Oxidative folding of proteins in the mitochondrial intermembrane space of *Leishmania tarentolae*

vom Fachbereich Chemie der Universität Kaiserslautern zur Verleihung des akademischen Grades „Doktor der Naturwissenschaften“ genehmigte Dissertation

vorgelegt von

Luzia Schneider

Wissenschaftliche Aussprache: 02.08.2022

Betreuer: Prof. Dr. Marcel Deponte

Promotionskommission:

Vorsitzender: Prof. Dr. Antonio Pierik

1. Gutachter: Prof. Dr. Marcel Deponte

2. Gutachter: Prof. Dr. Jörg Fahrer

Kennung der Universitätsbibliothek Kaiserslautern: DE-386

Erklärung

Ich versichere hiermit, dass ich die anliegende Dissertation mit dem Thema: " Oxidative folding of proteins in the mitochondrial intermembrane space of *Leishmania tarentolae*" selbständig verfasst und keine anderen Hilfsmittel und Quellen als die angegebenen benutzt habe. Die Stellen, die anderen Werken (einschließlich des Internets und anderer elektronischer Text- und Datensammlungen) dem Wortlaut oder dem Sinn nach entnommen sind, habe ich in jedem einzelnen Fall durch Angabe der Quelle bzw. der Sekundärliteratur als Entlehnung kenntlich gemacht. Ergebnisse etwa beteiligter Mitarbeiter und anderer Autoren sind klar gekennzeichnet. Weiterhin versichere ich hiermit, dass die o.g. Arbeit noch nicht anderweitig eingereicht wurde. Ich versichere, dass ich mich zuvor an keiner Hochschule um einen Doktortitel beworben habe.

_____

(Luzia Schneider)

_____

(Ort, Datum)

Danksagung

Viele Menschen haben mich bei meiner Arbeit unterstützt. Ihnen zu danken ist mir ein besonderes Anliegen.

Ich danke Prof. Marcel Deponte für die Möglichkeit, meine Doktorarbeit in seiner Arbeitsgruppe anfertigen zu können. Danke für die Überlassung dieses spannenden Themas und die gute Betreuung.

Mein Dank gilt auch Prof. Antonio Pierik, für die Übernahme des Vorsitzes der Prüfungskommission und Prof. Jörg Fahrer für die Berichterstattung.

Dr. Markus Räschle bin ich dankbar für die massenspektrometrischen Analysen, für die Hilfe bei der Probenvorbereitung, für die Messungen und die Hilfe bei der Auswertung.

Bei Miro und Tarik möchte ich mich für die Entwicklung der drop dilution assays bedanken.

Für die gute (Arbeits-) Gemeinschaft danke ich Sophie, Gino, Fabian, Robin, Lukas, Eileen, Britta, Felix und Ann-Cathrin. Danke für die gute Zusammenarbeit, die wissenschaftlichen Diskussionen und die guten Gespräche.

Meinen Eltern, meiner Schwester und meinem Freund Jochen danke ich für ihre Unterstützung.

Abstract

Mitochondrial genes encode for a few proteins. Thus, the majority of proteins has to be imported to the organelles, which is only possible in the unfolded state. The subsequent folding guarantees functionality. One of the proteins responsible for folding in the intermembrane space is Mia40, which is known in opisthokonts. No ortholog for Mia40 is known in kinetoplastida such as *Leishmania tarentolae*. First, already known candidates for Mia40 orthologs were investigated. In previous work Mic20 had been identified in *Trypanosomes*.[1–4] Gene editing cassettes to knock-out or modify the gene *LtaPh_3313851,* which encodes the Mic20 ortholog, could not be inserted homozygously. Thus, the gene is assumed to be essential. Another protein that plays an important role in mitochondrial protein import is Erv, a known interactor of Mia40. Erv is also found in *Leishmania*. Two proteins of so far unknown function had been identified as potential interactors of Erv and could be candidates for Mia40 orthologs.[5] Potential knock-out strains of one protein-encoding gene each were investigated. The knock-out of *LtaP32.0380* was assumed to be complete and the gene dispensable. The knock-out cassette for *LtaP07.0980* could be shown to be inserted heterozygously, which could indicate the essentiality of the gene. To identify new candidates for Mia40 orthologs in *Leishmania tarentolae*, potential substrates[6] of the Mia40/Erv pathway were used as baits in the present work. Gene editing via CRISPR/Cas9 included attempts to insert knock-out or tagging cassettes to five different genes. Homozygous insertion succeeded for the C-terminal His_8-tagging cassettes for *LtaP19.1110*, and for the N-terminal His_8-tagging cassettes for *LtaP25.1620* and *LtaP09.1390*. No homozygous gene editing could be observed for LtaP35.0210. The knock-out of *LtaP04.0060* was assumed to be complete. The presence of the N-terminal His_8-tagged substrate 4 (LtaP09.1390) could be shown in cell lysates. The correct position of tagged substrate 4 in the cell was confirmed. Further cell lysates were purified in pull-downs on Ni-NTA to obtain tagged substrate 4 with its interaction partners. The presence of tagged proteins in the eluates could be confirmed. To identify interacting proteins, mass spectrometry analysis was performed. In further experiments, DTT and TMAD were used to alter the redox conditions in the cells before lysis and purification. The evaluation of the data included the comparison of the proteins identified in different experiments and the comparison with potential interactors of Erv.[5,7] Also, properties of Mia40 that might be

conserved were considered. Two characteristic motifs of known Mia40 orthologs are a CPC and a twin CX_9C motif. Thus, proteins with these or similar motifs were specifically searched for. Different candidates for Mia40 orthologs were identified and discussed.

Zusammenfassung

Mitochondriale Gene codieren für wenige Proteine. Daher muss die Mehrheit der Proteine in diese Organellen importiert werden. Dies ist nur in ungefaltetem Zustand möglich. Nachfolgendes Falten garantiert die Funktionalität der Proteine. Ein Protein, das für die Faltung im Intermembranraum verantwortlich ist, ist Mia40, das aus Opisthokonta bekannt ist. Für Mia40 ist kein Ortholog in Kinetoplastida, wie beispielsweise *Leishmanien* bekannt. Zunächst wurden aus der Literatur bekannte Kandidaten für Orthologe untersucht. Als solches wurde Mic20 in *Trypanosoma* identifiziert.[1–4] Kassetten für den Knock-out oder die Modifizierung des Gens *LtaPh_3313851*, das für das Mic20 Ortholog kodiert, konnten nicht homozygot eingeführt werden. Daher wurde angenommen, dass das Gen unverzichtbar ist. Ein anderes Protein, das eine wichtige Rolle im mitochondrialen Proteinimport spielt ist Erv, von dem bekannt ist, dass es sich um einen Interaktionspartner von Mia40 handelt. Erv wurde auch in *Leishmanien* identifiziert. Zwei Proteine mit bisher unbekannter Funktion wurden als Interaktoren von Erv identifiziert und könnten Kandidaten für Mia40 Orthologe sein.[5] Stämme in denen je eines der beiden Proteine potentiell ausgeknockt werden sollte wurden untersucht. Der Knock-out des Gens *LtaP32.0380* wurde als vollständig und das Gen als entbehrlich interpretiert. Es konnte gezeigt werden, dass die Knock-out-Kassette für *LtaP07.0980* heterozygot eingefügt war. Dies deutet auf eine Unverzichtbarkeit des Gens hin. Um neue Kandidaten für Orthologe von Mia40 in *Leishmania tarentolae* zu identifizieren wurden in der vorliegenden Arbeit potentielle Substrate[6] des Mia40/Erv-Importwegs als Köder eingesetzt. Die genetische Manipulation mittels CRISPR/Cas9 umfasste das Einfügen von Knock-out- oder Tagging-Kassetten an fünf verschiedenen Genen. Homozygote Insertion gelang für die C-terminalen His_8-Tagging-Kassetten für *LtaP19.1110* und für die N-terminalen His_8-Tagging-Kassetten für *LtaP25.1620* und *LtaP09.1390*. Keine homozygote Genmanipulation konnte für *LtaP35.0210* beobachtet werden. Als vollständig wurde der

Knock-out von *LtaP04.0660* angesehen. Das N-terminal His_8-getaggte Substrat 4 (LtaP09.1390) konnte in Zelllysaten nachgewiesen werden. Die korrekte Lokalisierung von getaggtem Substrat 4 in der Zelle wurde bestätigt. Weitere Zelllysate wurden aufgereinigt in Pull-downs an Ni-NTA, um das getaggte Substrat 4 mit seinen Interaktionspartnern zu erhalten. Getaggte Proteine konnten in den Eluaten nachgewiesen werden. Um die Interaktoren zu identifizieren wurden massenspektrometrische Analysen durchgeführt. In weiteren Experimenten wurden DTT und TMAD verwendet um die Redoxbedingungen der Zellen zu manipulieren, bevor die Zellen lysiert und aufgereinigt wurden. Die Auswertung der identifizierten Proteine beinhaltete den Vergleich verschiedener Experimente untereinander und mit den Interaktoren von Erv.[5,7] Auch Eigenschaften von Mia40, die konserviert sein könnten wurden berücksichtigt. Zwei charakteristische Motive in den bekannten Mia40 Orthologen sind ein CPC- und ein doppeltes CX_9C-Motiv. Daher wurde gezielt nach Proteinen mit diesen oder ähnlichen Motiven gesucht. Verschiedene Kandidaten für Mia40 Orthologe wurden identifiziert und diskutiert.

Contents

Abbreviations and units

°C	degree celsius
µ	mikro; 10^{-6}
3’	three prime
5’	five prime
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BHI	brain heart infusion
bp	base pair
c	centi; 10^{-2}
ca.	circa
c(digitonin)	concentration of digitonin
Cas9	clustered regularly interspaced short palindromic repeats associated protein 9
CHCHD4	coiled-coil-helix-coiled-coil-helix domain-containing protein 4
CRISPR	clustered regularly interspaced short palindromic repeats
C-terminus	carboxy terminus
Da	Dalton
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
Erv	essential for respiratory and vegetative growth protein
FBS	fetal bovine serum
g	gravitational force equivalent; $g \approx 9.81\ m/s^2$
g	gram
h	hour
H. sapiens	*Homo sapiens*
HA	human influenza hemagglutinin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HiFi	High Fidelity
HPLC	high-performance liquid chromatography
HRP	horseradish peroxidase
h*Sp*Cas9	humanised *Streptococcus pyogenes* Cas9 nuclease
ID	identification number
IEP	isoelectric point
k	kilo; 10^3
kb	kilobase pair
KISS	kinetoplastida-specific second
KO	knock-out
L	liter
L. tarentolae	*Leishmania tarentolae*
LB	lysogeny broth (Luria Bertani)
Lt	*Leishmania tarentolae*
m	meter
M	molar; mol / liter; 1 mol = 6.022 x 10^{23}
m (prefix)	milli; 10^{-3}
m/v	mass per volume
MEM	minimal essential medium
MeOH	methanol
Mia40	mitochondrial intermembrane space import and assembly protein 40
Mic20	mitochondrial contact site and cristae organizing system protein 20
MICOS	mitochondrial contact site and cristae organizing system
min	minute
MOPS	3-(*N*-morpholino)propanesulfonic acid
mRNA	messenger ribonucleic acid
MS	mass spectrometry
n	nano; 10^{-9}
N. crassa	*Neurospora crassa*
NEM	*N*-ethylmaleimide
Ni-NTA	nickel-nitrilotriacetic acid agarose
nLC	nano-liquid chromatography

nt	nucleotide
N-terminus	amino terminus
OD_{600}	optical density at 600 nanometers wavelength
P value	*probabilitas* (probability) value
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pH	*pondus hydrogenii*
PVDF	polyvinylidene difluoride
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
rpm	revolutions per minute
S	Siemens
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulfate
sgRNA	single guide ribonucleic acid
STAGE	stop and go extraction
TAE	Tris-acetate-EDTA buffer
sTim	small translocase of the inner membrane
TBS	tris(hydroxymethyl)aminomethane buffered saline
TBS-T	tris(hydroxymethyl)aminomethane buffered saline 0.05% (m/v) Tween 20
TEMED	*N,N,N′,N′*-tetramethyl ethylenediamine
TFB	transformation buffer
Tim	translocase of the inner membrane
TM	transmembrane
TMAD	*N,N,N′,N′*-tetramethylazodicarboxamide
TOM	translocase of the outer membrane
Tris	tris(hydroxymethyl)aminomethane
Triton	Triton X-100
UF	unknown function
UF2	unknown function protein 2
UF3	unknown function protein 3

UTR	untranslated region
v/v	volume per volume
w/v	weight per volume
WT	wild type

Amino acids[8] as well as nucleic acids[9] and derivatives are abbreviated with one-letter symbols according to the International Union of Pure and Applied Chemistry.

Figures

Tables

1. Introduction

1.1. Basics

1.1.1. Mitochondria

Mitochondria are essential components in eukaryotic cells.[10–12] Two of their central functions are the synthesis of adenosine triphosphate (ATP) and the synthesis of iron-sulphur clusters.[13–16] According to the current state of knowledge, the evolutionary origin of mitochondria is described as symbiogenesis.[17–20] Derived from this, mitochondria evolved from aerobe bacteria that were taken up by a host cell. From the two originally separated cells arose a single organism. The evolutionary origin of the mitochondrion is also reflected in its structure. Mitochondria are surrounded by a double membrane which evolved from the two membranes of the incorporated bacterium. The inner membrane forms one of the characteristics of mitochondria: its invaginations are called cristae. They provide a large surface for respiration and ATP synthesis.[21–24] By their membranes, the segmentation of mitochondria is defined. The outer membrane limits the organelle. Between the two membranes the intermembrane space is located. The sector surrounded by the inner membrane is called matrix. Figure 1 shows the structure of a mitochondrion schematically.

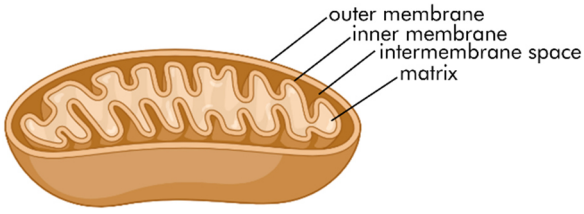


Figure 1: Schematic representation of a mitochondrion. Two membranes separate the compartments. The outer membrane separates the mitochondrion from the cytoplasm. The inner membrane is located between the intermembrane space and the matrix. Adapted from biorender.[25]

In living cells, mitochondria are not static. Their localisation and form can fluctuate, as well as their division or fusion with other mitochondria.[26–28] Having evolved from independent cells, mitochondria also contain their own deoxyribonucleic acid (DNA) and ribosomes for

protein synthesis. Since the genome of mitochondria itself encodes only for a few proteins, there is a need for efficient protein import.[29–31] Different properties of proteins as well as different destinations require corresponding mechanisms, so that each protein is functional and can be located at its target site.[32] For the present work, the oxidative folding of proteins after mitochondrial import to the intermembrane space via the Mia40/Erv pathway is of particular interest.

1.1.2. Mia40 and its substrates

The mitochondrial intermembrane space import and assembly protein 40 (Mia40) is known from opisthokonts such as *Saccharomyces cerevisiae* and its orthologs are found in many species.[33–36] The human coiled-coil-helix-coiled-coil-helix domain-containing protein 4 (CHCHD4) is one such ortholog.[30,37] The function of Mia40 is associated with mitochondrial protein import.[38–40] As an oxidoreductase it oxidises and thus folds proteins that are imported to the mitochondrial intermembrane space. Orthologs of Mia40 are not known for kinetoplastids as *Trypanosoma brucei* or *Leishmania*.[1,41–43] However, another component of mitochondrial import of oxidatively folded proteins is found in kinetoplastids. It is called essential for respiratory and vegetative growth (Erv).[43–46] From *Arabidopsis thaliana*, it is suggested that Erv alone can fold imported proteins.[47–49] Data from Turra et al. indicate that another component is necessary for oxidative folding in *Leishmania tarentolae*.[36,42] The task of the current work is to identify candidates for Mia40 orthologs in *Leishmania tarentolae*. Properties known from Mia40 orthologs of other organisms can help identify such candidates. One of the vital components of Mia40 is its active site: a CPC motif that is responsible for disulphide exchange with imported proteins.[38] Adjacent to this motif, a hydrophobic pocket is located, which is important for the binding of the substrates.[40,50–54] A twin CX_9C motif[38,52,55,56] is characteristic here (see also the supplements, S1). Figure 2 schematically shows the import of proteins with disulphide bridges that are oxidatively folded in the mitochondrial intermembrane space.

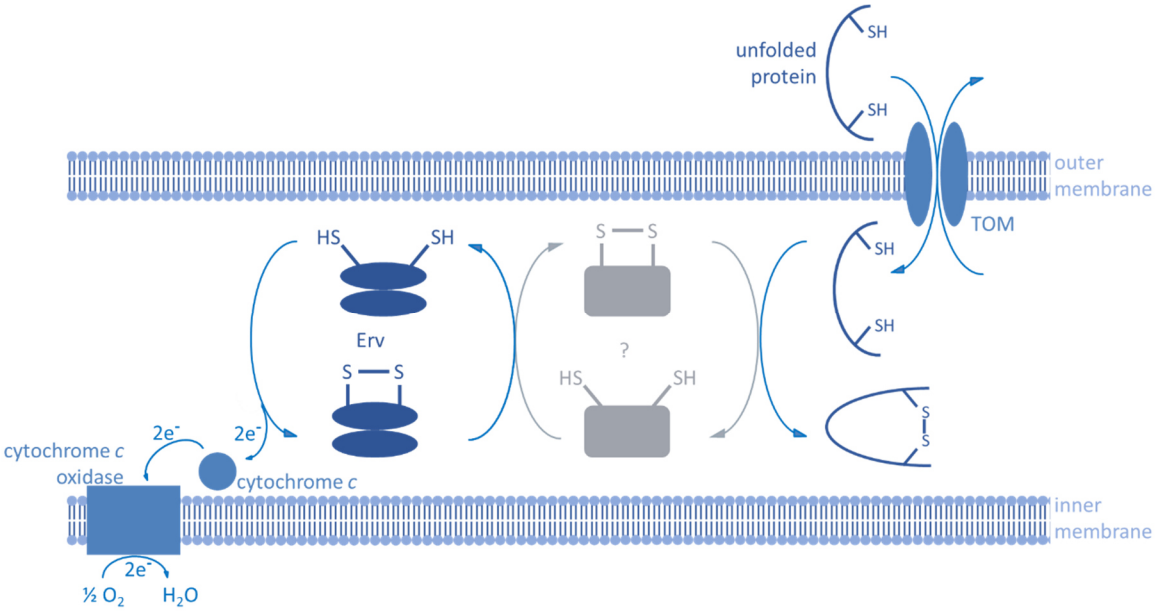


Figure 2: Schematic representation of hypothetical protein import to mitochondrial intermembrane space for oxidatively folded proteins. These are imported through the TOM in an unfolded, reduced state. In the intermembrane space, the proteins are oxidatively folded by an unknown enzyme. The electron flow enters the respiratory chain via Erv and cytochrome *c*. The figure was adapted from Deponte and Hell.[57]

The cysteine-containing proteins are imported to mitochondria through the translocase of the outer membrane (TOM) in an unfolded state. To gain functionality, oxidative formation of disulphide bonds has to take place. For example in *Leishmania tarentolae*, it is not yet known which oxidoreductase interacts directly with the imported proteins. It is very likely that electron flow continues via Erv and enters the respiratory chain via cytochrome *c*.

