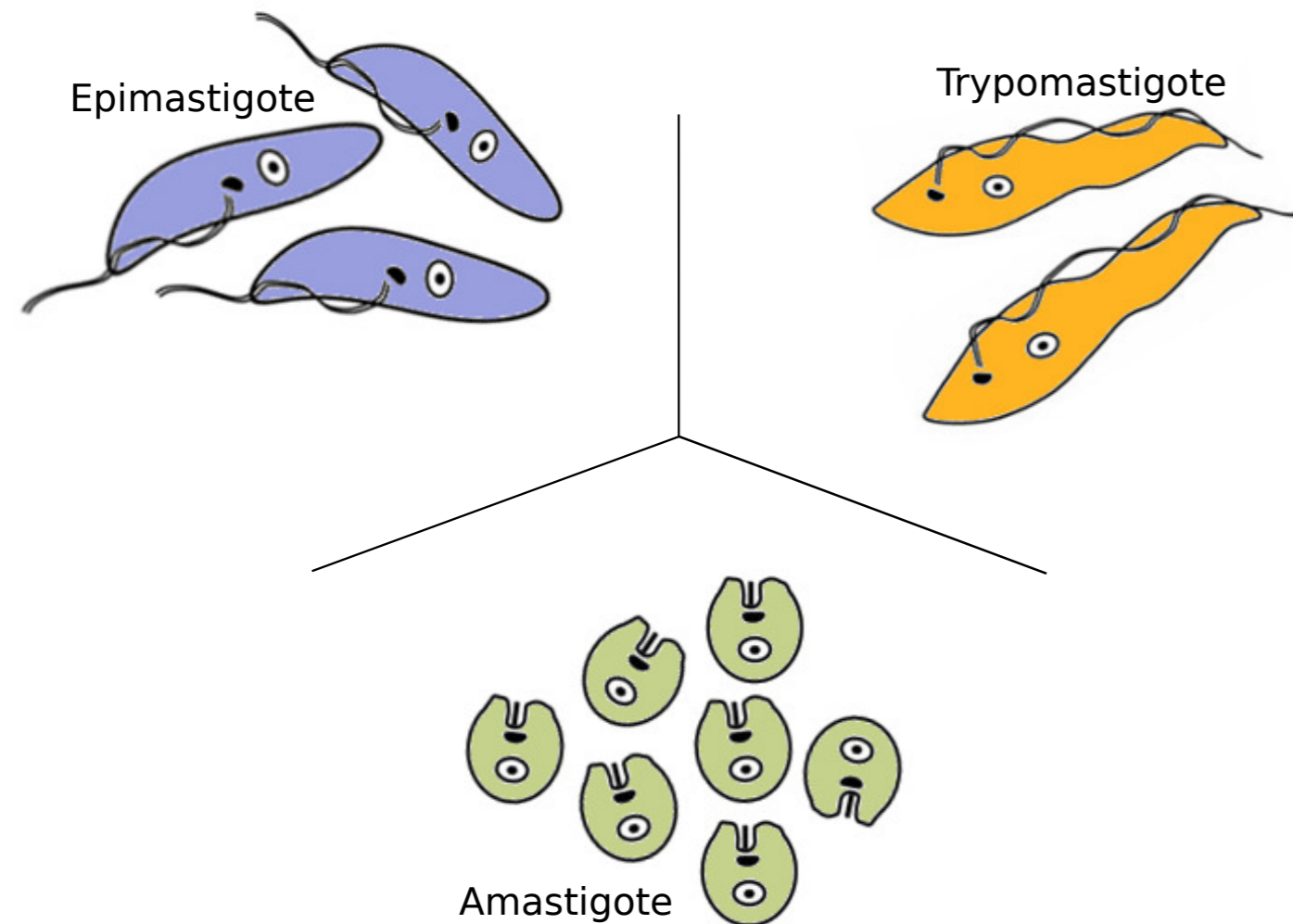
Expression levels overview of *Trypanosoma cruzi*

A) Diagram of the *Trypanosoma cruzi* stages and the major findings of transcriptoma analysis. B) Gene ontology (GO) enrichment analysis, showing GO terms exhibiting statistical significant differences (Fisher Exact Test, filtering p-values for multiple testing using False Discovery Rate) for the most expressed genes specific to amastigote (green), epimastigote (blue) and trypomastigote (orange).



