Supporting Data

Supporting Results and Discussion 1. Identification and classification of novel small Tims. Tim8/Tim13 and Tim9/10 form $\alpha_3\beta_3$ hexamers and guide proteins across the IMS to the TOB and the TIM22 complex (Figure 1B) (Baker et al., 2009; Chacinska et al., 2009; Endo and Yamano, 2009; Neupert and Herrmann, 2007). The small Tims have two characteristic Cx_3C motifs (a so-called twin Cx_3C motif) which are recognised and oxidised by the Mia40/Erv1 couple (Figure S1) (Deponte and Hell, 2009). As reported previously (Gentle et al., 2007), homologs of all four opisthokont small Tims are present in some apicomplexan parasites whereas others might have lost up to three of the proteins. In kinetoplastids, we found four candidates for small Tims (Table 1), which is one more than in previous studies (Gentle et al., 2007). The sequence similarity of the fourth protein is lower, and the loop connecting the two predicted Cx_3C-containing α-helices is enlarged (Figure S1), which might be the reason why it has been overlooked so far. Kinetoplastids furthermore have a small Tim-like protein lacking the first disulfide bridge (Q4DDD2 and Q4Q3B2 in *T. cruzi* and *L. major*, respectively). The alignment of the small Tims in Figure S1 reveals that some of the candidates cannot be unambiguously assigned: for example, the protein Q4QAR1 from *L. major* was previously classified as a homolog of Tim9 (CAJ03937) (Gentle et al., 2007), and the first motif (Ex*C*FNL*C*x_2E) indeed resembles Tim9 from yeast. However, the second motif (KxEx$_2$*C*IDR*C*x_2RY) is far more similar to Tim10/Tim12 and both motifs are separated by sixteen instead of fifteen residues. In summary, the unusual repertoire of small Tims and the absence of Mia40 suggest that the IMS import machineries of parasitic protists differ significantly from their vertebrate hosts.

Supporting Results and Discussion 2. The molecular mechanism of Tim17. So far, only few data on the Tim17-dependent MPI mechanism is available. Two functionally highly relevant negative charges at the N-terminal arm of yeast Tim17 were suggested to interact with two

positive charges of an internal loop in the IMS (Meier et al., 2005). The intramolecular ionic interaction could then be replaced by the interaction with the positive charges of matrix precursor proteins during import. Indeed, the positive charges of residues Arg^{83} and Arg^{85} in yeast Tim17 are highly conserved and are also found in homologs from kinetoplastid and apicomplexan parasites (peptide 3 in Table 2). The acidic residues at the N-terminus of Tim17 are also conserved and are in front of a proline residue in a $(D/E)x_3(D/E)P$ motif in opisthokonts and apicomplexan parasites. In kinetoplastid parasites, the motif is replaced by the sequence $(D/E)x_5(D/E)P$ (peptide 1 in Table 2). In summary, MPI into the matrix could be functionally as well as mechanistically conserved.

Table S1 Primers used for cloning of *L. tarentolae* candidate genes.

Construct	Annotation	Primer	Sequence
LTTOB55/pDrive	JN380346	Sense	5'-ATGACCGACACTATGCAACAAACGG TAAACATTTGTGAGG-3'
		Antisense	5'-CTAGAACGAGAAATTGGATGACCAA ACCAAACCAAACCGGAACCGATC-3'
LTERV/pDrive	JN380347	Sense	5'-ATGTCGGACGACGACGTACACGAAC GCCTCACCACCATCCC-3'
		Antisense	5'-CTAGAGCTTGAGTTCTTCGTCCTCTG GGCAGTACACTTG-3'
LTTIM17/pDrive	JN380349	Sense	5'-ATGACATCCATCTTGGACCCTAGGC-3'
		Antisense	5'-TTAGTGCTGGGCCATGCCCATGGC-3'
LTsTIM1/pDrive	JN380348	Sense	5'-ATGCAGCCGGTGCAGTCGAATCCGA GCCTCATGGGGCTGACGC-3'
		Antisense	5'-TCACATTTTACCCGCTGCTGCGTCTT TCATCCACTGATACGG-3'
LTHSP60/pDrive	JN380350	Sense	5'-ATGCTCCGCTCCGCTGTGTGTCTTGC AG-3'
		Antisense	5'-CTAGAAGCCCATGCCGCCCATGCCG CC-3'

Table S2 Peptide sequences used for rabbit immunization and antibody purification. If necessary, an additional cysteine residue (underlined) was added to the N-terminus of the synthetic peptide for peptide coupling before affinity chromatography.