Substrates of Mia40 are cysteine-containing proteins of the mitochondrial intermembrane space. Often these exhibit targeting information within the mature protein, not as cleavable presequence.[6,32,38,55] Cysteines exchanging a disulphide bond with Mia40 are part of the signal, as well as hydrophobic residues that are important for recognition by the hydrophobic binding pocket of Mia40.[32,38] One putative targeting signal could possess nine aminos acids upstream or downstream of any cysteine of a twin CX_9C motif.[58] Four and seven amino acids distant from the cysteine, an aromatic and a hydrophobic amino acid or two aromatic amino acids are located.[58] Common cysteine motifs in substrates of Mia40 are twin CX_3C, twin CX_9C or CX_2C.[38,55] CX_9C motifs form a coiled-coil–helix–coiled-coil–helix structure, stabilized by disulphide bonds of its cysteine residues.[58] Other substrates of Mia40, such as small translocase of the inner membrane (sTim) proteins exhibit CX_3C motifs. But also Erv is one of the substrates of Mia40 in opisthokonts.[58]

For the functionality of Mia40, its composition plays an important role. The proximity of hydrophobic substrate binding site and catalytic disulphide allows the initial formation of a dynamic enzyme-substrate complex that is not covalently bound.[52,59]

The import of Mia40 itself is also noteworthy. In animals, it is imported without presequence[58] as was described for its substrates. Thus, Mia40 itself is a substrate of the Mia40/Erv pathway. In fungi, on the other hand, Mia40 is imported via the presequence pathway.[58]

1.1.3. Selection of potential substrates of a Mia40 ortholog

Since potential substrates of an unknown oxidoreductase are well characterised (see also Chapter 1.1.2), they are the ideal starting point for the search for Mia40 orthologs. However, the idea to find the oxidoreductase via its substrates is not new in the Deponte research group. In her dissertation, Linda Liedgens identified suitable potential substrates of a Mia40 ortholog.[6] Based on the work of Peikert et al,[7] performing knock-down of Erv in *Trypanosoma brucei*, proteins related to the Mia40/Erv pathway were identified. Criteria were chosen by Liedgens to select suitable subtrates as baits. For example, molecular mass, presence of cysteine motifs, total number of cysteines and phylogenetic conservation in kinetoplastida, apicomplexa and opisthokonts were taken into account.[6] For instance, a molecular mass up to 24 kDa is appropriate to distinguish the substrate from Erv in size-dependent analytics such as sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Table 1 lists the selected substrates of Liedgens that were also used in the present work.

Table 1: List of potential Mia40 substrates used in the present work with gene identification number, molecular mass and cysteine motifs.[6,60] The potential substrates were identified in the work of Liedgens.[6]

Substrate	Gene identification number	Molecular weight / [kDa]	Cysteine motifs
substrate 1	*LtaP04.0660*	28	twin CX_9C
substrate 2	*LtaP35.0210*	24	$CX_{11}C$
substrate 3	*LtaP19.1110*	27	$CX_6VCVCVCVC$, twin CX_9C
substrate 4	*LtaP09.1390*	22	CC, CX_9C
sTim1	*LtaP25.1620*	12	twin CX_3C

Liedgens used episomally encoded constructs of the substrates to perform pull-down experiments with Erv and sTim1. Sandra Specht also used the identified substrate candidates for her dissertation.[36,61] She introduced point mutations and different tags and used episomal constructs for expression in *Leishmania tarentolae*. The present work on the other hand uses chromosomal tagging and knock-out of the substrate encoding genes, which is enabled by the adaption of a gene editing system for *Leishmania tarentolae* (Chapter 1.1.5). This offers the advantage that no additional copy of the gene is created experimentally. In the case of a complete editing, there are no unaltered genes present and no competition between two different expressed genes.

1.1.4. Leishmania tarentolae

Leishmania are unicellular parasites. *Leishmania tarentolae* is a non-pathogenic species. This fact and its easy handling in culture makes it an ideal model organism and one of the best studied *Leishmania* species.[62–66] Figure 3 shows a microscopy image of *Leishmania tarentolae*.

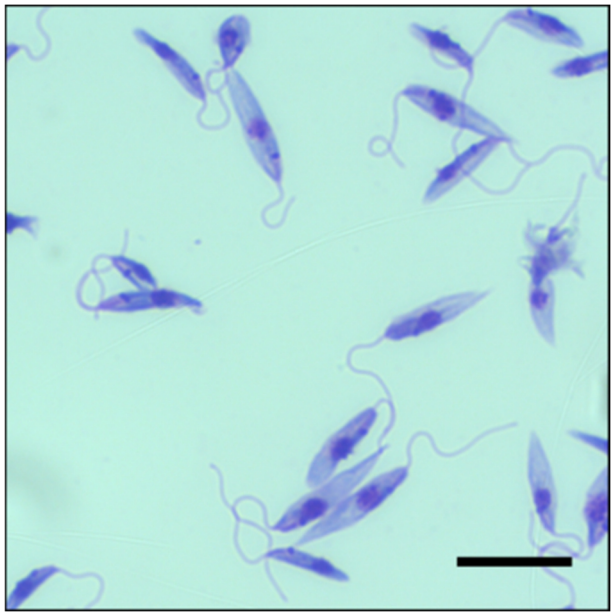


Figure 3: Image of *Leishmania tarentolae*, parental strain. The cells were fixed with MeOH and stained with Giemsa. The scale bar corresponds to 5 µm. Nucleus and kinetoplast appear as dark spots.

Nevertheless, the life cycle of *Leishmania tarentolae* is not yet fully known.[62,67] The two hosts of this species are sand flys (*Sergentomya*) and geckos (*Tarentola*).[67,68] Figure 4 shows the different life stages assumed.

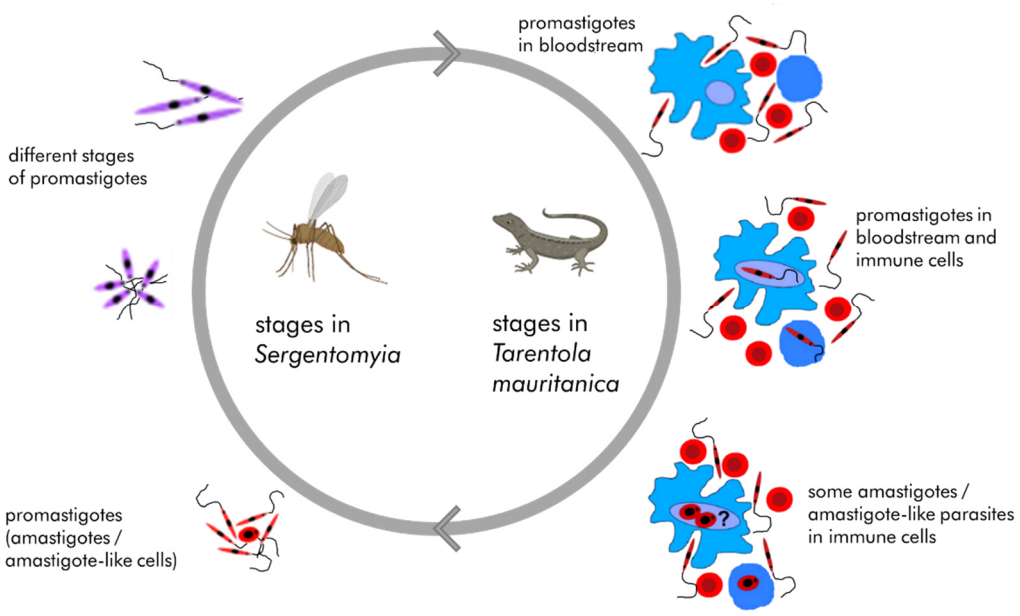


Figure 4: Schematic representation of the life cycle of *Leishmania tarentolae*. Parasites occur in two hosts: *Sergentomyia* and *Tarentola*. In both, promastigotes are the dominating stage. In sand fly, different stages of promastigotes are passed. In gecko, *Leishmania tarentolae* enters the blood stream and immune cells. Formation of amastigotes in immune cells is not fully understood yet. The figure was adapted from Klatt et al.[25,62]

Promastigotes are the dominating stages in both hosts, the role of non-motile amastigotes of *Leishmania tarentolae* is still unclear. Promastigotes and amastigotes or amastigote-like cells are transferred to a sandfly, taking blood from a gecko. Different stages of promastigotes are passed. When an infected sand fly bites a gecko, promastigotes are transferred and enter the blood stream of the reptile. Immune cells are occupied by promastigote cells developing to amastigotes or amastigote-like cells. Axenic cultures of *Leishmania* that are used in this work and many applications of *Leishmania* consist of promastigotes.

Leishmania tarentolae are of special interest because of the similarity to pathogenic *Leishmania* species. Leishmaniosis is a wide-spread disease in humans and other mammals, transmitted by the bite of a sand fly.[69] Especially tropics, subtropics, and the Mediterranean basin are affected.[70] Up to one million infections per year are counted. 20000 – 30000 deaths are caused by leishmaniosis in the same period.[71] Leishmaniosis is also included in a project of the World Health Organization to combat neglected tropical diseases[72] and thus, in the Kigali declaration, which was drafted by an alliance of global health and development organisations, and industry partners to support these efforts.[73] Thus, understanding the parasite and its metabolism is important, also to identify differences to the host as well as other parasite species.

On the other hand, applications of *Leishmania tarentolae* in protein production are useful, since the posttranslational modifications are similar to those in human cells. For example, the production of recombinant proteins in *Leishmania tarentolae* yields similar glycosylation patterns.[62,74–76]

1.1.5. Gene editing of *Leishmania tarentolae* via CRISPR/Cas9

For chromosomal gene editing, the modular system, published by Beneke and Madden et al. was used.[77] Transfer of this method to *Leishmania tarentolae* was established by Gino Turra et al.[5,42,78] in the Deponte research group. The efficient, modular system from Beneke and Madden et al. is suitable for tagging and knock-out experiments as needed for the present work.[79,80] DNA fragments are available without cloning. Polymerase chain reaction (PCR) is performed to amplify the composite fragments specific to each target gene.

The basis of the system is a cell line with the episomal plasmid pTB007[77]. This plasmid contains genes for humanised *Streptococcus pyogenes* clustered regularly interspaced short palindromic repeats associated protein 9 nuclease (h*Sp*Cas9) and T7 RNA polymerase. In the present work, the *Leishmania tarentolae* strain with episomal pTB007 plasmid is referred to as the parental strain. The parental strain was already available in the Deponte research group, based on the work of Turra.[5]

To guide the double-strand breaks caused by Cas9, single guide ribonucleic acid (sgRNA) fragments are needed. These are *in vivo* transcribed from DNA templates by T7 ribonucleic acid (RNA) polymerase. In practise, the DNA templates are composed of two parts and amplified by PCR[77]. One part consists of the T7 promoter and the 20 nt homology region for the target site and a second homology region for the sgRNA scaffold. This scaffold is the second part for the sgRNA template and is the same for all applications. Its primer is called G00[77] (primer 44, Table 3).

To repair the precisely controlled DNA cut from Cas9, DNA donor cassettes are used. The cassettes exhibit a homology region of 30 nt that ensures correct position of the donor cassette[77]. Another vital part of the donor cassettes is the drug-selectable marker gene, which encodes for example Blasticidine-*S* deaminase and Puromycin *N*-acetyltransferase. Other parts of the cassettes are sequences for untranslated regions from *Leishmania mexicana* (*LmxM*) that promote robust expression of the resistance genes[77]. Also, intergenic regions from *Crithidia fasciculata*[81] are used, which contain messenger ribonucleic acid (mRNA)

processing signals. These unspecific parts of the cassettes are encoded in the commercially available plasmids pT for knock-out and pPLOT for tagging[77] (see also the Figure 5). Specificity for the target genes is reached via primers for PCR, which contain two homology regions each: one for the target gene and one for the cassette on the plasmid. Due to the modular system, the same primers can be used for pT and pPLOT plasmids. Sequences encoding for protein tags can be included in primers and target carboxy (C-) or amino (N-) terminus. All plasmids that are used in the present work are based on the work of Turra[5]. With LeishGEdit[82], automated primer design is available[77]. Figure 5 summarises the components for gene editing by Beneke and Madden et al.[77]

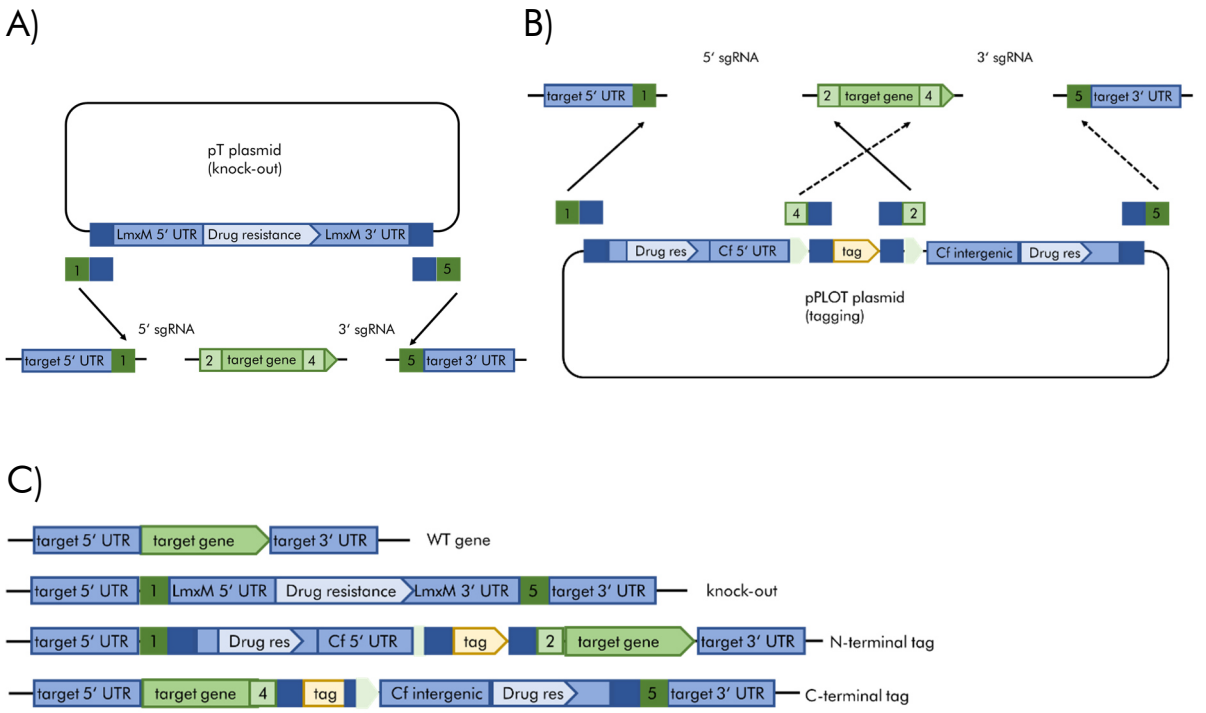


Figure 5: Strategy for genome editing of kinetoplastids by Beneke and Madden et al. The figure was adapted from Beneke, Madden et al.[77] Panel A) shows the pT plasmid for knock-out experiments. The drug resistance cassette is flanked by untranslated regions from *Leishmania mexicana* (*LmxM*) that promote robust expression of the resistance genes. Panel B) shows the pPLOT plasmid for tagging experiments. The resistance cassette is present twice. The geometry of the amplicons depends on the position of the tag. Panel C) shows the resulting constructs in the chromosome. The wild-type (WT) gene is the not altered version. The knock-out gene exhibits an insert from a pT plasmid. The inserts encoding for N- and C-terminal tags derived from a pPLOT plasmid. Primer numbers 1 – 5 correspond to p1 – p5 in Table 3 (see the summary column).

To generate cassettes for knock-out experiments, pT plasmids are used. These exhibit the drug resistance flanked by the *LmxM* untranslated regions. The pPLOT plasmid for the generation of tagging cassettes exhibits two copies of the drug resistance, since the geometry of the cassettes for N- or C-terminal tagging are mirror-inverted. Only one

cassette per tag is used, depending on the desired position. The constructs resulting from genome editing are also shown in Figure 5.

1.1.6. Characteristics of the DNA of *Leishmania tarentolae*

A peculiarity of kinetoplastida is the kinetoplast mitochondrial DNA, which consists of two types of circular DNA molecules, minicircles and maxicircles.[83–85] The kinetoplast is located in the mitochondrion and contains many copies of mitochondrial genes. In images of *Leishmania tarentoale* with stained DNA, the kinetoplast can be seen as small spot at the base of the flagellum (compare Figure 3). Since the present work deals with proteins imported to mitochondria, the kinetoplast is not in focus.

Dealing with genetic manipulation, it is important to consider the organisation of the DNA of the target organism. It was previously assumed that *Leishmania tarentolae* as most eukaryotes, are generally diploid. New data, however, suggest that they exhibit aneuploidy.[78,86–89] This means that several individual chromosomes are present in a number that differs from the usual number in the chromosome set. One explanation for the presence of aneuploidy could be rapid adaption to changing environments.[87] In *Leishmania tarentolae* mosaic aneuploidy is observed. This means that the number of chromosomes can differ between individual cells.[86] However, Beneke and Madden et al. showed that gene editing and the generation of a null mutant are possible in a single round of transfection.[77] With the presented procedure, using two selection markers, disomic chromosomes are covered. There is evidence that chromosomal gene editing experiments can lead to an increase of the copy number of the targeted gene.[5] The presence of unaltered copies of a gene is excluded by genotyping after transformation. If the characteristic PCR amplicon for the parental strain is not present, all copies of a gene were processed.

1.2. Aim of this work

This work deals with the import of cysteine-containing proteins in *Leishmania tarentolae* to mitochondrial intermembrane space. Cysteine-containing proteins are imported in an unfolded, reduced state to mitochondrial intermembrane space. To be functional, they must be oxidised, forming disulphide bonds between certain cysteine residues. In opisthokonts, Mia40 is the oxidising agent. Mia40 itself is reduced by Erv, which, among other characteristics of the Mia40/Erv pathway, is also found in kinetoplastida. However, no homolog for Mia40 is identified so far. Mic20 was considered as a candidate in kinetoplastida.[1–3,41] To investigate the role of its CIPC motif, insertion of mutations was a task of the present work and attempts were made to knock out the corresponding gene. Other previous attempts tried to identify interaction partners of Erv in *Leishmania tarentolae*.[7,78] Two proteins with so far unknown function were identified and knock-out attempts for the corresponding genes (*LtaP32.0380*, *LtaP07.0980*) were present.[5] Genotyping these strains with potential knock-outs was included in the present work. Also, potential substrates of Mia40 in *Leishmania tarentolae* were previously identified.[6,61] The major task of the present work was the identification of interaction partners of these substrates and to find out whether candidates for a Mia40 ortholog are present. Comparisons with findings from the literature were conducted to support the evaluation.

2. Materials and Methods

General

All experiments with cells that were subsequently stored under physiological conditions were always carried out under sterile conditions. If not stated otherwise, pure water was obtained from an OmniaTap device from Stakpure with a conductivity of 0.055 µS/cm.