A

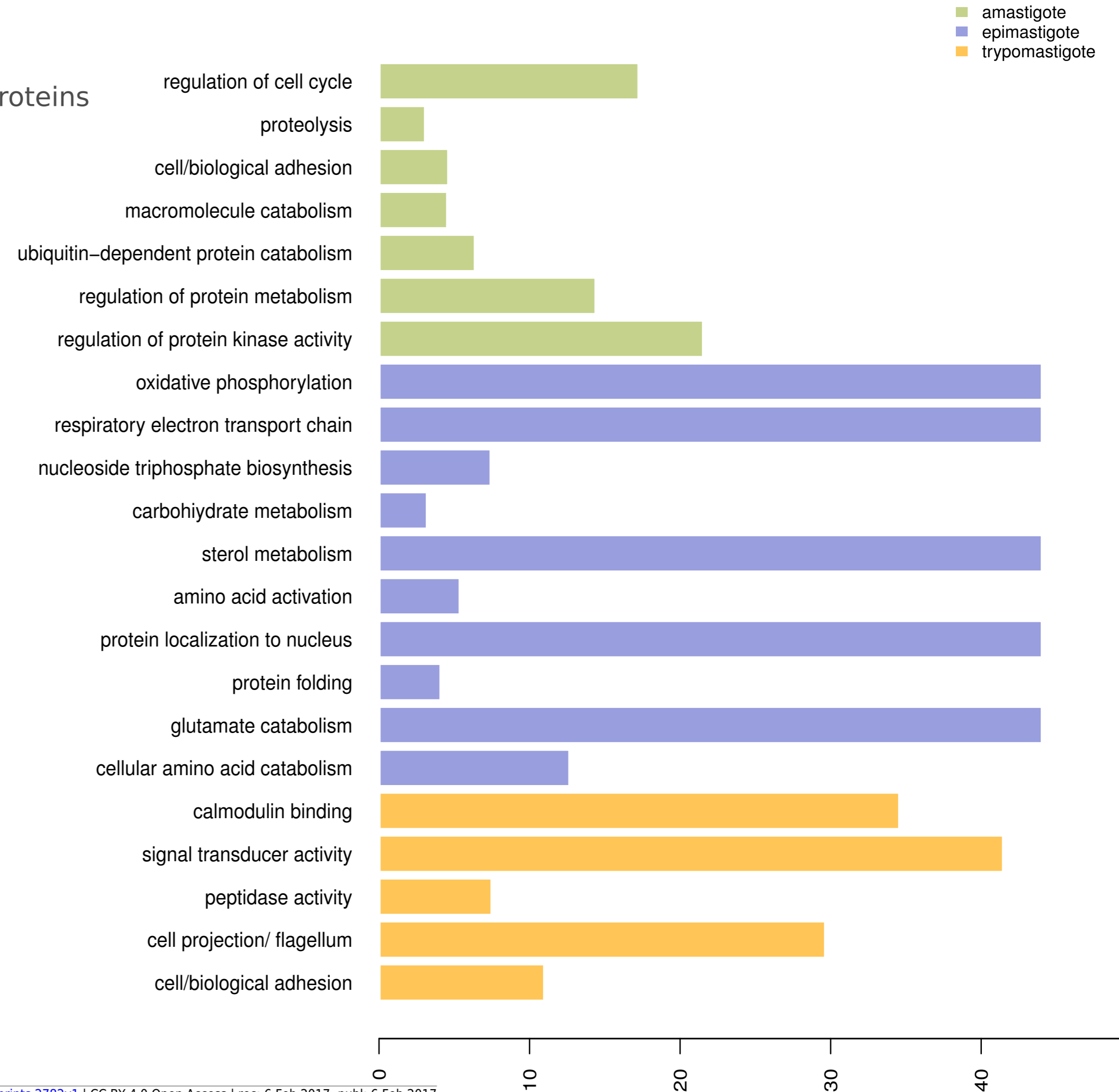
↑ anabolism  
 ↑ electron transport chain  
 ↑ NADPH generation  
 ↑ nucleotide biosynthesis  
 ↓ cell adhesion