Protein	**Annotation**	**Position**	**Sequence**
*Lt*Tob55	JN380346	Residues 182-200 of 473	H_2N-<u>C</u>RVEEVKATTTNRKGKLASE-$CONH_2$
*Lt*Erv	JN380347	Residues 115-133 of 312	H_2N-<u>C</u>LRRWHPGYPNKMEDTPTIE-$CONH_2$
*Lt*Tim17	JN380349	Residues 61-78 of 152	H_2N-<u>C</u>TADFFRHSLRSAHRLGGS-$CONH_2$
*Lt*sTim1	JN380348	Residues 40-57 of 102	H_2N-CITHYGDDAIPYHPGEKA-$CONH_2$

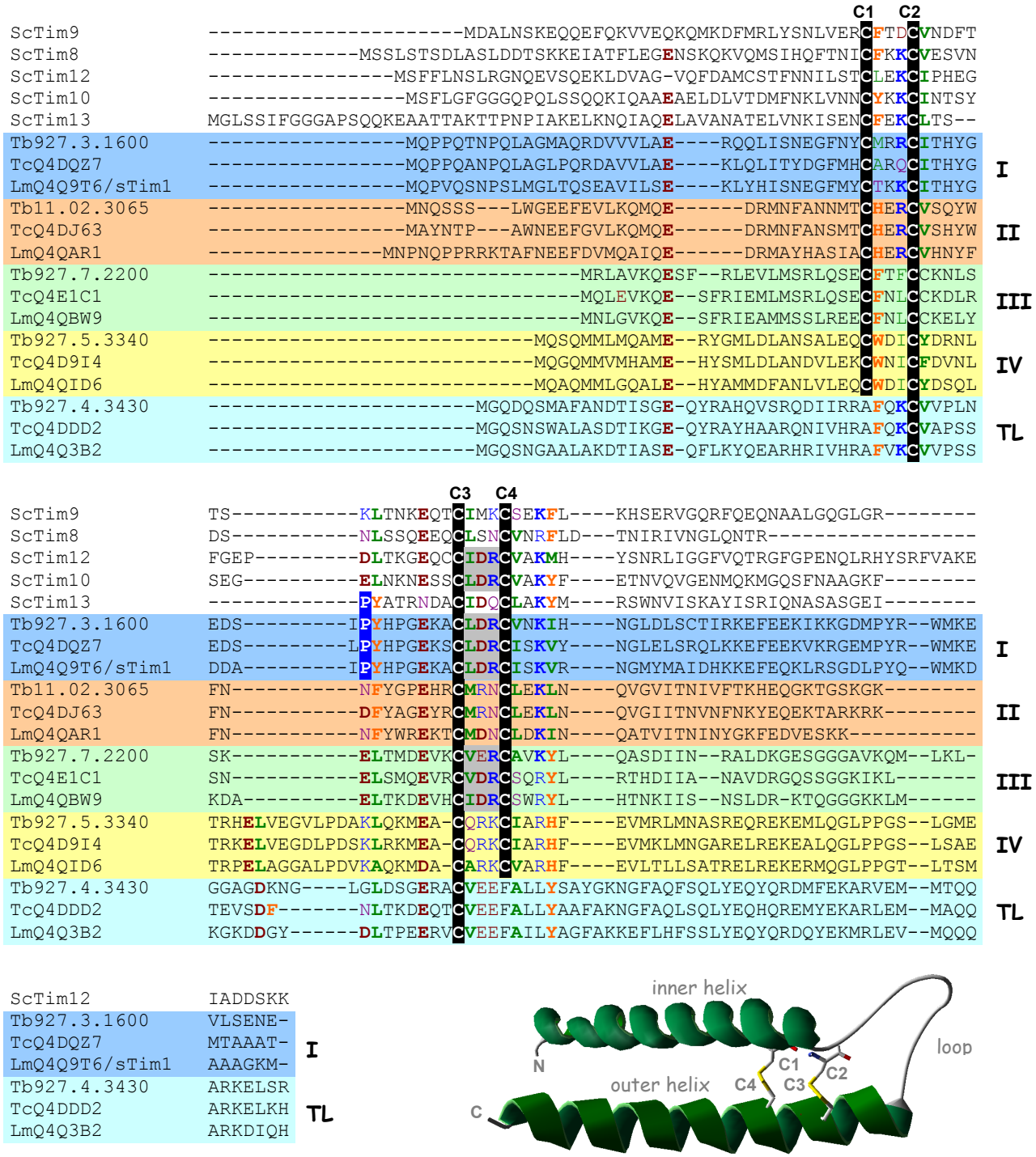

Figure S1 Sequence alignment of small Tims from yeast and kinetoplastid parasites. Four different groups of small Tims (I-IV) were found in the parasite genomes. The twin Cx_3C motifs are shaded in black. In addition, a novel group of small Tim-like proteins (TL), lacking cysteine residues C1 and C4, was identified. Residues which are characteristic for different subgroups are

highlighted. The structure of monomeric yeast Tim10 is shown for comparison (PDB entry 3DXR (Baker et al., 2009)). A significantly altered loop (comprising residues EL...LPDx) connects the predicted inner and outer α-helices in the newly identified group IV. *Lt*sTim1 described in this study is 97% identical to annotation Q4Q9T6 from *Leishmania major* and therefore belongs to group I. Further details are outlined in the Supplementary Results and Discussion 1. Accession numbers are from UniProtKB (http://www.uniprot.org/uniprot/) and GeneDB (http://www.genedb.org/). Tc, *Trypanosoma cruzi*; Tb, *Trypanosoma brucei*, Lm, *Leishmania major*.