2.1. Materials

2.1.1. Companies

ACROS ORGANICS: Acros Organics B.V.B.A., part of Thermo Fisher Scientific

BD: Becton, Dickinson and Company, Franklin Lakes, United States

BERND KRAFT: Bernd Kraft GmbH, Duisburg, Germany

biostep: biostep GmbH, SARSTEDT Group, Sarstedt AG & Co. KG

Bio-Rad: Bio-Rad Laboratories, Inc., Hercules, California, United States

Biotium: Biotium, Inc., Fremont, CA 94538, United States

BTX: BTX, division of Harvard Bioscience, Inc, Holliston, Massachusetts, Unites States

Carl Roth: Carl Roth GmbH + Co. KG, Karlsruhe, Germany

cell projects: cell projects, Harrietsham, Maidstone ME17 1AB, United Kingdom

Cell Signaling Technology: Cell Signaling Technology, Inc, Danvers, Massachusetts, Unites States

CHEMSOLUTE®, TH. Geyer: Th. Geyer GmbH & Co. KG, Renningen, Germany

Corel Corporation: Corel Corporation, Ottawa, Ontario, Canada

Eppendorf: Eppendorf SE, Hamburg, Germany

Eurofins Genomics: Eurofins Genomics Germany GmbH, Ebersberg, Germany

Fisher BioReagents™: Trademark of Thermo Fisher Scientific

Fisher Chemicals: see Thermo Fisher Scientific

GE Healthcare: GE Healthcare Chicago, Illinois, United States

GenScript: GenScript Biotech (Netherlands) B.V., Leiden, Netherlands

Gibco®: Trademark of Thermo Fisher Scientific

Greiner: Greiner Bio-One GmbH, Kremsmünster, Austria

Honeywell: Honeywell International Inc., Morristown, United States

InvivoGen: InvivoGen, San Diego, California, United States

Lonza: Lonza Group AG, Basel, Switzerland

Merck: Merck KGaA, Darmstadt, Germany

metabion: metabion international AG / metabion GmbH, Planegg, Germany

Microsoft: Microsoft Corporation, Redmond, Washington, United States

Motic: Motic (Xiamen) Electric, China

New England Biolabs: New England Biolabs GmBH, Ipswich, Massachusetts, United States

PanReac AppliChem: AppliChem GmbH, part of ITW - Illinois Tool Works Inc., Chicago, Illinois, USA

Promega: Promega Corporation, Madison, Wisconsin, United States

Qiagen: Qiagen N.V., Venlo, Netherlands

Roche: Roche Holding, Basel, Switzerland

SARSTEDT: SARSTEDT AG & Co. KG, Nümbrecht, Deutschland

SERVA: SERVA Electrophoresis GmbH, Heidelberg, Germany

Seq It: Seq It GmbH & Co.KG, Kaiserslautern, Germany

Sigma-Aldrich: Sigma-Aldrich, St. Louis, Missouri, United States, subsidiary of Merck KGaA

Simport: Simport Scientific, Saint-Mathieu-de-Beloeil, QC J3G 4S5, Canada

Stakpure: Stakpure GmbH, Niederahr, Germany

STARLAB: STARLAB GmbH, Hamburg, Germany

Thermo Fisher Scientific: Thermo Fisher Scientific Inc., Waltham, Massachusetts, U.S.

Waldeck: Waldeck GmbH & Co. KG, Münster, Germany

Vilber: Vilber Lourmat Deutschland GmbH, Eberhardzell, Germany

VWR Chemicals: see VWR international

VWR international: VWR International, Radnor, Pennsylvania, United States, part of Avantor

Zeiss: Carl Zeiss AG, Oberkochen, Germany

2.1.2. Equipment with manufacturer

Axiocam 105 color	Zeiss
Axio Lab.A1 Binocular Microscope, light field	Zeiss
Binocular AE30 Inverted Microscope	Motic
EASY-nLC™ 1200 HPLC	Thermo Fisher Scientific
FUSION SL documentation imaging system, Vilber Lourmat Peqlab	Vilber
Gel Electrophoresis Tank Horizontal	biostep
Mini-PROTEAN® electrophoresis cell	Bio-Rad
NanoDrop™ 1000 Spectrophotometer	Thermo Fisher Scientific
Q Exactive HF™ Mass Spectrometer	Thermo Fisher Scientific
Thermal Cycler Mastercycler® Nexus	Eppendorf
Transfection Device Nucleofector™ 2b Device	Lonza
Wet Tank Mini-Trans Blot® cell system	Bio-Rad

2.1.3. Disposables with manufacturer

6-well plates	Greiner
Centrifuge tubes (15 and 50 mL)	Greiner
Cryovials Cryos 2 mL	Greiner
Electroporation Cuvettes Plus 2 mm gap	BTX®
Electroporation Cuvettes 2 mm gap	cell projects
Empore™ C18 extraction disks	Thermo Fisher Scientific
Inoculation loop 10 µL	Simport
Micro Bio-Spin™ Chromatography Columns	Bio-Rad
Micro reaction tubes (1.5 and 2 mL)	SARSTEDT
PCR reaction tubes	SARSTEDT
Petri dishes 85/13 mm	Waldeck
Polyvinylidene difluoride (PVDF) membrane	Merck
Serological pipettes (1, 5, 10, 25 mL)	SARSTEDT
Sterile syringes, BD Plastipak™ (20, 60 mL Luer lok™, 2 mL Discardit™ II)	BD

Syringe Filter, PVDF (Sterile) (0.22 µm)	STARLAB
Tissue culture (TC) flasks	Greiner
Whatman paper	GE Healthcare

2.1.4. Chemicals with supplier

Acetic acid Rotipuran® 100% p.a.	Carl Roth
Acetone ≥99,8%	VWR Chemicals
Acrylamide/Bis Solution (30%) - Mix 37.5 : 1, for molecular biology	PanReac AppliChem
Agar granulated (BP9744-500)	Fisher BioReagents
Agarose Basic	PanReac AppliChem
Ammonium persulfate ≥98%	Sigma-Aldrich
Brain heart infusion (BHI)	VWR Chemicals
Bromophenol blue	Waldeck
Calcium chloride	Merck
cOmplete™ EDTA-free protease Inhibitor	Roche
Deoxyribonucleoside triphosphate (dNTPs)	Thermo Fisher Scientific
Digitonin	PanReac AppliChem
Dithiothreitol (DTT) ≥99% p.a.	Carl Roth
Dimethyl sulfoxide (DMSO) for PCR	New England Biolabs
DNA ladder (100 bp, 1kb)	New England Biolabs
Ethanol absolute p.a.	CHEMSOLUTE®, TH. Geyer
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
N-ethylmaleimide (NEM) ≥98% (HPLC)	Sigma-Aldrich
Fetal bovine serum (FBS) (heat inactivated)	Sigma-Aldrich
Folic acid ≥96% for biochemistry	Carl Roth
Gel loading dye, purple 6x	New England Biolabs
GelRed® dye, 6x	Biotium
Giemsa stock solution	Carl Roth
Glycerol for analysis	PanReac AppliChem
Glycine 99+% for analysis	ACROS ORGANICS
Hemin chloride	Calbiochem®, MerckMillipore

Hydrochloric acid 37%	BERND KRAFT
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], (HEPES)	Merck
Imidazole	Merck
Isopropanol ≥99,7%	VWR Chemicals
Lysogeny broth (LB) agar	Carl Roth
LB medium	Carl Roth
Manganese(II) chloride x2H_2O 99%	Merck
β-mercaptoethanol ≥99%	Sigma-Aldrich
Methanol ≥99.9%	Fisher Chemicals
Milk powered, skimmed blotting grade	Carl Roth
Minimal essential medium (MEM)	Gibco
3-(*N*-morpholino)propanesulfonic acid, (MOPS) analytical grade	SERVA
Nickel-nitrilotriacetic acid agarose beads (Ni-NTA)	Qiagen
Paraformaldehyde (PFA) reinst	Carl Roth
Ponceau S	SERVA
Potassium acetate puriss.	Honeywell
Potassium chloride	AppliChem
Potassium dihydrogen phosphate	ACROS ORGANICS
Prestained protein ladder	Thermo Fisher Scientific
Roti-Lumin® plus	Carl Roth
Rubidium chloride ≥99% p.a.	Carl Roth
Sodium azide	PanReac AppliChem
Sodium chloride ≥99.5%	Carl Roth
Sodium dihydrogen phosphate xH_2O p.a.	Merck
Sodium dodecyl sulphate (SDS) research grade	SERVA
Sodium hydroxide puriss. p.a.	Sigma-Aldrich
di-Sodium monohydrogen phosphate	Merck
N,*N*,*N*',*N*'-tetramethyl ethylenediamine (TEMED)	SERVA
N,*N*,*N*′,*N*′-tetramethylazodicarboxamide	Sigma-Aldrich
Tris Base (tris(hydroxymethyl)aminomethane)	Fisher BioReagents

Triton™ X-100, (t-Octylphenoxypolyethoxyethanol), for analysis	Merck
Tween® 20 (Polysorbate 20), for molecular biology	PanReac AppliChem
Urea	SERVA

2.1.5. Selection marker with organism, application concentration and supplier

Ampicilin	*E. coli*	100 µg/mL	PanReac AppliChem
Blasticidine	*L. tarentolae*	10 µg/mL*	InvivoGen
Hygromycin	*L. tarentolae*	100 µg/mL	Carl Roth
Puromycin	*L. tarentolae*	20 µg/mL*	InvivoGen

* twice this concentration was used in BHI agar.

2.1.6. Enzymes with supplier

Expand™ High Fidelity (HiFi) polymerase	Roche
Phusion® polymerase	New England Biolabs
Restriction enzymes (*Nde*I and *Bam*HI)	New England Biolabs
T4 DNA ligase	New England Biolabs
Taq polymerase	New England Biolabs

2.1.7. Kits

DNeasy® Blood & Tissue Kit	Qiagen
Plasmid Midi Kit	Qiagen
Plasmid Mini Kit	Qiagen
Wizard® SV Gel and PCR Clean-Up System	Promega

2.1.8. Software and online resources with source and version

Biorender	https://biorender.com/	
BLAST®[90]	https://blast.ncbi.nlm.nih.gov/Blast.cgi	
Cas-OFFinder[91]	http://www.rgenome.net/cas-offinder/	
Chromas	http://technelysium.com.au/wp/chromas/	Version 2.6.6
Corel Draw 2021	Corel Corporation*	23.1.0.389

DeepL	https://www.deepl.com/en/translator	(used for single word spell check or vocabulary)
ImageJ[92]	https://imagej.nih.gov/ij/	v1.53k
LeishGEdit[77]	http://www.leishgedit.net/	updated on 5th August 2020
molbiotools	https://molbiotools.com/	
Microsoft Office	Microsoft*	version 2202
Perseus[93]	https://maxquant.net/perseus/	Perseus 1.6.15.0
TMHMM[94]	https://services.healthtech.dtu.dk/service.php?TMHMM-2.0	
TriTrypDB[95]	https://tritrypdb.org/tritrypdb/app	Release 56, 15 Feb 2022 and Release 57, 21 Apr 2022
TrypTag[96]	http://tryptag.org/?pageType=landing	
VEuPathDB[95]	https://veupathdb.org/veupathdb/app	Release 56, 15 Feb 2022 and Release 57, 21 Apr 2022
VisionCapt	Vilber	for Fusion Solo 3
ZEN 2 core	Zeiss	v2.4.lnk
Zotero	https://www.zotero.org/	Zotero 5.0.96.3

* license via TU Kaiserslautern

2.1.9. Antibodies

Table 2: List of antibodies used in the present work.

	sales name	host	source	order number	dilution
anti-HA antibody	HA-Tag (C29F4) Rabbit mAb	rabbit	Cell Signaling Technology	#3724	1:1000
anti-His antibody	6x-His Tag Monoclonal Antibody (HIS.H8)	mouse	Thermo Fisher Scientific	MA121315	1:1000
anti-Erv antibody		rabbit	Eckers et al.[37]		1:500
anti-mouse antibody	Goat Anti-Mouse IgG (H + L)-HRP Conjugate 1	goat	Bio-Rad	1706516	1:5000
anti-rabbit antibody	Goat Anti-Rabbit IgG (H + L)-HRP Conjugate 1	goat	Bio-Rad	1706515	1:5000

All antibodies are diluted in milk in tris(hydroxymethyl)aminomethane buffered saline (TBS).

Milk: 5% (w/v) milk powder in TBS

TBS buffer: 10 mM Tris, 0.9% (w/v) NaCl, pH = 7.6

2.1.10. Bacterial strain with genotype and source

XL-1 Blue

recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F´ proAB lacIqZΔM15 Tn10 (Tetr)]

From Qiagen

2.1.11. *Leishmania tarentolae* strains with source

wild-type strain

Parrot TarII/UC

From André Schneider, University of Bern Switzerland

parental strain

derived from wild type, expressing pTB007 plasmid

From Gino Turra[5], Deponte research group, TU Kaiserslautern

transgenic strains

derived from parental strain, introduction of cassettes for chromosomal tagging N- or C-terminal, mutant gene or knock-out

From this work

2.1.12. Plasmids with selection marker and reference

pTB007	AmpR, Hygro	Beneke, Madden et al.[77]
pTPuro_v1	AmpR, PuroR	Beneke, Madden et al.[77]
PTBlast_v1	AmpR, BlastR	Beneke, Madden et al.[77]
pPLOTv1 puro-mCherry-puro	AmpR, PuroR	Beneke, Madden et al.[77]
pPLOTv1 blast-mNeonGreen-blast	AmpR, BlastR	Beneke, Madden et al.[77]
pPLOT-His_8-Ervrecodon-His_8-Puro	AmpR, PuroR	Turra[5]
pPLOT-His_8-Ervrecodon-His_8-Blast	AmpR, BlastR	Turra[5]
pPLOT-HA-mic20recodon-HA-Puro	AmpR, PuroR	this work
pPLOT-HA-mic20recodon-HA-Blast	AmpR, BlastR	this work

2.1.13. Primers

All primers were ordered from metabion international AG, Planegg/Steinkirchen Germany.

Table 3: List of primers used in the present work. The numbers in the first column are used for identification in the present work. A lab code was assigned for frequently used primers. It is indicated on the lid of the tube to enable fast identification. The summary gives information about the target gene and the direction of the primer.

number	lab code	summary	sequence
1	LS001	p4/Plot/Chis8subs2/Fw	GGCTTTTTTGCTGGTCAGGAGGTGGAGTACCACCACCACCACCACCACCACCACtaaccaccaccaccactgag
2	LS002	p5/subs02KO/Rw	CTACCCTCCTACACATACACACACACTTATccaatttgagagacctgtgc
3	LS003	p/subs2/5'UTR/Fw	CACATTTACTGCCTCGTTTATTAG
4	LS004	p/subs2/3'UTR/Rw	AAAACAGGCTGGGGACAGGTG
5	LS005	P4/Plot/Chis8subs3/Fw	CCGGACGGATATGAGAAGGAGAAGGGGGCCCACCACCACCACCACCACCACCACCACtaaccaccaccaccactgag
6	LS006	p5/subs03KO/Rw	CCCTCCTCTGACCGCCTCTCGTGCTCAGTAccaatttgagagacctgtgc
7	LS007	p/subs3/5'UTR/Fw	CTCACCGAGATCGAAGGTGAC
8	LS008	p/subs3/3'UTR/Rw	AGCTGTACCTGCATGTACGTG
9	LS009	P4/Plot/Chis8subs4/Fw	GAGGCCTTTGCCAACGTCATGAAGGCGCAGCACCACCACCACCACCACCACCACCACtaaccaccaccaccactgag
10	LS010	p5/subs04KO/Rw	CCCGGGTGAAGGTGAGTATGGGAAACACGAccaatttgagagacctgtgc
11	LS011	p/subs4/5'UTR/Fw	CGGAGGGCCCGAATCGGC
12	LS012	p/subs4/3'UTR/Rw	GTCATCGCACACAAGGGAGTG
13	LS013	P4/Plot/Chis8subs1/Fw	CAGAGAGAGCACCCGATGCAGCCTCCACAGCACCACCACCACCACCACCACCACtaaccaccaccaccactgag
14	LS014	p5/subs01KO/Rw	CACACAGAGTCCTATGTGCGAGGGCATCCCccaatttgagagacctgtgc
15	LS015	p/subs1/5'UTR/Fw	CGCTTCCCCCTCGTGCTTC
16	LS016	p/subs1/3'UTR/Rw	CTTCCGCTGTATCATCAAAACG
17	LS017	P4/Plot/Chis8LtTIM1/Fw	TGGATGAAAGACGCGGCAGCAGGTAACATGCACCACCACCACCACCACCACCACCACtaaccaccaccaccactgag
18	LS018	p5/LtTIM1KO/Rw	GAAACACGTGTGCGTGTGCCTTTGAGTGGGccaatttgagagacctgtgc
19	LS019	p/LtTIM1/5'UTR/Fw	CAGGTCTGTCGGCTCCAGTC
20	LS020	p/LtTIM1/3'UTR/Rw	CACCCGTCGGTAGCAAGATG
21	LS021	p/ subs02sgRNA3'/01	gaaattaatacgactcactataggTTCCTTGACGGTAGGGTTCAgttttagagctagaaatagc
22	LS022	p/ LtTIM1sgRNA3'/01	gaaattaatacgactcactataggTCAGGTTCCATCTCCTCTGTgttttagagctagaaatagc
23	LS023	p/ subs03sgRNA3'/01	gaaattaatacgactcactataggGGAGGGGAAGGGGAGACGCGgttttagagctagaaatagc
24	LS024	p/ subs04sgRNA3'/01	gaaattaatacgactcactataggCGAGAGGACGCGGGGCGGGGgttttagagctagaaatagc

number	lab code	summary	sequence
25	LS025	p/ subs01sgRNA3'/01	gaaattaatacgactcactataggGCAGGTATGGAGGTGAAAGAgttttagagctagaaatagc
26	LS026	p1/subs02KO/Fw	CAACACGTAGTGCACATATTCCCCCTCCCAgtataatgcagacctgctgc
27	LS027	p1/subs03KO/Fw	ATGCAGATCGCTTCTAGTAGGTAACGAAACgtataatgcagacctgctgc
28	LS028	p1/subs04KO/Fw	CTTGCTCTTCACACACACACACAGAGACCAgtataatgcagacctgctgc
29	LS029	p1/subs01KO/Fw	AGTGTGGCACCTCGCTATTTACTCGCTCCTgtataatgcagacctgctgc
30	LS030	p1/sLtTIM1KO/Fw	CAGGCACTGCGGCTTGCAAGCACATATCCAgtataatgcagacctgctgc
31	LS031	p/ subs02sgRNA5'/01	gaaattaatacgactcactataggGTGTATGAAATGAGCTGCGAgttttagagctagaaatagc
32	LS032	p/ subs03sgRNA5'/01	gaaattaatacgactcactataggTGACTCCCCTCAAGCACGCCgttttagagctagaaatagc
33	LS033	p/ subs04sgRNA5'/01	gaaattaatacgactcactataggACAGCTCTACGGCTGAAGAGgttttagagctagaaatagc
34	LS034	p/ subs01sgRNA5'/01	gaaattaatacgactcactataggCGGAGGAGGTGGAGTTAGGAgttttagagctagaaatagc
35	LS035	p/ LtTIM1sgRNA5'/01	gaaattaatacgactcactataggTTGCTATAGGTGCAAGATTGgttttagagctagaaatagc
36	LS036	P2/PLOT/NHis8sTim1/Rv	GAGGCTCGGATTGGGCTGCACCTGCTGGTGGTGGTGGTGGTGGTGGTGGTGcatgcttgacaagtggaaga
37	LS037	P2/PLOT/NHis8Subs1/Rv	GGTACGCGGAGCTGCACGGGAGCGAGCGTGGTGGTGGTGGTGGTGGTGGTGcatgcttgacaagtggaaga
38	LS038	P2/PLOT/NHis8Subs2/Rv	CATGAAGTTAGGGGATGTTGGCTTGTGGTGGTGGTGGTGGTGGTGGTGcatgcttgacaagtggaaga
39	LS039	P2/PLOT/NHis8Subs3/Rv	TTGCGTTACCCTTCCTACATCTATGTGGTGGTGGTGGTGGTGGTGGTGcatgcttgacaagtggaaga
40	LS040	P2/PLOT/NHis8Subs4/Rv	CGACGACGTACTTGTCTTGGGAGCCTGGTGGTGGTGGTGGTGGTGGTGcatgcttgacaagtggaaga
41	LS041	p/seqKOBlast/Fw	CGGATGAAAACGAGAGAGAGAC
42	LS042	p/seqKOPuro/Fw	cgtctatacgtgagtacgttcg
43	LS045	pshort/subs1/5'UTR/Fw	CGCTTCCCCCTCGTGC
44	GT132	G00	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC
45	LS058	p1/mic20KO/Fw	GTTTGCGCTGAACACGGTTATGTAGAACCCgtataatgcagacctgctgc
46	LS060	p/mic20/sgRNA3'	gaaattaatacgactcactataggATGCCCATCGCTGCACGCTCgttttagagctagaaatagc
47	LS061	p5/mic20KO/Rw	GAGAAAAACGAGATGCAAAAAAACGGCCCCccaatttgagagacctgtgc
48		p/mic20/sg5'	gaaattaatacgactcactataggTCTTGCTTTCGTGCGTCTGTgttttagagctagaaatagc
49		UF2 UTR Fw:	CATATTGTATCCGCCTCAGAGGCTG
50		UF2 UTR Rv	CAGAGACACCTCTCGAAGCC
51		UF3 UTR Fw	CAGGAAAGTTGGTGAGGTGAG
52		UF3 UTR Rv	ACCAAGACGTCTTCCTTATGC
53		p1mic20Fw2	gtttgcgctgaacacggttatgtagaacccgtataatgcagacctgctgc
54		p5mic20Rv2	GAGAAAAACGAGATGCAAAAAAACGGCCCC ccaatttgagagacctgtgc

number	lab code	summary	sequence
55		p4mic20HisFw2	gtttgcgctgaacacggttatgtagaacccATGCACCACCACCACCACCACCACCA CTCGATGACACAGAGCATCGT
56		p2mic20HisRv2	GAGAAAAACGAGATGCAAAAAAACGGCCCCCTAGTGGTGGTGGTGG TGGTGGTGGTGAGAGGTGGATTTGGGCACGC
57		p4mic20Fw2	gtttgcgctgaacacggttatgtagaacccATGTCGATGACACAGAGCATCGT
58		p2mic20Rv2	GAGAAAAACGAGATGCAAAAAAACGGCCCCCTAAGAGGTGGATTTG GGCACGC
59		Mic205UTRFwok	CTCATTACGATCTACGGTTTCCTC
60		mic203UTRRvok	GCTGTGAAGAAAGAAGAAATAATACC
61	GT140	p/blasticidin/Rw	CCGTTGCTCTTTCAATGAGGGTG
62	GT160	p/puromycin/Fw	ATGACTGAATACAAGCCAACGG
63	GT161	p/blasticidin/Fw	ATGCCTTTGTCTCAAGAAGAATC
64	GT196	p/NTtagsSEQ/Fw	acgcgttcgagaccgacaagac
65		mic20/C82S/fw	ctggaagcacggcagcatcccgtgcctcac
66		mic20/C82S/rv	gtgaggcacgggatgctgccgtgcttccag
67		mic20/C85S/fw	cacggctgcatcccgagcctcaccttcgccg
68		mic20/C85S/rv	cggcgaaggtgaggctcgggatgcagccgtg
69		mic20/C82+85S/fw	cacggcagcatcccgagcctcaccttcgccg
70		mic20/C82+85S/rv	cggcgaaggtgaggctcgggatgctgccgtg