↑ surface remodeling  
 ↑ immune evasion (TS, CRP)  
 ↑ adhesion/invasion related proteins  
 (GP63, mucin, MASP, TS)  
 ↑ signalling  
 ↓ nucleic acid metabolism

↑ transmembrane transport  
 ↑ macromolecule metabolism  
 ↑ DNA replication  
 ↓ carbohydrate catabolism  
 ↓ flagellar structural proteins

## GO – Most expressed genes



**Table 1** (on next page)

Table 1

RNA-seq mapping statistics

**Table 1. RNA-seq mapping statistics.**

Library	Readlength h	Total reads	Alignedreads	Alignedreads %	readcounts in CDS*	% readcounts in CDS*
A3	36	37.972.908	21.083.527	55,5	10.150.097	48,1
A7	72	6.011.870	2.644.677	44,0	1.314.640	49,7
T3	36	37.549.496	24.880.138	66,3	10.941.002	44,0
T7	72	2.976.919	1.604.848	53,9	716.431	44,6
E3	36	35.740.671	24.024.322	67,2	9.482.211	39,5
E7	72	8.540.435	5.496.063	64,4	2.192.983	39,9

\* CDS extended 200bp at both sides

A: amastigotes, T: tryomastigotes, E: epimastigotes

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**Table 2** (on next page)

Table 2

Gene Ontology enrichment of commonly highest expressed genes

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**Table 2. Gene Ontology enrichment of commonly highest expressed genes**

GO term	% genes present	Fold enrichment	p value
microtubule-based movement	21.0	5.4	2.73e-6
cellular component movement	20.3	5.2	3.72e-6
microtubule-based process	18.3	4.7	1.01e-5
chromosome organization	35.3	9.1	1.46e-4
DNA conformation change	31.3	8.0	8.54e-4
organelle organization	19.4	5.0	9.16e-4
DNA packaging	44.4	11.4	1.03e-3
cellular component organization	12.3	3.2	5.39e-3
cellular component organization at cellular level	12.7	3.27	7.75e-3
cellular component organization or biogenesis	10.6	2.7	7.86e-3
chromosome condensation	100.0	25.7	7.94e-3
sister chromatid cohesion	100.0	25.7	7.94e-3
chromosome segregation	100.0	25.7	7.94e-3
response to stress	12.1	3.1	9.99e-3

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**Table 3** (on next page)

Table 3

Membrane components in *T. cruzi* and those differentially expressed in Dm28c

**Table 3. Membrane components in *T. cruzi* and those differentially expressed in Dm28c**

	TS	MASP	mucin	DGF-1	GP63	CRP
CL BrenerGenome:						
Brener	419	345	230	205	93	1
Non-Esmeraldo	579	501	321	186	160	2
Esmeraldo-like	526	531	339	174	172	0
<i>T. marinkellei</i>	841	337	69	709	129	15
Sylvio	1112	249	76	984	126	4
Dm28c	659	311	116	69	60	13
<b>DEG</b>	295 (45%)	205 (66%)	25 (22%)	1 (1%)	26 (43%)	9 (69%)
Upregulated in trypanomastigote	268 (92%)	198 (97%)	25 (100%)	0 (0%)	10 (38%)	9 (100%)

1