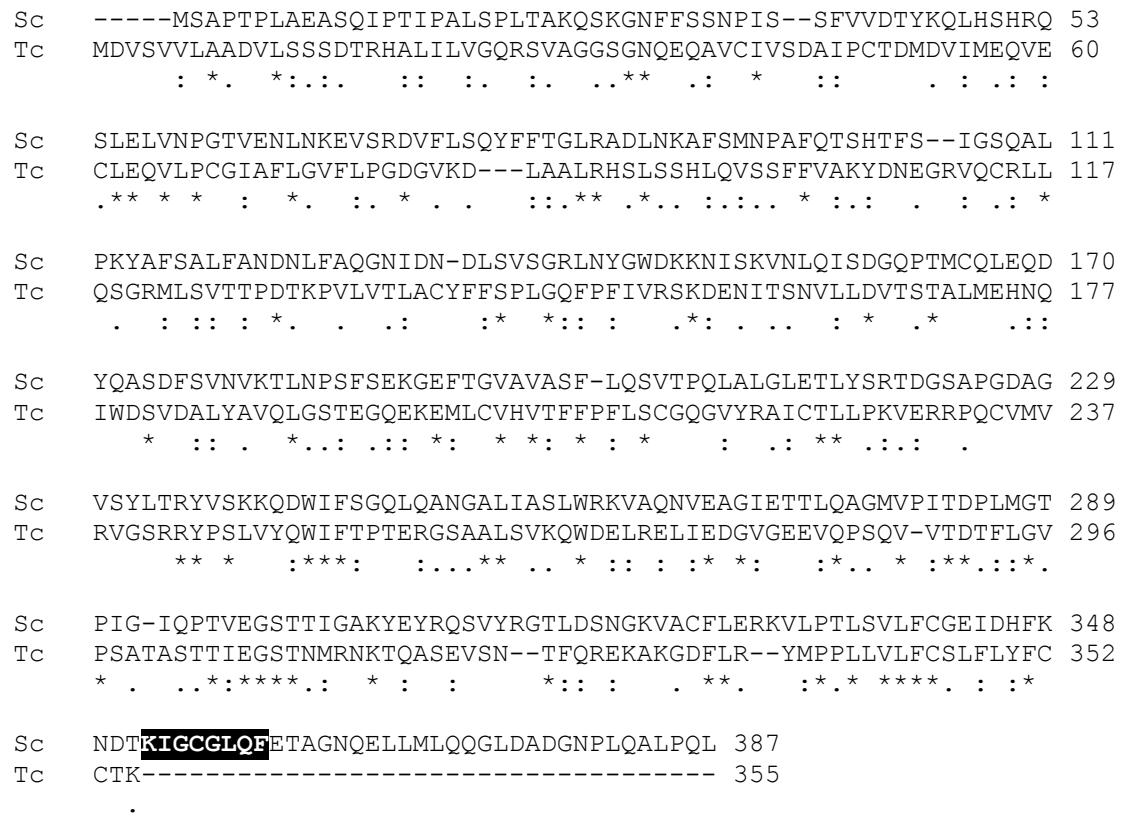

Figure S2 Sequence alignment of Tom40 from *S. cerevisiae* and a putative candidate protein from *T. cruzi*. The *T. cruzi* sequence corresponds to hypothetical protein Q4DKQ4 (UniProtKB annotation, http://www. uniprot.org/uniprot/). The targeting sequence of yeast Tom40 is shaded black (Kutik et al., 2008). The signal is absent in the parasite candidate.

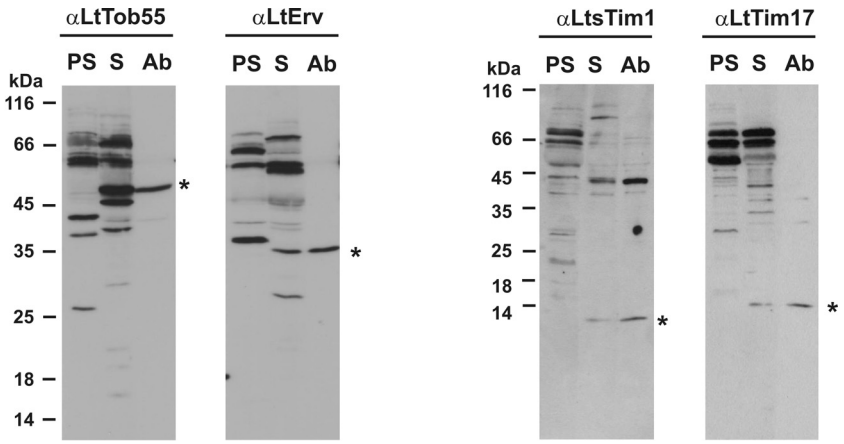

Figure S3 Purification of antibodies against mitochondrial marker proteins from *L. tarentolae*. Antibodies against the proteins *Lt*Tob55, *Lt*Erv, *Lt*sTim1 and *Lt*Tim17 were purified by affinity chromatography using the respective peptides listed in Table S2. The quality of the preparations was monitored by western blot analyses using preimmune serum (PS), serum (S), and affinity purified antibodies (Ab). In each lane 50 µg of mitochondrial proteins from *L. tarentolae* were separated by gel electrophoresis using either 12% SDS-polyacrylamide gels (left panels) or 8 M urea gels (right panels). Mitochondrial proteins were subsequently detected by western blotting. Preimmune sera, sera and purified antibodies were diluted 1:500, 1:2000 and 1:500, respectively. The calculated molecular masses of *Lt*Tob55, *Lt*Erv, *Lt*sTim1 and *Lt*Tim17 are 52.6, 34.7, 11.6 and 16.3 kDa, respectively. Bands with expected protein sizes are labelled with an asterisk.