2.2. Molecular biology

2.2.1. Chemically competent XL1-Blue cells

The preparation of chemically competent cells was performed on ice. XL-1 Blue cells with OD_{600} = 0.5 were resuspended in transformation buffer 1 (TFB1) and incubated for 15 min. After centrifugation (2600*g*, 15 min, 4°C), the pellet was resuspended in TFB2 and incubated for several minutes. The cells were frozen in liquid nitrogen and stored at -80 °C.

TFB1: 30 mM potassium acetate, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 100 mM RbCl, 15% (v/v) glycerol, pH = 5.8 at room temperature

TFB2: 10 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl, 15% (v/v) glycerol, pH = 6.5 at room temperature.

Chemically competent XL1-Blue cells were prepared in-house for all users in the Deponte research group.

2.2.2. Transformation of *E. coli*

Chemically competent *Escherichia coli* (*E. coli)* cells were prepared in the Deponte research group. The cells were stored at -80 °C and thawed on ice. 0.5 – 10 µL of DNA solution were added according to the DNA content. The mixture was incubated on ice for 30 min. Subsequently, a heat shock was performed for 90 s at 42 °C, followed by incubation on ice for 5 min. 10 volumes of LB medium (RT) were added. The cells were incubated for up to 1 h at 37 °C. 20 - 200 µL of the cell suspension were spread on LB-agar plates, containing the appropriate antibiotic for selection. Plates were incubated overnight at 37 °C upside down, sealed with parafilm. If colonies were not pecked immediately, the plates were stored at -4 °C for up to 4 weeks.

2.2.3. *E. coli* cell culture

2 - 10 mL of LB medium were completed with ampicillin to a final concentration of 100 µg/mL. The medium was inoculated with *E. coli* pecked from a transfection plate. Incubation was performed at 37 °C, shaking at 160 rpm overnight.

2.2.4. Plasmid preparation

Plasmid preparation from *E. coli* cultures was performed with QIAprep Miniprep Kit or QIAprep Midiprep Kit to yield high purity plasmids.

Alternatively, the Deponte research group used the following protocol for plasmid preparation: 1 – 2 mL *E. coli* culture were harvested at 13000*g* for 30 s at 4 °C. The pellet was resuspended in 100 µL buffer P1. 200 µL of buffer P2 were added and the tube was inverted several times, followed by incubation for 5 min at room temperature. 150 µL of ice-cold buffer P3 were added, followed by inverting the tube. Centrifugation was performed at 13000*g* for 10 min at 4 °C. The supernatant was removed and mixed with 600 µL isopropanol by inverting the tube. Subsequently, centrifugation was performed at 13000*g* for 5 min at 4 °C. The supernatant was discarded. The pellet was washed with 600 µL 70% (v/v) ethanol (precooled to -20 °C), then dried under red light and resuspended in 30 µL water.

Buffer P1: 50 mM Tris, 10 mM EDTA, pH = 8 at room temperature, 0.1 mg/mL ribonuclease A, stored at 4 °C.

Buffer P2: 0.2 M NaOH, 1% (w/v) SDS, stored at room temperature.

Buffer P3: 1.8 M KOAc/HOAc, pH = 5.2, stored at 4 °C.

2.2.5. Sequencing

Sequencing of DNA was commercially done by Seq It or Eurofins Genomics.

2.2.6. Restriction digest

The conditions for a restriction digest were always adapted for the enzymes used. Buffer and time of digest were chosen according to the enzyme supplier's recommendations. A common mixture for restriction digest contained 1 µg DNA and 1 µL (20 units) enzyme for 50 µL reaction volume and was incubated at 37 °C. Heat inactivation was performed according to the properties of the enzyme.

2.2.7. Agarose gel electrophoresis

0.7 – 2% (w/v) agarose were dissolved in Tris-acetate-EDTA buffer (TAE), boiling in a microwave oven. 0.01% (v/v) GelRed were added, the mixture was cooled until the flask could comfortably held in hand, poured in a gel tray equipped with a comb, and let solidify at room temperature. The agarose gel was transferred to a gel chamber and covered with TAE. 5 µL of 1 kb or 100 bp DNA ladder was loaded according to the expected fragment sizes. DNA samples were supplemented with loading dye and loaded on the gel. 70 – 100 V, depending on the gels size were applied until the desired resolution of the DNA fragments was reached. The results were documented by UV transilluminator and a camera, or a documentation imaging system.

TAE: 40 mM Tris/HOAc, 1 mM EDTA, pH = 7.6.

2.2.8. Purification of DNA from agarose gel or from PCR products

DNA from agarose gels or PCR products were purified using the Promega Wizard SV Gel and PCR Clean-Up Kit.

2.2.9. Ligation

5 µL of insert solution, 5 µL of vector solution, 2 µL of T4 ligase buffer and 7 µL water and 1 µL of T4 ligase were mixed and incubated at room temperature for 1 h or overnight at 16 °C. Heat inactivation was done for 20 min at 65 °C.

2.2.10. Construction of pPLOT with recodonised gene for Mic20

A recodonised DNA sequence encoding for the mitochondrial contact site and cristae organizing system protein 20 (Mic20) was ordered commercially in a pUC57 vector from GenScript. Mutations that should lead to the production of serines instead of certain cysteines were introduced. The plasmids pPLOT-His_8-ERVrecodon-His_8-PURO and pPLOT-His_8-ERVrecodon-His_8-BLAST were available from the work of Turra.[5] *Nde*I and *Bam*HI were used for subcloning the recodonised sequence to these pPLOT plasmids.

2.2.11. Mutagenesis

30 – 60 ng DNA template, 200 µM dNTPs, 0.5 µM forward primer, 0.5 µM reverse primer and 1 unit phusion polymerase were mixed in 1x phusion HF buffer complemented to 50 µL. The conditions for the PCR were: 30 s 98 °C; 30x: 10 s 98 °C, 1 min 72 °C; 10 min 72 °C. The PCR product was digested with 40 units *Dpn*I at 37 °C for 1 h, followed by heat inactivation at 80 °C, for 20 min. Transformation to competend XL1-Blue was subsequently performed.

The performance of mutagenesis was assisted by Tarik Begic as part of an internship.

2.3. Protein biochemistry

2.3.1. SDS-PAGE

Separation of proteins by size was performed via SDS-PAGE. The Mini-PROTEAN system was used. 15% resolving gels contained: 15% (w/v) acrylamide/bis solution, 375 mM Tris (pH = 8.8), 1% (w/v) SDS, 0.01% (w/v) ammonium persulfate and 0.04% (v/v) *N*,*N*,*N*',*N*'-tetramethyl ethylenediamine (TEMED).

5% stacking gels contained: 5% (w/v) acrylamide/bis solution, 125 mM Tris (pH = 6.8), 1% (w/v) SDS, 0.01% (w/v) ammonium persulfate and 0.1% (v/v) TEMED.

For separation, an initial current of 40 mA was chosen. Running time 45 min – 60 min.

SDS running buffer: 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, pH = 8.3

Protein samples (cell pellets or eluates from pull-down experiments) were heated to 95 °C for 5 min in Lämmli-buffer.

Lämmli-buffer: 50 mM Tris/HCl pH = 6.8, 5% (w/v) glycerol, 2% (w/v) SDS, 0.02% (w/v) bromophenol blue

2.3.2. Western blotting

After separation via SDS-PAGE, proteins were transferred to polyvinylidene difluoride (PVDF) membrane via western blot. The Mini Trans-Blot cell was used for blotting. PVDF membranes were activated in methanol and equilibrated in blotting buffer at 4 °C. Transfer was performed at 100 V for 1 h 30 min starting at 4 °C. Ponceau staining of the membrane was performed to monitor the transfer. Membranes were incubated in Ponceau S solution for 30 s and washed in water. After documentation, destaining of the membranes was performed in TBS. The membranes were blocked in milk in TBS for 1 h at room temperature. Incubation in primary antibody (diluted in milk in TBS) was performed with agitation at room temperature for 1 h or at 4 °C overnight. The membranes were washed four times in tris(hydroxymethyl)aminomethane buffered saline 0.05% (m/v) Tween 20 (TBS-T) for 10 min and once in TBS for 10 min. Incubation in a secondary antibody (horseradish peroxidase (HRP)-conjugate, diluted in milk in TBS) was performed for 1 h at room temperature, followed by washing in TBS. For detection, the decorated membranes were incubated in Roti-Lumin plus and signals were detected with a documentation imaging system.

Blotting buffer: 20 mM Tris, 150 mM glycine

Ponceau S solution: 0.1% (w/v) Ponceau S, 1% (v/v) acetic acid

Milk: 5% (w/v) milk powder in TBS

TBS buffer: 10 mM Tris, 0.9% (w/v) NaCl, pH = 7.6

TBS-T buffer: 10 mM Tris, 0.9% (w/v) NaCl, 0.05% (m/v) Tween 20, pH = 7.6

2.3.3. Pull-downs

2 x 10^9 cells were harvested (ca. 50 mL cell culture) by centrifugation at 1000*g*, 4 °C, 30 min. The cell pellets were washed with 3 mL phosphate buffered saline with *N*-ethylmaleimide

(PBS/NEM) (incubation 10 min), followed by centrifugation (100*g*, 4 °C, 5 min). If necessary, pellets were stored at -80 °C. The pellets were resuspended in 1 mL denaturing buffer with 50 mM *N*-ethylmaleimide (NEM), the SDS-stock solution was added to 2% (w/v) final concentration and the mixture was heated to 95 °C for 20 min, then kept on ice. (Precipitation of SDS on ice for 2 h, followed by centrifugation at 30000*g*, 4 °C, 30 min was performed at the pull-downs for the first mass spectrometry, the results of which are described in Chapter 3.3.8.) 100 µL of nickel-nitrilotriacetic acid agarose beads (Ni-NTA) slush were washed twice in 500 µL denaturing buffer, resuspended in 200 µL denaturing buffer and added to the sample mixture. Incubation was performed tumbling for at least 2 h up to overnight. The mixture was loaded on Micro Bio-Spin Columns and washed 8 times (pull-down for first mass spectrometry) or 3 times (pull-downs from redox treated cells) with 500 µL washing buffer (centrifugation 800*g*, 1 min, 4 °C). Elution was performed with 200 µL elution buffer. Samples were stored at -80 °C until used for SDS-PAGE and western blot or mass spectrometry.

PBS: 1.84 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH = 7.4

Denaturing buffer: 30 mM Tris, 150 mM NaCl, 0.01 mM EDTA, pH = 8

PBS/NEM: 50 mM *N*-ethylmaleimide (NEM) in PBS

Denaturing buffer/NEM: 50 mM NEM in denaturing buffer

SDS-stock: 8% (w/v) SDS in denaturing buffer

Washing solution: 10 mM imidazole in denaturing buffer.

Elution solution: 200 mM imidazole in PBS

2.3.4. Acetone precipitation of proteines

Protein samples were precipitated as required. Acetone was added at fourfold sample volume and samples were precipitated at -20 °C overnight, followed by centrifugation at 30000*g* at 4 °C for 30 min. The pellets were air dried for 15 min.

2.4. *Leishmania tarentolae*

2.4.1. Standard culture

Leishmania tarentolae was cultured in brain heart infusion (BHI) medium with 10 µg/mL hemin at 27 °C. 10 mL cultures were grown in tissue culture (TC) flasks with continuous shaking at 20 rpm. Cultures were split 2 – 3 times a week, diluting 1:10 – 1:20, to keep the cells growing in mid-log phase (5 x 10^7 cells/mL).

For determination of cell density cells were counted in a Neubauer chamber. To this purpose, a sample of the cell culture was diluted with twice its volume of fixation solution.

Fixation solution: 150 mM NaCl, 15 mM sodium citrate, 10% (w/v) paraformaldehyde, pH = 7.4

2.4.2. Freeze-down

Cultures in mid-log phase (5 x $10^{\circ 7}$ cells/mL) were mixed with the same volume of freezing solution in cryo tubes. After incubation at -80 °C overnight, the cells were stored in liquid nitrogen.

After thawing a tube at 27 °C, the mixture was washed in 10 mL BHI medium and resuspended in 5 mL BHI medium. When the mid-log phase was reached, the standard culture procedure continued.

Freezing solution: 30% (v/v) glycerol, 10 µg/mL hemin in BHI medium

2.4.3. Giemsa staining

For visualisation of cells, a cell culture in mid-log phase was concentrated 10 times and smeared on glass slides. Samples were fixed for 10 s in methanol, dried and then stained for 30 min in 10% (v/v) Giemsa solution. The slides were washed with water and dried. Using a light microscope with 100-fold magnification (oil immersion), pictures were taken with a Zeiss Axiocam.

2.4.4. Gene editing

For gene editing in *Leishmania tarentolae*, a modular system published by Beneke, Madden et al.[77] and adapted by Turra[5] was used. The system was based on the technology of clustered regularly interspaced short palindromic repeats / clustered regularly interspaced short

palindromic repeats associated protein 9 (CRISPR/Cas9). Starting point was a cell line with pTB007 plasmid, available from the work of Turra. All original plasmids (pT, pPLOT and pTB007) were a gift from Eva Gluenz (University of Oxford, United Kingdom). The primer design followed the requirements defined by Beneke, Madden et al.[77] For automated primer design, the online resource LeishGEdit[82] was available.

Preparation of cassettes for gene editing

1.875 mM $MgCl_2$, 3% (v/v) DMSO, 30 ng plasmid DNA, 0.2 mM dNTPs, 2 µM forward primer, 2 µM reverse primer and 1 unit HiFi Polymerase were mixed in 1x HiFi reaction buffer with $MgCl_2$ to a volume of 40 µL. The conditions for the PCR were: 5 min 94 °C; 40x: 30 s 94 °C, 30 s 65 °C, 2 min 15 s 72 °C; 7 min 72 °C.

Preparation of sgRNA templates for gene editing

0.2 mM dNTPs, 2 µM primer G00 (primer 44, Table 3), 2 µL forward primer and 1 unit HiFi Polymerase were mixed in 1x HiFi reaction buffer with $MgCl_2$ to a volume of 20 µL. The conditions for the PCR were: 30 s 98 °C; 35x 10 s 98 °C, 30 s 60 °C, 15 s 72 °C.

Sterilisation of DNA for gene editing

PCR products were heated to 94 °C for 5 min.

Transfection for chromosomal gene editing

1 x 10^7 cells for a knock-out experiment or 0.5 x 10^7 cells for a tagging experiment (parental cells harboring pTB007 plasmid) were washed in 1 mL transfection buffer and resuspended in 150 µL transfection buffer. 80 µL PCR product including cassettes (for example 40 µL from amplification of cassette with puromycin resistance and 40 µL from amplification of cassette with blasticidine resistance) and 40 µL PCR product including sgRNA template were mixed in an electroporation cuvette. The cell suspension was added and briefly mixed. Electroporation was performed in Lonza Nucleofector 2b with 1 pulse in program X-001. The mixture was resuspended in 1 mL BHI medium and incubated at 27 °C overnight, followed by selection.

Transfection buffer: 111 mM Na_2HPO4, 38.9 mM NaH_2PO4, 8.33 mM KCl, 83.3 mM HEPES, 0.25 mM $CaCl_2$, pH = 7.3

Selection of *Leishmania tarentolae*

BHI agar was poured into petri dishes and solidified with open lid for 10 min. Cells from one transfection were resuspended in 200 µL BHI medium and spread on a prepared BHI agar plate. After 1 - 3 weeks first colonies were expected to appear. Colonies were pecked and resuspended in 5 mL BHI medium with antibiotics. When the mid-log phase was reached, the standard culture procedure continued.

BHI agar: 3.7% (w/v) BHI, 0.8% (w/v) agar, 0.08% (w/v) folic acid, 10% (v/v) heat-inactivated FBS, 20 µg/mL hemin.

In BHI agar: 40 µg/mL puromycin, 20 µg/mL blasticidine.

In BHI medium: 20 µg/mL puromycin, 10 µg/mL blasticidine.

Genotyping of clones

Extraction of DNA from *Leishmania tarentolae* was performed with the DNeasy Blood and Tissue kit from Qiagen.

100 ng DNA from extraction, 0.2 mM dNTPs, 2 µM forward primer, 2 µM reverse primer and 1 unit Roche HiFi polymerase were mixed in 1x Roche HiFi reaction buffer with $MgCl_2$ to a volume of 20 µL. The conditions for the PCR were: 5 min 94 °C; 35x: 30 s 94 °C, 5 s 60 °C, 50 s 72 °C; 7 min 72 °C. The amplicons were separated and visualised on agarose gels.

Also with *Taq* polymerase, genotyping PCRs were performed: 100 ng DNA from extraction, 0.2 mM dNTPs, 1 µM forward primer, 1 µM reverse primer and 0.5 units *Taq* polymerase were mixed in 1x Thermopol buffer to a volume of 20 µL. The conditions for the PCR were: 5 min 95 °C; 30x: 30 s 95 °C, 30 s 45 - 68 °C, 3 min 68 °C; 7 min 68 °C. The annealing temperature was always set according to the primers used.

2.4.5. Fractionation of cells

2 x 10^8 cells per sample were harvested and washed with PBS. The pellets were resuspended in 200 µL of digitonin solution in PBS (0, 0.01, 0.1, 1 or 5 mg/mL digitonin in PBS, control: 2% (v/v) Triton X-100). The mixture was incubated for 20 min, followed by centrifugation at 20000*g*, 4 °C for 20 min. The supernatant was complemented 4:1 with 5x Lämmli-buffer and analysed via SDS-PAGE and western blotting.

PBS: 1.84 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH = 7.4

2.4.6. Treatment of cells with azide

50 mL cell culture in minimal essential medium (MEM), with 5% (v/v) FBS, 10 µg/mL hemin, with or without 5 mM NaN_3 were kept in the fume hood overnight with continuous shaking in the dark. The cells were harvested, washed with PBS/NEM and stored at -80 °C until pull-downs were performed.

PBS: 1.84 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH = 7.4

PBS/NEM: 50 mM NEM in PBS

2.4.7. Treatment of cells with DTT or TMAD

For cell treatment, 10x stock solutions of additives in water were regularly added to the cell cultures six times within 30 minutes to give a final concentration of 1 mM of dithiotreitol (DTT) or *N*,*N*,*N*′,*N*′-tetramethylazodicarboxamide (TMAD) after each addition. As a control, the same volume of water was added to cultures of the same cell density and volume. Immediately after the last addition of additive, NEM was added to 50 mM. The cells were harvested and washed in PBS with 50 mM NEM. Pellets of 10^7 cells were stored at -80 °C until pull-downs were performed.

PBS: 1.84 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, 2.7 mM KCl, pH = 7.4

2.4.8. Sample preparation and mass spectrometry

All steps to prepare the eluates of pull-down experiments (precipitated as indicated) for the mass spectrometry experiments were carried out in the Center for MS Analytics, Division of Molecular Genetics, TU Kaiserslautern under the guidance of Dr. Markus Räschle.

For sample preparation for mass spectrometry ms grade components were used.

Reduction, alkylation and digest

Eluates from pull-down experiments were used. After addition of DTT to 10 mM, the mixture was incubated for 30 min at room temperature. Then, chloroacetamide solution was added to 55 mM and the mixture was incubated at room temperature in the dark. After precipitation with 4 volumes of acetone for 2 h at -20 °C, centrifugation at 1600*g* for 15 min at 4 °C was performed. The pellets were washed with 80% (v/v) acetone, resuspended in

urea buffer and sonicated for 15 min on ice. 1 µg endoproteinase Lys-C was added and incubated for 1 h at room temperature. 50 mM ammonium bicarbonate solution was used to dilute the samples to 2 M urea. 1.6 µg trypsin were added and incubated at 37 °C overnight. Trifluoroacetic acid was added to 1% (v/v). STAGE protocol followed afterwards.

DTT stock: 500 mM

Chloroacetamide stock: 550 mM

Urea buffer: 100 µL 8 M urea in 10 mM HEPES, pH = 8.0.

STAGE

For stop and go extraction (STAGE), a protocol adapted from Rappsilber et al. was used.[97] As a matrix three layers of C18 resin were used, which were activated with 100 µL methanol. For equilibration of the column, first 100 µL buffer B, then 100 µL buffer A were used. After the sample load, one washing step was performed with 100 µL buffer A, elution with 60 µL buffer B. The sample was dried *in vacuo* (30 mbar, 40 °C for 1 h.).

Analysis in mass spectrometer was performed by Dr. Markus Räschle.

Buffer A: 0.1% (v/v) formic acid.

Buffer B: 0.1% (v/v) formic acid in 80% (v/v) acetonitrile.

Mass spectrometry analysis

Analysis in the mass spectrometer was performed by Dr. Markus Räschle in the Center for MS Analytics, Division of Molecular Genetics, TU Kaiserslautern. HPLC was performed on reverse phase columns with 75 µm inner diameter, packed in-house with ReproSil-Pur 120 C18-AQ. The loading was performed in buffer A, the elution in a gradient of buffer B (250 nL/min, 50 °C). The separated samples were directly injected to the mass spectrometer.

Buffer A: 0.1% (v/v) formic acid.

Buffer B: 0.1% (v/v) formic acid in 80% (v/v) acetonitrile.

3. Results

3.1. Candidate from the Literature: Mic20

While suitable candidates for an oxidoreductase were sought in *Leishmania*, a proposal was made for *Trypanosma brucei*.[1–3] Indications pointed at Mic20, a component of the mitochondrial contact site and cristae organizing system (MICOS). Therefore, the ortholog of Mic20 in *Leishmania tarentolae* was investigated.

For chromosomal manipulation of kinetoplastida, CRISPR/Cas9 genome editing was available, based on the work of Beneke, Madden et al.[77] This modular system was adapted for *Leishmania tarentolae* by Turra[5] and was chosen for gene editing in the present work. Starting point of this method was a strain of *Leishmania tarentolae*, expressing the plasmid pTB007, which enables the expression of components as Cas9 and T7 RNA polymerase. This strain was called parental strain. Since *Leishmania tarentolae* was assumed to be a predominantly diploid organism, the insertion of two DNA cassettes with distinguishable selection markers was chosen to ensure genetic manipulation of at least two copies of a gene.

3.1.1. Knock-out attempt of the gene encoding Mic20

In a first step, an attempt was made to knock-out (KO) the gene *LtaPh_3313851*, which encodes the Mic20 ortholog in *Leishmania tarentolae*. The insertion of two knock-out cassettes with different selection markers was performed simultaneously. As Figure 6 shows, in several attempts no living parasites could be selected after transfection.

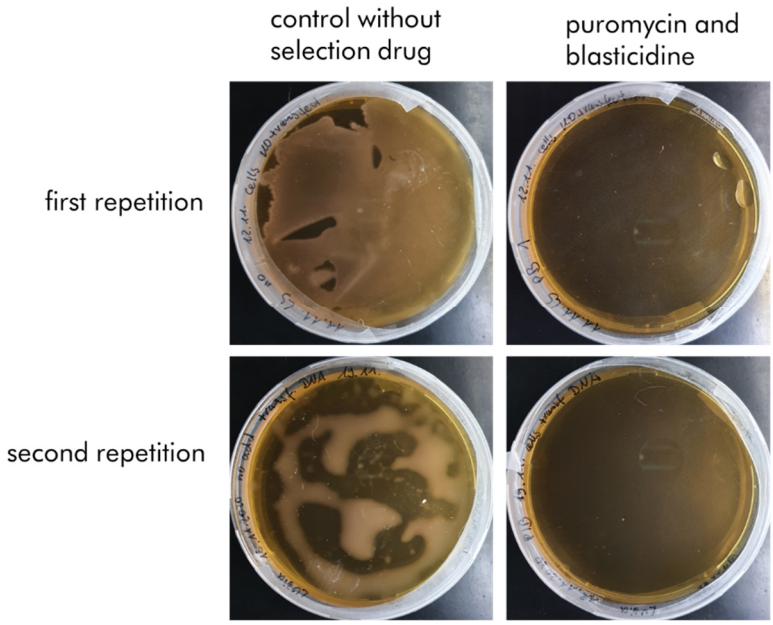


Figure 6: Images of selecting plates for knock-out attempts of the gene *LtaPh_3313851* (encoding Mic20). Two replicates are shown. The selection was ongoing for 26 days. Left panels: the control without selection drugs shows cell growth. Right panels: with selection drugs, no growth could be observed.

The left panels show a control for cell viability 26 days after transfection. The cells were transfected as for the knock-out, but the agar plates did not contain any drug for selection. Cells on these plates showed strong growth. The panels on the right show plates with selection drugs. No cell growth was visible here after 26 days. The fact that no living parasites were obtained could be a hint for a failed integration of the knock-out cassettes or for essentiality of the gene *LtaPh_3313851*, which encodes the Mic20 ortholog.

3.1.2. Constructs for mutations of the gene encoding Mic20

Since a knock-out of the gene encoding for the Mic20 ortholog was not possible, the function of the individual cysteine residues was investigated in the next step. The cysteine residues included in the CIPC motif (Figure 7) were mutated to serine residues to inhibit the formation of disulphide bonds. The structuring properties of both amino acids are comparable and little impact on other protein characteristics was expected. To circumvent repair mechanisms of point mutations, the parental gene sequence of *LtaPh_3313851* encoding the Mic20 ortholog was replaced by a recodonised sequence, containing the different mutations. The plasmids for this strategy corresponded to the editing strategy of Beneke and Madden et al.[77] and were modified by Turra[5] for insertion of recodonised genes equipped with 3x human influenza hemagglutinin (HA)-tag. The recodonised sequence

encoding the Mic20 ortholog was commercially synthesised. (For the recodonised sequence encoding the Mic20 ortholog with tag, linker and restriction sites, see the supplements, S2). Figure 7 shows the amino acid sequence of the Mic20 ortholog.

MSMTQSIVRRIFGDRKLPENLSNEEYDKYMQDNFPKWMKEFEDGGFLEKTKLPPIKSEEEFIT
KLQEHKDELMVIKYWKHGCIPCLTFAEMYKEAAERCCKEKKKIVWYSVDTKALNTRQLIDY
QLISGTPTIQTFTGQKQVGNEIRAVSTEELLSELDKRVPKSTS-

Figure 7: Amino acid sequence of Mic20 ortholog from *Leishmania tarentolae*.[60] Cysteine residues were highlighted in dark yellow for the CIPC motif, and in light yellow for all other cysteines.

For mutation the two cysteines in the CIPC motif were chosen. C82 was expected to be the essential cysteine, C85 the resolving cysteine. The recodonised sequence without mutation (abbreviated as WT) was used as control. Figure 8 summarises the constructs schematically.

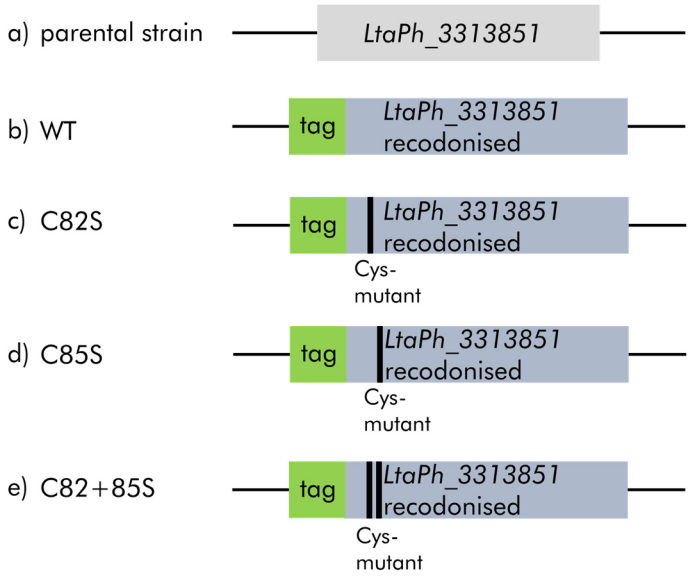


Figure 8: Schematic representation of constructs for chromosomal editing of *LtaPh_3313851* encoding the Mic20 ortholog. a) represents the parental gene, b) the recodonised sequence of the parental gene with the sequence for the N-terminal 3xHA-tag, c) the C82S mutant inserted to the recodonised sequence, d) the C85S mutant inserted to the recodonised sequence and e) the C82S and C85S mutant inserted to the recodonised sequence.

The parental strain (a) exhibited the unedited gene. In the WT mutant (b), the recodonised sequence was inserted, and the sequence encoding the N-terminal 3x HA-tag was also present in the cassette. The resulting protein had the same sequence as the parental strain, except for the tag. In mutant C82S (c) the codon for cysteine 82 was mutated to a codon

for serine additionally. In mutant C85S (d) the codon for the cysteine at position 85 was converted to a codon for serine and in C82+85S (e) both mutations were present. Figure 9, shows the mutagenesis primers that were used to introduce the mutations. Also, one sequencing result for each construct is shown. Puromycin and blasticidine cassettes were used for each. All constructs exhibited the expected sequence for cysteine to serine mutations. The corresponding codons were changed from tgc to agc. Since the constructs were correct, they could be used for amplification of cassettes followed by transfections to *Leishmania tarentolae*.

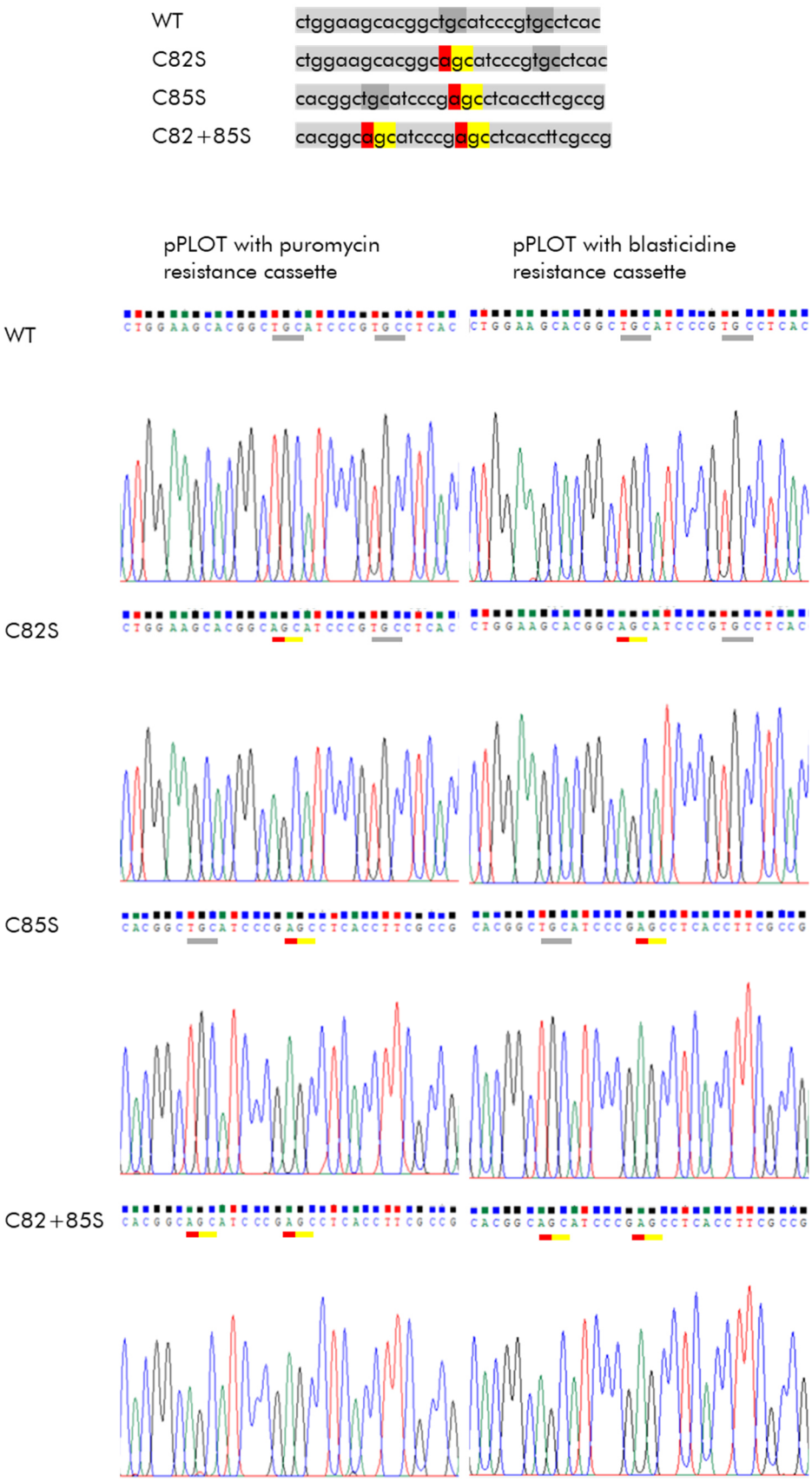


Figure 9: Chromatograms from the sequencing confirming the cysteine mutants in pPLOT plasmids. The top panel shows the sequences of the mutagenesis primers. By exchanging the codon tgc with agc, cysteine should be converted to serine (highlighted in yellow and red). The bottom panels show the chromatograms for the constructs, the mutants are indicated. The colours coding the nucleobases are: blue for cytosine, C; red for thymine, T; green for adenine, A; and black for guanine, G. For the sequencing, primer 64 was used, the numbers of the mutagenesis primers were 65, 66, 67, 68, 69 and 70 (Table 3).

3.1.3. Mutations of the gene encoding Mic20

Attempts to simultaneously introduce two different cassettes did not succeed. Therefore, a stepwise strategy was chosen. First, a cassette selected with blasticidine was introduced to produce the C82S, C85S and C82+85S mutants. Figure 10 shows the genotyping of the resulting strains.

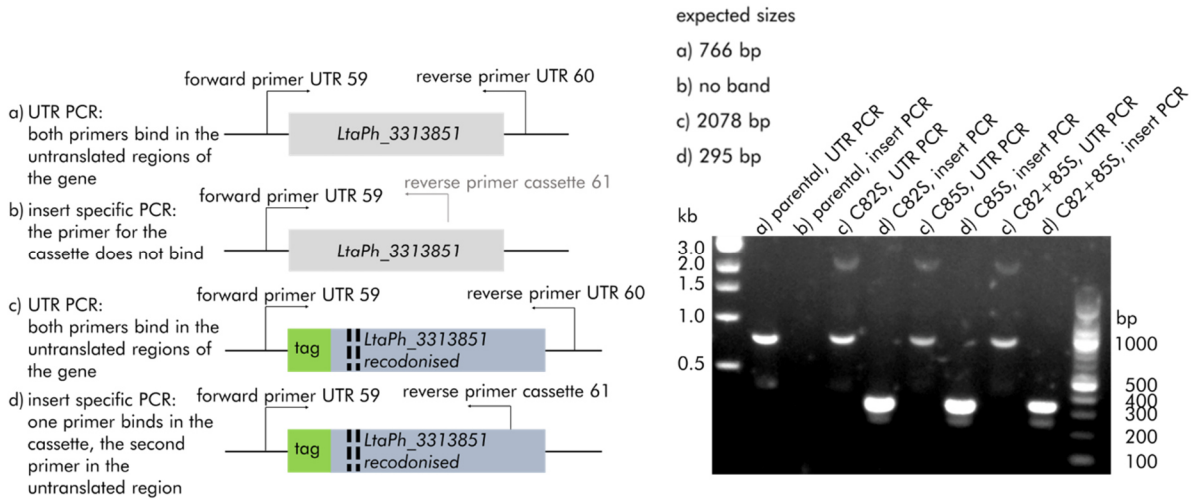


Figure 10: Genotyping mutants with one insert of a cassette for mutation of *LtaPh_3313851* enconding the Mic20 ortholog. Cysteine mutants are present as indicated. A N-terminal 3x HA-tag was encoded by the tagging cassette. Insertion of the knock-out cassettes should lead to blasticidine resistance. The left panel schematically shows the position of the primers. The right panel shows an image of the agarose gel separating the amplicons of the PCRs. Each PCR from the left panel was represented by one sample. The numbers of the primers used for the genotyping are: 59, 60, 61 (Table 3).

In general, correct gene editing was confirmed by genotyping PCRs. Primers for the genotyping could bind in the untranslated region (UTR) next to a gene, or in the inserted cassettes. Thus, different types of PCRs were possible. If one primer bound in the 5' UTR and one primer in the 3' UTR, the amplicon covered the whole gene of interest, with or without any manipulation by gene editing. If one primer was specific for the inserted cassette, and one for an UTR, an amplification product could only be obtained if the cassette was inserted correctly to the target gene. Schemes attached to the results for each modified gene explain the position of the primers and the expected sizes of the amplification products.

In the course of genotyping the mutants of *LtaPh_3313851* encoding the Mic20 ortholog, examination of the parental strain showed the expected band at 766 bp (a). As expected, the insert specific PCR b) did not result in an amplification product. The PCR with primers

in the UTRs (c) for a clone with C82S mutations showed the band specific for the parental gene at 766 bp and for insertion at 2078 bp. PCR d) confirmed the presence of the insert for C82S with a band at 295 bp. The same pattern for PCRs c) and d) was received for C85S and C82+85S mutants. This suggested the successful insertion of the cassette with simultaneous presence of the parental gene. The next step was the proof of the presence of the 3x HA-tag. For this purpose, cell lysates were analysed via SDS-PAGE and western blot, with the membrane decorated with anti-HA antibody (Figure 11).

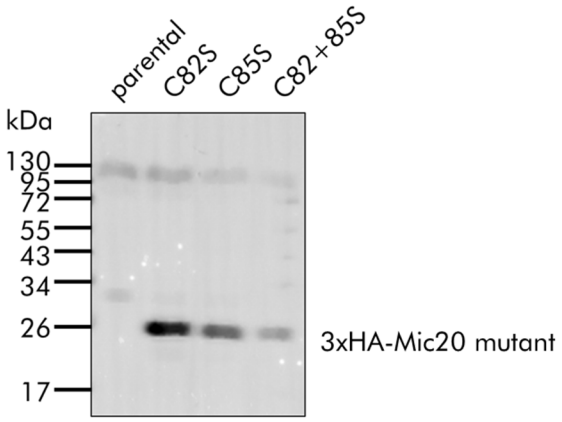


Figure 11: Image of western blot membrane, decorated with anti-HA antibody. Cell lysates from heterozygous clones with mutant Mic20 N-terminally 3x-HA tagged were separated by SDS-PAGE before western blotting. Parental cells did not show the specific band at 27 kDa, whereas the clones edited to produce C82S, C85S and C82+85S did.

A weak band showed a protein with a mass between 95 and 130 kDa in all samples, including the parental strain. Thus, it was considered to be unspecific. The presence of HA-tagged Mic20 ortholog could be shown in C82S, C85S and C82+85S manipulated strains by a band at 27 kDa. Since the presence of the tagged protein could be shown, the fitness of the parasites was monitored. Growth behaviour was comparable to parental cells for all strains. As an example one image of the strain with the C82S manipulation is shown in Figure 12.

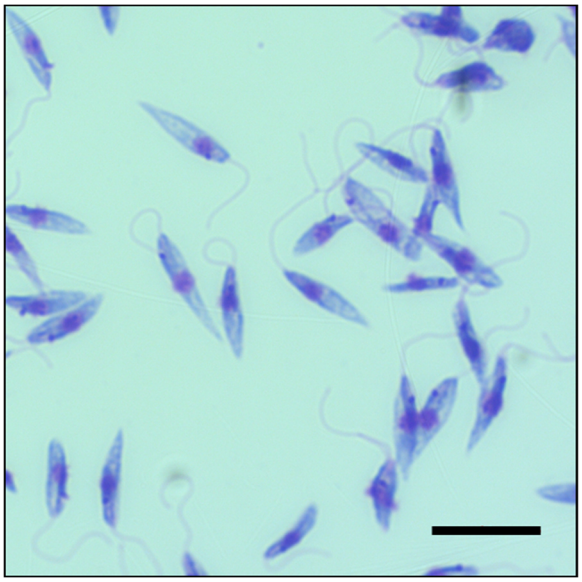


Figure 12: Image of *Leishmania tarentolae*. The cells were derived from a clone with an insert for C82S editing of Mic20. The cells were fixed with MeOH and stained with Giemsa. Scale bar: 5 µm. The cells showed the usual shape and size.

Compared with the parental cells, the mutated strain showed the same morphology. Thus, the second transfection was subsequently continued. For this transfection, the WT cassette was chosen as a control. For each of the strains with one mutation insert, two transfections were performed: one with a WT cassette, and one with the original mutation in the cassette. For C82S and C82+85S manipulations, colonies were obtained for both transfections each. No living cells were obtained for transfections based on C85S editing. This showed the sensitivity of the gene editing system. A transfection is a highly stressful procedure for the cells and low survival rates can occur. For future transfections higher cell numbers and high repetition rates are proposed. For the living clones, Figure 13 shows the genotyping.

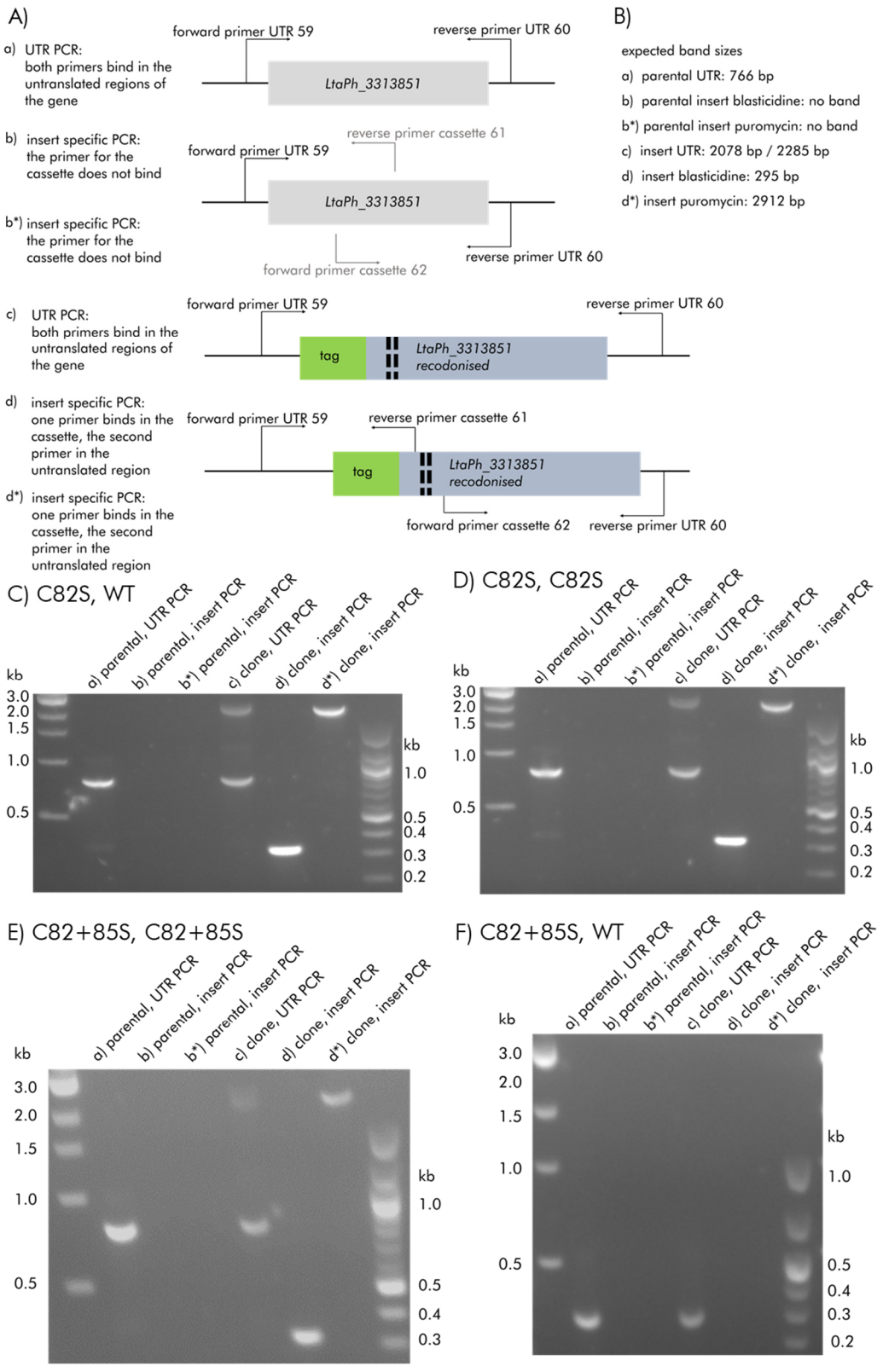


Figure 13: Genotyping of edited *LtaPh_3313851* (encoding Mic20) with two inserts. Cysteine mutants are present as indicated. A 3x-HA tag is encoded by the cassettes. Panel A) schematically shows the positions of the primers. Panel B) shows the expected sizes of the amplicons. Panels C-F) show images of the agarose gels separating the amplicons of the PCRs. C) shows the genotyping of a clone from a first transfection with C82S editing insert and a second transfection with WT insert. D) shows the genotyping of a clone from a first transfection with C82S editing insert and a second transfection with C82S editing insert. E) shows the genotyping of a clone from a first transfection with C82+85S editing insert and a second transfection with C82+85S editing insert. F) shows the genotyping of a clone from a first transfection with C82+85S editing insert and a second transfection with WT insert. The numbers of the primers used for the genotyping are: 59, 60, 61, 62 (Table 3).

The scheme of the primer positions is given in panel A), expected band sizes in panel B). Panels C) – F) show the genotyping agarose gels. PCRs for the parental strain were included on each gel and are labelled by letters a), b) and b*). Part a) showed the band for the UTR PCR at 766 bp, while insert specific PCRs b) and b*) did not show any amplicon. Panel C) corresponds to the clone with the C82S blasticidine cassette and the WT puromycin cassette, which exhibited a band for the presence of the parental gene in the UTR PCR at 766 bp as well as the insert specific bands at 2078 bp and 2285 bp, which could not be resolved (c). PCRs d) and d*) confirmed the presence of both inserts via DNA amplicons at 295 bp and 2912 bp respectively. The same pattern is given for panel D) corresponding to a clone with C82S blasticidine cassette and C82S puromycin cassette and panel E) corresponding to a clone with C82+85S blasticidine cassette and C82+85S puromycin cassette. All these clones exhibited both inserts, but parental copies of the gene were also present. Panel F) shows the genotyping of a clone transfected with C82+85S blasticidine cassette and WT puromycin cassette. For this clone only amplicons specific for the parental gene could be observed at 766 bp in the UTR PCRs a) and c). Thus, the presence of any insert remained unclear in this clone.

Editing the gene *LtaPh_3313851* was challenging, and genotyping revealed evidence that multiple copies of the gene may be present. The failed knock-out attempt could be a hint for the essentiality of the gene *LtaPh_3313851*. Furthermore, no homozygous clones could be obtained for the insertion of the cassettes for the mutations. Heterozygous clones were obtained for the modifications C82S, C85S and C82+C85S. It should also be mentioned, that a recent publication questions the function of Mic20 as an oxidoreductase.[41] However, the gene *LtaPh_3313851* could have an essential function.

3.2. Candidates from Erv pull-downs: UF2 and UF3

The present work was based on the substrates of a potential oxidoreductase, i.e. cysteine-containing proteins that are imported to mitochondria and need to be oxidatively folded. Turra approached the unknown protein on the side of Erv.[5] C-terminally His_8-tagged Erv was used, which lacked a domain specific for kinetoplastids called the kinetoplastida-specific second (KISS) domain.[42] Pull-down assays with this version of Erv were performed. In this way, two proteins of hitherto unknown function (UF) were identified as candidates for an oxidoreductase. For unknown function protein 2 (UF2) and unknown function protein 3 (UF3), knock-out attempts for the corresponding genes were performed by Turra. A complete knock-out could allow conclusions about the dispensability of the genes. The characterisation of the received strains was included in the present work. The genotyping followed a comparable procedure as before (Figure 14).

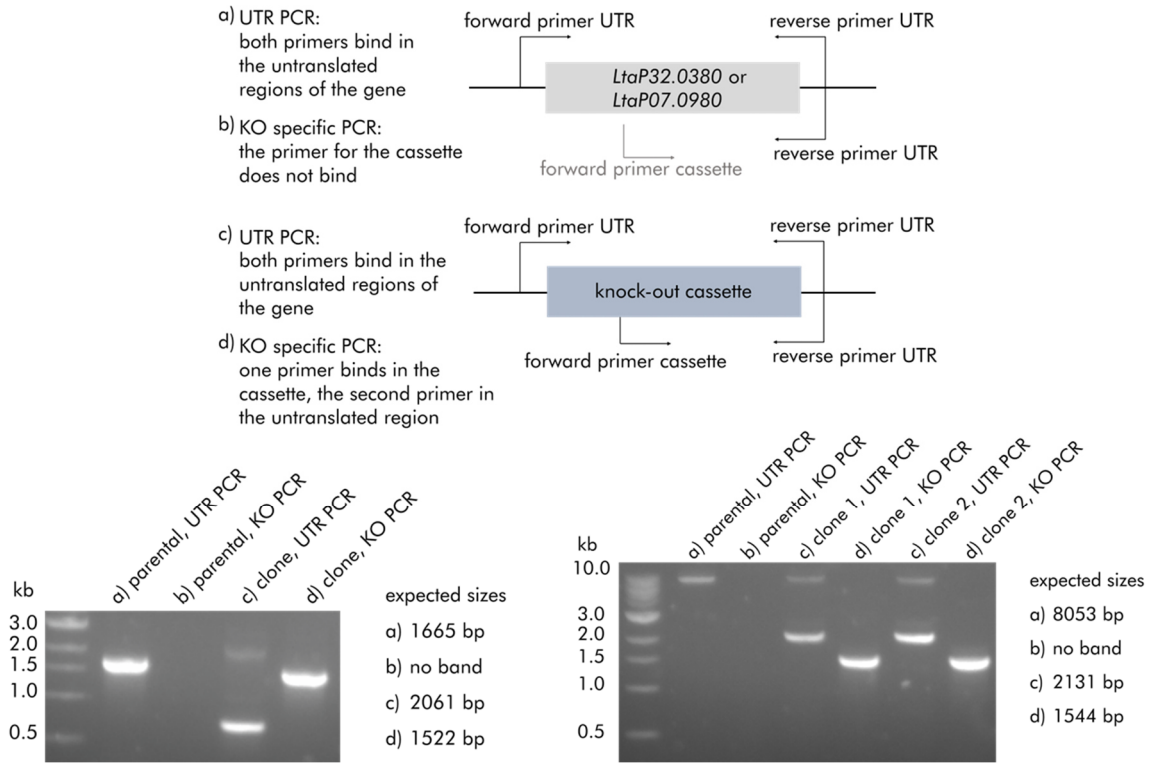


Figure 14: Genotyping strains from knock-out attempts for the genes encoding UF2 and UF3. The top panel schematically shows the position of the primers. The bottom panels show images of the agarose gels, separating the amplicons of the PCRs. The bottom left panel shows the genotyping of a clone from a knock-out attempt of the gene *LtaP32.0380* encoding UF2. The bottom right panel shows the genotyping of two clones from a knock-out attempt of the gene *LtaP07.0980* encoding UF3. Insertion of the knock-out cassettes should lead to puromycin resistance. The numbers of the primers used for the genotyping are: 49, 50, 62 (Table 3) for the editing of *LtaP32.0380* encoding UF2 and 51, 52, 62 (Table 3) for the editing of *LtaP07.0980* encoding UF3.

The scheme in Figure 14 visualises the primer positions in the untranslated regions or specific for the knock-out cassettes. The left agarose gel shows the amplicons of the genotyping results for one clone of the UF2 gene knock-out, the gel on the right for two different clones of the UF3 gene knock-out. Examination of the parental strain revealed the expected band at 1655 bp (a) in the PCR with primers in the untranslated regions and no band in the knock-out specific PCR (b). This excluded unspecific binding of the knock-out specific primers. For the investigated clone, two DNA fragments were amplified with the primers in the untranslated region (c). The first band corresponded to the expected size of the amplicon for the correct insert at 2061 bp. The second band between 500 bp and 1000 bp did not match with any expectation for parental or knock-out strain. The knock-out specific amplicon at 1522 bp confirmed the presence of an insert on the other hand (d). Thus, the genotyping of the clone from the knock-out attempt of gene *LtaP32.0380*, which encodes UF2 indicates a complete knock-out. Further investigations would include sequencing of the edited gene region.

Since the expected amplicons of UTR PCRs for UF3 gene knock-out were exceptionally large, the amplifications were performed with HiFi polymerase. Characterisation of the parental strain visualised the expected amplicons, 8053 bp for UTR PCR (a) and no amplification for the knock-out specific PCR (b). Clone 1 and 2 exhibited the knock-out specific amplicon at 1544 bp (d). For the UTR PCR both clones showed two different bands, one at 2131 bp being specific for correct insert and the band at 8053 bp specific for the parental gene. This result corresponded to a correct knock-out in one copy of *LtaP07.0980* and a second unmodified, parental gene. This could be explained by an increase in the copy number of the target gene in the genome due to editing. In this context, it is also pointed out that Turra only used one cassette with one selection marker.[5] A possible next step would therefore be the insertion of a second knock-out cassette, selected with a different marker. In addition, rescue studies with episomal *LtaP07.0980* could be performed to investigate the dispensability of the gene. In summary, heterozygous clones for the knock-out of *LtaP07.0980* encoding UF3 could be confirmed. This could be a hint for the essentiality of the gene. The knock-out of *LtaP32.0380* encoding UF2 could be assumed to be complete, which would indicate the dispensability of the gene. Notable cysteine motifs are, for example, a twin CX_9C motif in UF3 and CX_2C motifs in UF2.

3.3. Candidates identified as interactors of potential substrates

To find candidates for the protein folding oxidoreductase, one strategy of the present work was based on its substrates. Proteins with a certain pattern of cysteines and with properties suitable for purification were identified in the work of Liedgens[6] (see also Chapter 1.1.3). The idea was the co-purification of disulphide bridged intermediates of the substrates and the oxidoreductase. For this, tagged substrate proteins were needed. To introduce tags to the proteins, a chromosomal manipulation of the encoding genes was chosen in order to minimise the risk of the presence of unmodified proteins.

3.3.1. Chromosomal gene editing for the introduction of His_8-tags to potential substrates

To identify interaction partners of the potential substrates, tagged versions of the proteins were generated. These could be purified in later pull-down experiments together with disulphide bridged interaction partners. Since the functionality of internal targeting signals could be influenced by protein tagging, an attempt was made to modify either the C- or the N- terminus to improve the possibility for correct protein localisation. Not for all substrates both variants could be obtained.

Editing of the gene encoding sTim1 to produce a His_8-tag

The gene *LtaP25.1620* encoding the protein sTim1 could be modified at the N-terminus. As Figure 15 shows, the presence of both tagging cassettes was confirmed by the genotyping PCRs.

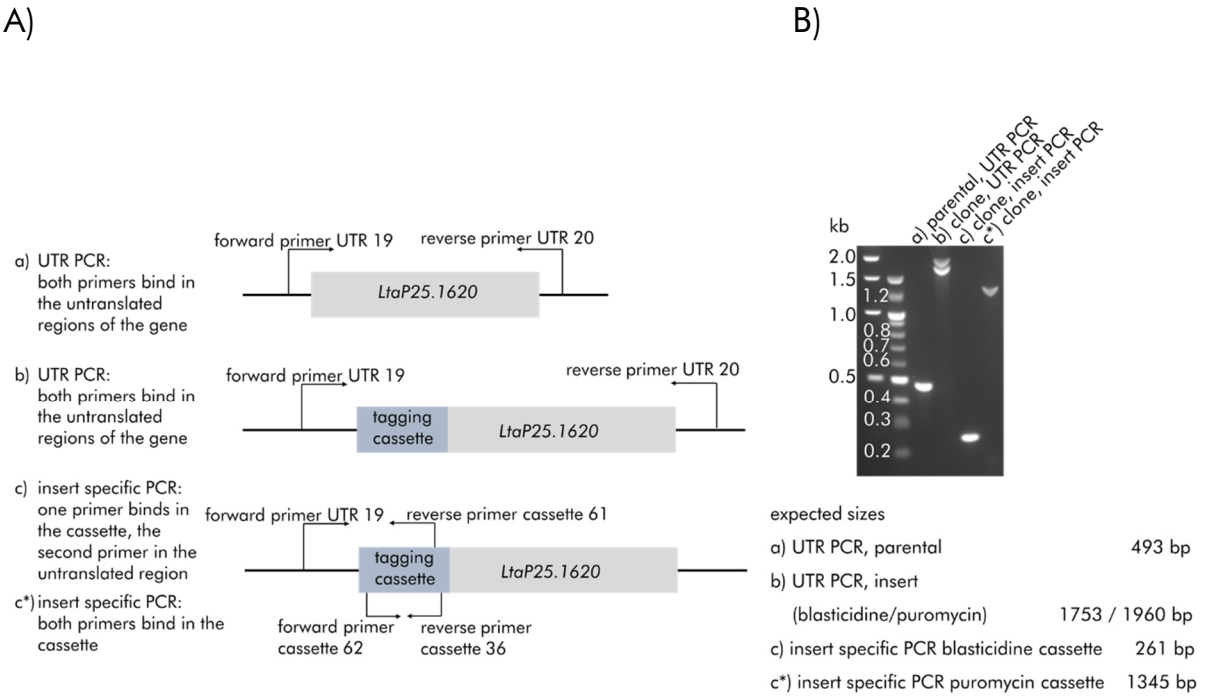


Figure 15: Genotyping of a clone from the N-terminal tagging attempt of *LtaP25.1620* encoding sTim1. A His_8-tag was encoded by the tagging cassette. Panel A) shows the schematic position of the primers. Panel B) shows an image of the agarose gel separating the amplicons. The numbers of the primers used for the genotyping are: 19, 20, 36, 61, 62 (Table 3).

The obtained band sizes were in line with the expectations. Figure 15 A) shows a scheme of the primer position for the performed PCRs. Figure 15 B) shows a picture of the agarose gel with the received DNA amplicons. PCR a) covered the whole locus of sTim1 with the primers binding in the UTRs adjacent to it. Investigation of the parental strain without genomic alteration showed a band at 493 bp for PCR a). For the clone with the tagging cassette inserted at *LtaP25.1620* (encoding sTim1), two slightly different sizes of DNA fragments were expected, since two different cassettes were inserted, one at each gene copy. Thus, the double band resolved at PCR b) showed the expected sizes of 1753 bp and 1960 bp. The absence of a band at 493 bp that would be expected for the parental strain, showed the complete alteration of all copies. To confirm the presence of the blasticidine selecting tagging cassette, PCR c) was performed with one primer binding specifically to the cassette and one primer in the untranslated region of the target gene. Thus, an amplicon did only arise if the cassette was located correctly in the gene. Specific for the correct integration of the blasticidine selecting cassette was the band at 261 bp in PCR c). The band at 1345 bp in PCR c*) confirmed the presence of the puromycin selection cassette. Since both primers were binding in the cassette, this PCR could not show the correct integration of the cassette, but in combination with the results of the UTR PCR (b) it was a strong

indication. In summary, it was assumed that the N-terminal editing of *LtaP25.1620* was complete.

Editing of the gene encoding substrate 1 to produce a His_8-tag

To achieve His_8-tagging of substrate 1, clones with C- and N-terminally edited gene *LtaP04.0660* could be generated (Figure 16).

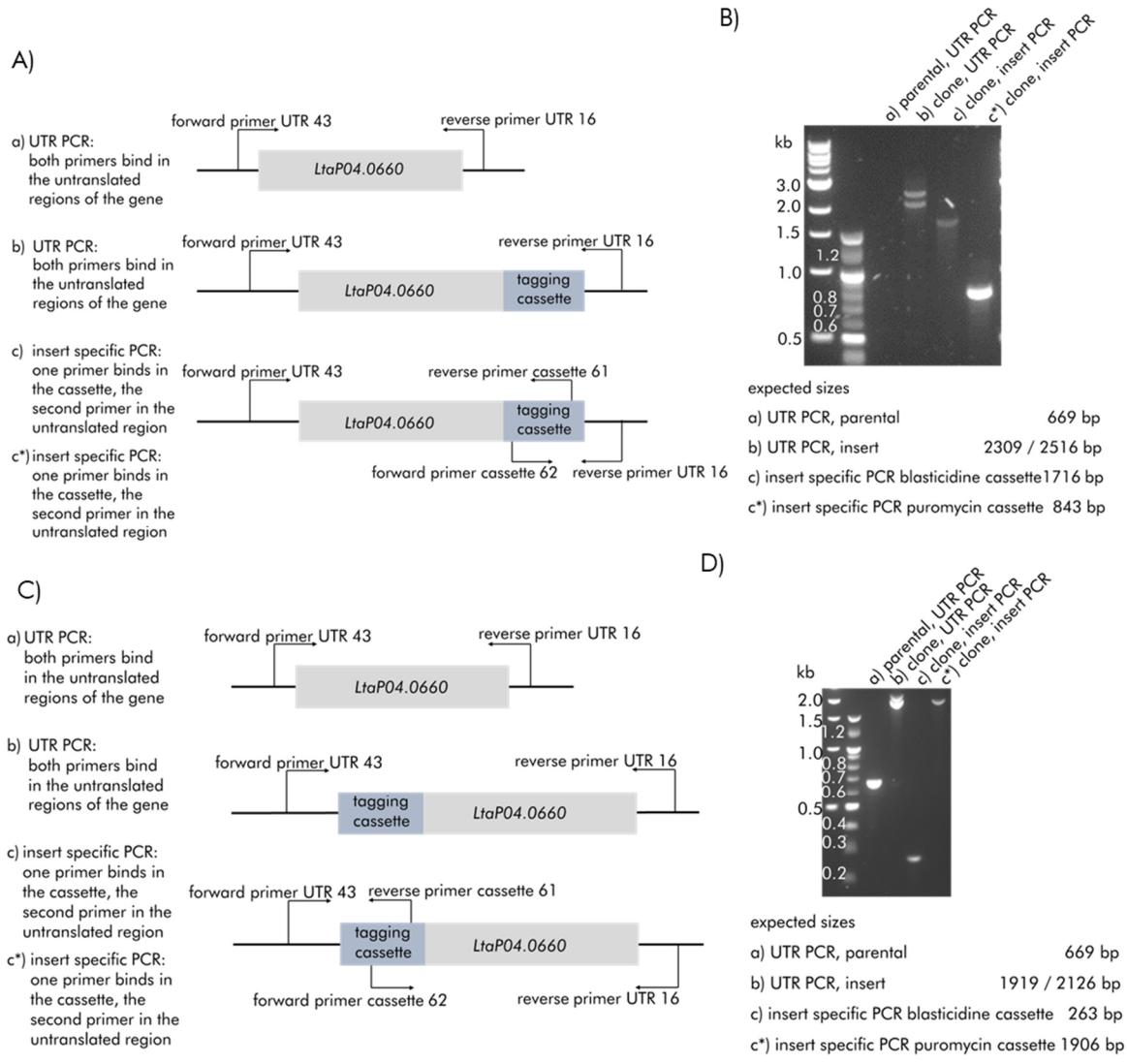


Figure 16: Genotyping of a clone from either C- or N-terminal tagging attempt of *LtaP04.0660* encoding substrate 1. A His_8-tag was encoded by the tagging cassettes. A) shows schematically the position of the primers used to verify the presence of the C-terminal tagging cassette. Panel B) shows an image of the agarose gel separating the amplicons for a clone with the C-terminal tagging cassettes. Panel C) shows schematically the position of the primers used to verify the presence of the N-terminal tagging cassette. Panel E) shows an image of the agarose gel separating the amplicons for a clone with the N-terminal tagging cassettes. The numbers of the primers used for the genotyping are: 16, 43, 61, 62 (Table 3).

Scheme A) explains the localisation of the primers for C-terminal editing. Picture B) shows the agarose gel for the amplicons received in these PCRs. When both primers in the untranslated region were chosen, a band at 669 bp was expected for the parental strain. Since this band was absent in the genotyping of the parental strain, PCR b) for the edited clone did not have any significance. However, the presence of all the insert specific bands at the expected sizes was a strong hint for the correct integration of both inserts. The DNA amplicons at 2309 bp and 2516 bp confirmed the presence of both inserts, as well as the specific PCRs. 1719 bp was the expected size for the presence of the blasticidine selecting insert and was received in PCR c). PCR c*) confirmed the presence of the puromycin selecting insert, since the expected amplicon at 843 bp was present.

N-terminal editing of *LtaP04.0660* (encoding substrate1) was confirmed by the agarose gel shown in Figure 16 D). The band specific for the unaltered gene of substrate 1 at 669 bp was present for genotyping the parental strain and absent in the manipulated clone. Specific bands for the presence of the blasticidine insert and the puromycin insert in PCR b) were present at 1919 bp and 2126 bp respectively. PCRs c) and c*) confirmed the presence of the inserts with amplicons at 263 bp and 1906 bp. Thus, the C-terminal editing of *LtaP04.0660* (encoding substrate 1) produced heterozygous clones, and the N-terminal editing could be considered homozygous.

Editing of the gene encoding substrate 2 to produce a His_8-tag

For *LtaP35.0210*, which encodes substrate 2, neither C- nor N-terminal editing was possible homozygously. Thus, substrate 2 was not suitable for the subsequently planned pull-down experiments, since the expression of the unmodified gene might be favoured over the edited gene and probably no tagged protein could be obtained. The genotyping PCRs with the primers binding in the UTRs showed heterozygous insertion for two clones with potentially inserted cassettes for C-terminal His_8-tags (see the supplements, S3). Substrate 2 was excluded as bait and no further investigations were made.

Editing of the gene encoding substrate 3 to produce a His_8-tag

From the genotyping PCRs of substrate 3 for C- terminal editing, the correct insertion of the cassettes could be concluded (Figure 17).

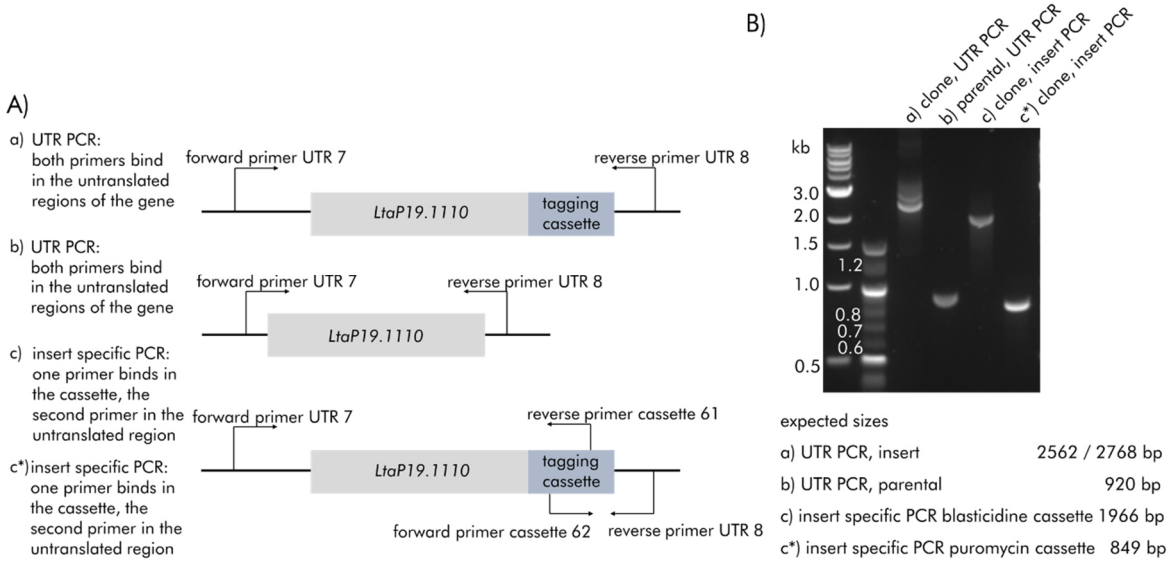


Figure 17: Genotyping of a clone from the C-terminal tagging attempt of *LtaP19.1110* encoding substrate 3. A His_8-tag was encoded by the tagging cassette. A) shows schematically the position of the primers used to verify the presence of the C-terminal tagging cassette. Panel B) shows an image of the agarose gel separating the amplicons for a clone with the C-terminal tagging cassette. The numbers of the primers used for the genotyping are: 7, 8, 61, 62 (Table 3).

For the PCR with both primers in the UTR (a), two amplicons at 2562 bp and 2768 bp were resolved for the clone, as expected. The amplicon derived from the parental strain had a size of 920 bp, and was only obtained in PCR b). The PCR specific for the blasticidine resistance cassette revealed the expected band at 1966 bp for the clone. The size of the amplicon specific for the puromycin resistance cassette could be observed for PCR c*). Thus, C-terminal tagging of substrate 3 was assumed to be complete.

Editing of the gene encoding substrate 4 to produce a His_8-tag

For editing the gene encoding substrate 4, colonies were obtained for the insertion of C- or N-terminal tagging cassettes. Figure 18 shows the results for the genotyping PCRs.

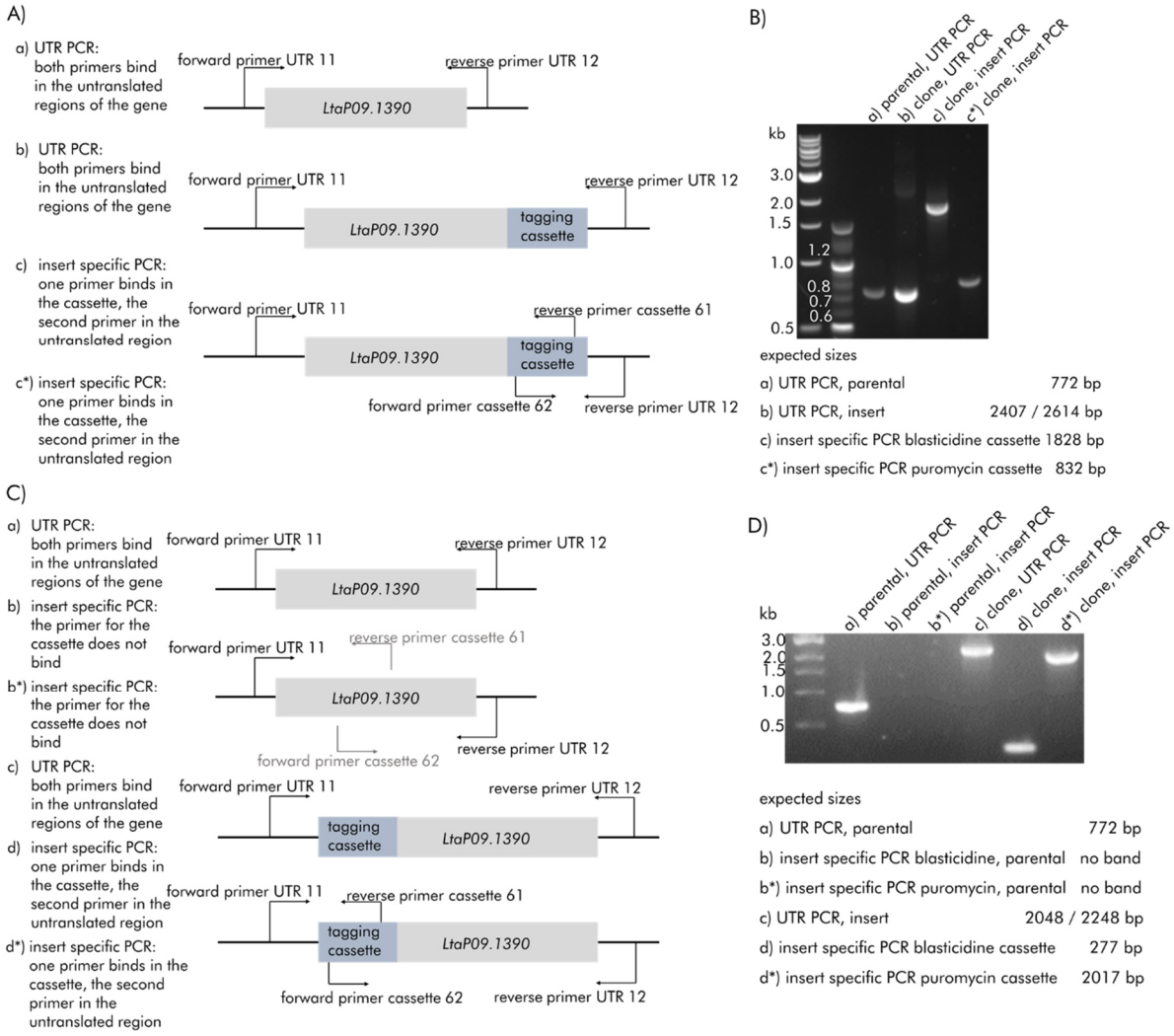


Figure 18: Genotyping of a clone from either C- or N-terminal tagging attempt of *LtaP09.1390* encoding substrate 4. A His_8-tag was encoded by the tagging cassettes. Panel A) shows schematically the position of the primers used to verify the presence of the C-terminal tagging cassette. Panel B) shows an image of the agarose gel separating the amplicons for a clone with C-terminal tagging cassette. Panel C) shows schematically the position of the primers used to verify the presence of the N-terminal tagging cassette. Panel E) shows an image of the agarose gel separating the amplicons for a clone with N-terminal tagging cassette. The numbers of the primers used for the genotyping are: 11, 12, 61, 62 (Table 3).

Scheme A) shows the positions of the primers for the genotyping of the C-terminal tagging attempt with the results represented in B). C) and D) refer to N-terminal tagging. Insert specific bands for the presence of the C-terminal tagging cassette were present (Figure 18 B), PCRs b), c), c*) at the expected band sizes of 2407 / 2614 bp, 1828 bp and 832 bp respectively. The PCR amplicon specific for the unaltered gene was present in PCR a) for

the parental strain, but also appeared in PCR b) for the manipulated strain. Thus, for C-terminal tagging of substrate 4, a clone with heterozygously present tagging cassette could be obtained.

To monitor the presence of the N-terminal tagging cassette, six PCRs were performed. PCR a) showed the parental typical band at 772 bp. PCRs b) and b*) were insert specific and were performed with DNA extracted from the parental strain. Thus, the absence of amplicons that is observed corresponds to the expectations. For PCR c) a double-band at 2048 /2248 bp was expected and an amplicon in this size range is present. The separation of the two bands could not be resolved on the agarose gel. Insert specific bands for PCRs d) and d*) at 277 bp and 2017 bp confirmed the presence of both tagging cassettes. In summary, C-terminal tagging of substrate 4 could not be confirmed to be complete. N-terminal tagging was successful.

3.3.2. Chromosomal knock-out of the genes encoding potential substrates

N- or C-terminal edited genes encoding for sTim1 and for substrates 1, 3 and 4 could be obtained. The tag was an important requirement to analyse protein interactions via pull-down assays. But also, the correct position in the intermembrane space was vital for the interaction with a potential oxidoreductase. However, this could be compromised by the introduction of a tag to the protein, interfering with targeting signals. The possibility for incorrect position was assumed to be higher for non-essential tagged proteins. An essential protein that does not reach its destination could prevent parasites from surviving. Therefore, living parasites with edited essential genes are a good starting point. Chromosomal knock-out attempts were made to analyse the essentiality of the substrates. For knock-out attempts for the genes encoding sTim1 and for substrates 2, 3, and 4 no living parasites could be obtained in the selection after transfection. Figure 19 shows the genotyping of a clone from the knock-out attempt of the gene for substrate 1.

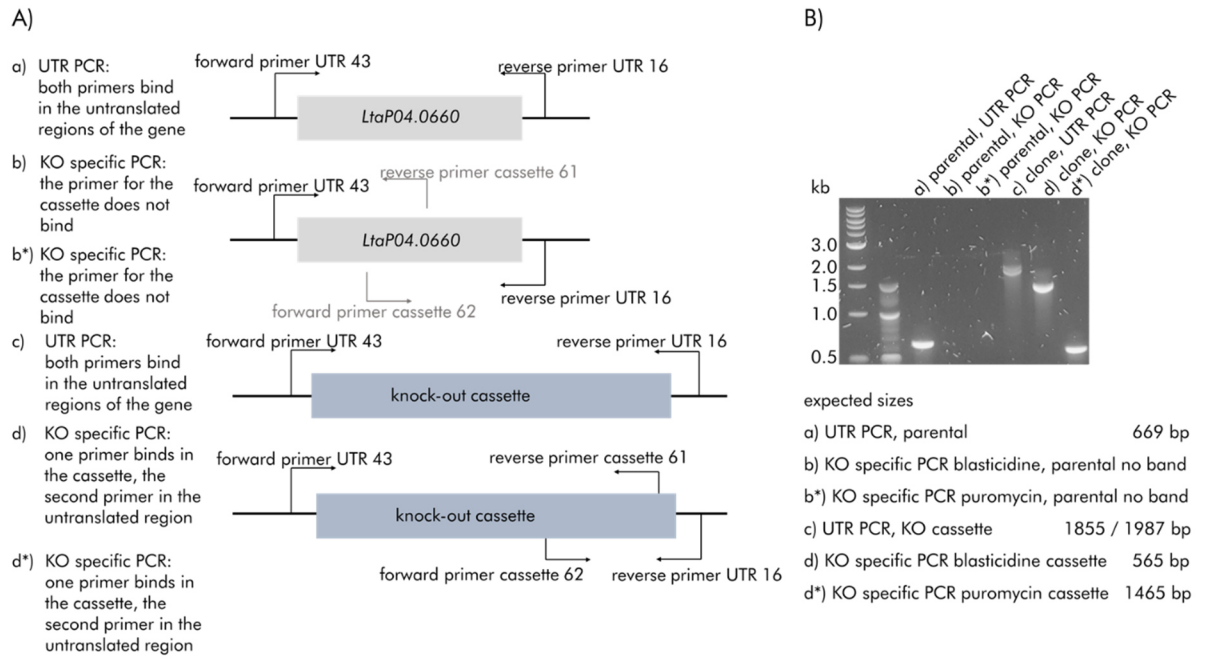


Figure 19: Genotyping of a clone from a knock-out attempt of *LtaP04.0660* (substrate 1). Panel A) shows schematically the positions of the primers used to verify the presence of the knock-out cassettes. Panel B) shows an image of the agarose gel separating the amplicons for a clone with the knock-out cassettes. The numbers of the primers used for the genotyping are: 16, 43, 61, 62 (Table 3).

PCRs a), b) and b*) (Figure 19) show the amplicons representative for the parental strain: 669 bp for primers in the UTR and no bands for insert specific PCRs. For the clone from the knock-out attempt, specific bands were visible at 565 bp (d) and 1465 bp (d*). The expected double-band at 1855 bp and 1987 bp in PCR c) was not resolved, but an amplicon in the expected size was present. In summary, the knock-out of *LtaP04.0660* (substrate 1) was assumed to be complete.

3.3.3. Summary of gene editing

Table 4 gives an overview of all the gene editing attempts described.

Table 4: Summary of gene editing attempts. The results of the experiments are shown in the columns. Fields without an indication mean that a corresponding experiment was not carried out.

Gene	Protein encoded	Knock-out of gene	tagging cassettes	mutation cassette
LtaPh_3313851	Mic20	no parasites	-	heterozygous for C82S, C85S and C82+C85S
LtaP32.0380	UF2	homozygous*,***	-	-
LtaP07.0980	UF3	heterozygous***	-	-
LtaP25.1620	sTim1	no parasites	N-terminal: homozygous	-
LtaP04.0660	substrate 1	homozygous	C-terminal: heterozygous, N-terminal: homozygous	-
LtaP35.0210	substrate 2	-	C-terminal: heterozygous**	-
LtaP19.1110	substrate 3	no parasites	C-terminal: homozygous	-
LtaP09.1390	substrate 4	no parasites	C-terminal: heterozygous, N-terminal: homozygous	-

*An additional band could not be explained. **Two clones were partially genotyped, the data are shown in the supplements (S1). ***The knock-out attempt was based on only one resistance cassette.

The gene *LtaPh_3313851* encoding for Mic20, could not be knocked-out and homozygous insertion of the mutation cassettes was not possible, but heterozygous mutants could be obtained. This could indicate the essentiality of the cysteines C82 and C85. For *LtaP32.0380*, a homozygous knock-out and thus dispensability is assumed. The knock-out attempt of *LtaP07.0980* yielded heterozygous clones. For all but one substrate, the corresponding genes could be edited homozygously with cassettes for C- or N- terminal tagging.

3.3.4. Confirmation of the presence of the His_8-tag

The presence of the tagging cassettes was investigated on the DNA level in Chapter 3.3.1. To confirm the presence of the tag in proteins, immunodetection of the His_8-tag after western blotting is suitable. Cell lysates of all strains with homozygous insertion of a tagging cassette were investigated. But only for the strain with the cassettes for the N-terminal tagging of substrate 4, the presence of a His_8-tag could be verified (Figure 20).

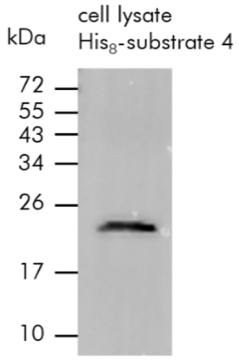


Figure 20: Image of the western blot membrane decorated with anti-His antibody. Cell lysates of a strain with homozygous insertion of a N-terminal His_8-tagging cassette at gene *LtaP09.1390* (encoding substrate 4) were investigated. The expected size for the tagged protein is 23 kDa.

Figure 20 shows a His_8-tagged protein with a size between 17 kDa and 26 kDa. This was consistent with the expected size of 23 kDa for the His_8-tagged substrate 4. Thus, substrate 4 was considered to be suitable for use in pull-down experiments to trap the sought oxidoreductase. The absence of a larger protein band indicating an intermediate was indicative of their low abundance. For none of the other strains with homozygously inserted tagging cassettes (see Table 4), a signal of a His_8-tag could be observed. As suspected, later pull-down experiments with other substrates also failed (see also Chapter 3.3.10).

3.3.5. Morphology of the cells with N-terminally His_8-tagged substrate 4

Since the exact function of substrate 4 was not known, the morphology of the cells was compared to parental cells, to confirm that cells with N-terminally His_8-tagged substrate 4 exhibit usual fitness. Figure 21 shows images of both cell lines.

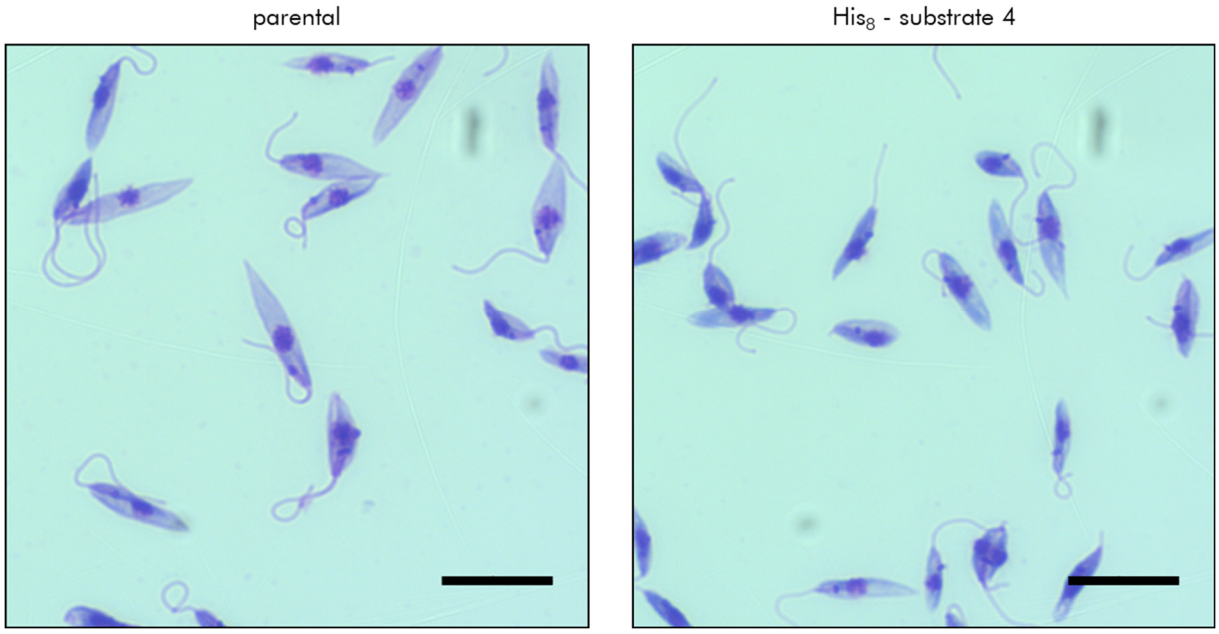


Figure 21: Image of *Leishmania tarentolae*. The left panel shows an image of the parental cells. The right panel shows an image of cells with N-terminally His_8-tagged substrate 4. All cells showed similar shape and size. The cells were fixed with MeOH and stained with Giemsa. The scale bar corresponds to a length of 5 µm.

Size and shape of the cells differed slightly between individual cells, but no significant difference between the two cell lines was observed. No different growth behaviour was observed either. In summary, cells with N-terminally His_8-tagged substrate 4 did not exhibit morphological particularities.

3.3.6. Localisation of N-terminally His_8-tagged substrate 4

Since cells with N-terminal His_8-tag at substrate 4 were shown to have usual fitness, the next step was to confirm the position of tagged substrate 4. Any interaction partner, and thus, the desired oxidoreductase could only be found if substrate 4 reaches its target locus in the mitochondrial intermembrane space. A prediction of the position for the N-terminally tagged ortholog of substrate 4 in *Trypanosoma brucei*[98] expects its presence in the mitochondrion[99]. This hint for a correct position was confirmed for the available N-terminally His_8-tagged substrate 4 in *Leishmania tarentolae*. Cells were fractionated with increasing concentrations of digitonin, to stepwise solubilise the membranes. For comparison, the presence of Erv was visualised. As control, the harsh detergent Triton X-100 was used (Figure 22).

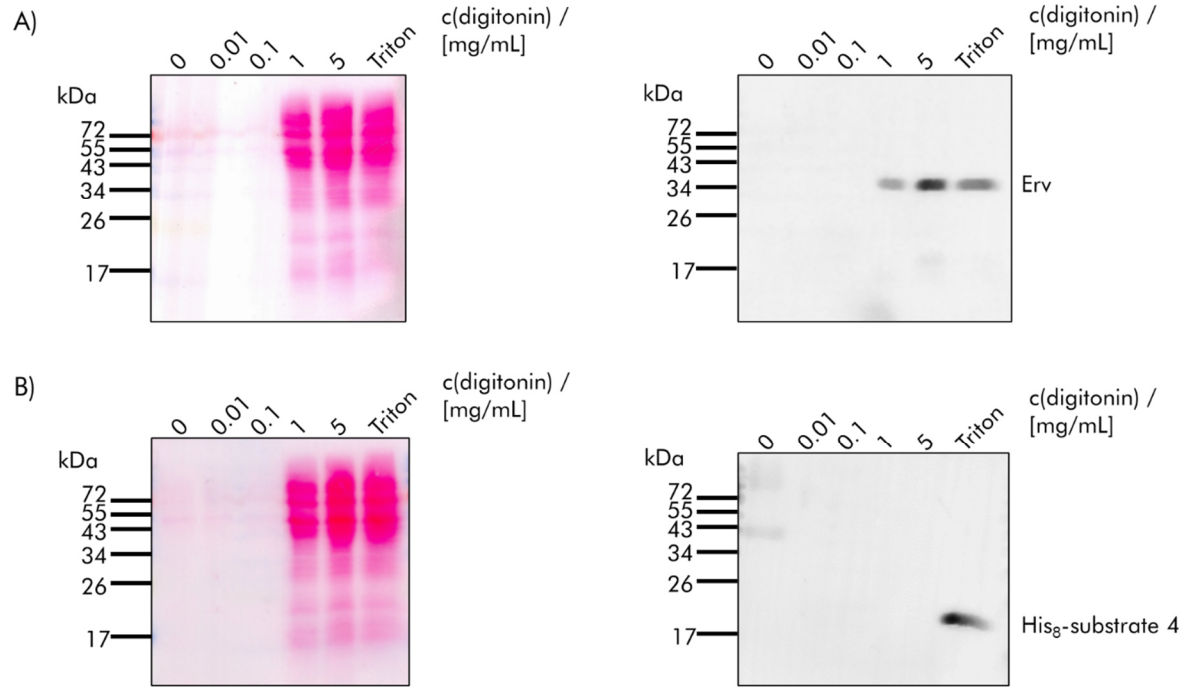


Figure 22: Fractionation of cells with N-terminally His_8-tagged substrate 4. The left panels show images of Ponceau stained western blot membranes. The right panels show images of western blot membranes decorated with antibodies: The membrane in panel A) was decorated with anti-Erv antibody, the membrane in panel B) was decorated with anti-His antibody. The expected band sizes were: 34 kDa for Erv and 23 kDa for N-terminally His_8-tagged substrate 4.

Since Erv was a direct interaction partner of Mia40 and thus, of the putative oxidoreductase in *Leishmania tarentolae*, it was a suitable indicator for localisation. Ponceau stainings showed uniform protein loading for both membranes (left panels). Bands for Erv at 34 kDa were detected in fractions with 1 and 5 mg/mL digitonin, as well as in the control fraction were all compartments were solubilised by 2% (v/v) Triton X-100 (top right). For the His_8-tagged substrate 4, a band at 23 kDa was visible only in the Triton fraction (bottom right). This could be explained by the presence of two transmembrane domains in substrate 4 (see the supplements, S4), possibly placing it in the mitochondrial inner membrane. Thus, substrate 4 was solubilised only in the presence of harsh detergents. It is important to note that tagged substrate 4 was not found in fractions without Erv. In summary it could be stated that the N-terminally His_8-tagged substrate 4 was localised in the mitochondrion.

3.3.7. Pull-down of N-terminally His_8-tagged substrate 4

Since the presence and correct position of the tagged substrate 4 could be confirmed, further investigation of this substrate was indicated. Pull-down experiments were performed to purify and enrich the substrate and possible disulphide bridged intermediates.

Screening for conditions for the pull-down experiments

The first step for a successful pull-down assay was to establish suitable experimental conditions. First, the influence of the pH value was investigated (Figure 23).

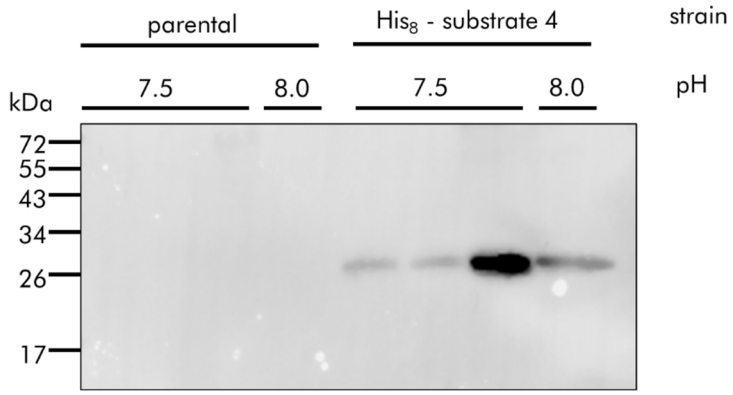


Figure 23: Comparison of different pH values for pull-down experiments. A triplicate was performed for pH = 7.5, a single experiment for pH = 8. The western blot membrane was decorated with anti-His antibody. The left part of the membrane shows four samples from parental cells. The right part shows four samples from cells with N-terminally His_8-tagged substrate 4 (expected: 23 kDa).

A pH value of 8 was desired for binding of proteins to the Ni-NTA beads, whereas lower pH values were preferred for the reaction of *N*-ethylmaleimide (NEM) with thiols. Therefore, both conditions were competing. Figure 23 shows that pH values of 7.5 likewise 8 could be successfully used in pull-down experiments. As expected, for pull-down assays performed with the parental strain, no signal was obtained, independent of the pH value. A triplicate of pull-down experiments at pH = 7.5 showed the presence of His_8-tagged substrate 4 in the eluate. Also, at pH = 8 a band at ca. 23 kDa showed the success of the pull-down assay.

To stabilize a potentially short-lived and low abundant disulphide bridged intermediate, the use of NEM is advantageous for stabilisation. NEM scavenges free thiols and prevents them from cleaving disulphide bridges by disulphide exchange. The first condition tested was 100 mM NEM during cell lysis and binding to Ni-NTA at pH = 7.5. These experiment did not result in successful pull-downs (data not shown).

A side reaction of NEM with the imidazoles of the His_8-tag could explain failure of this pull-down.[100] Thus, screening for suitable conditions was performed. Lower NEM concentrations were assumed to protect the tag. Repetition of experiments at other pH values or using other additives could be promising for future investigations. NEM concentrations of 0 mM, 5 mM and 50 mM were tested at pH = 8 (Figure 24). Also, the influence of a pre-treatment of the cells with 100 mM NEM before lysis was investigated.

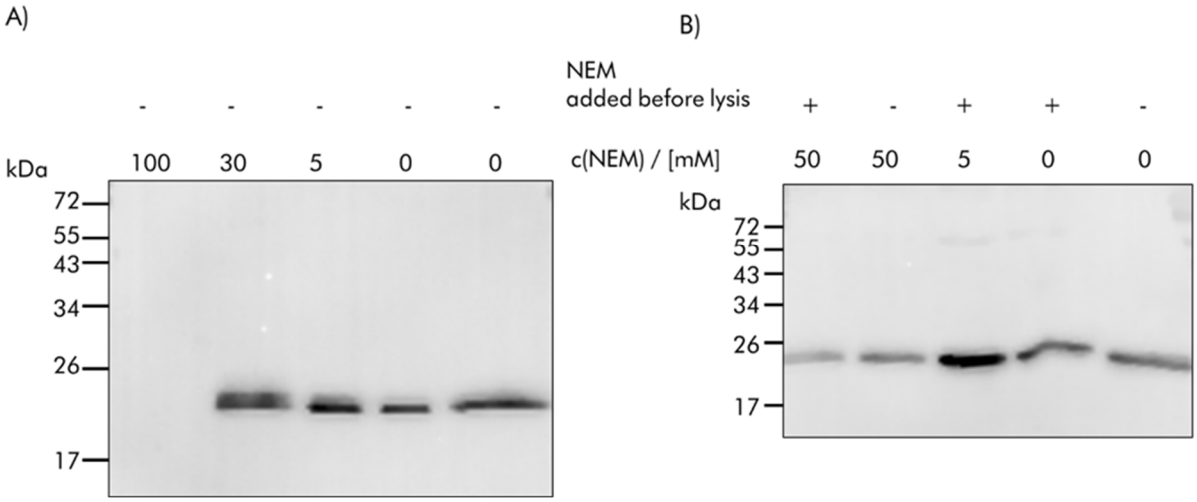


Figure 24: NEM concentrations in pull-down experiments and pre-treatment with 100 mM NEM. Different concentrations of NEM were used in the pull-downs experiments. Pre-treatment of the cells with NEM prior to lysis was performed as indicated. The western blot membranes were decorated with anti-His antibody. During the pull-down experiments pH = 8 was used. All experiments were carried out with cells with N-terminally His_8-tagged substrate 4. The expected band size was 23 kDa.

NEM concentrations up to 100 mM were tested (Figure 24, A). For 0 mM, 5 mM and 30 mM strong bands at 23 kDa for tagged substrate 4 could be obtained, but not for 100 mM NEM. Thus, an additional concentration was chosen at 50 mM (Figure 24, B), to approach the maximal NEM concentration. Figure 24 B) shows that NEM concentrations up to 50 mM at pH = 8 did not affect the efficiency of pull-down experiments, since His_8-tagged proteins at ca. 23 kDa were detected in all experiments. Thus, 50 mM NEM was chosen for further experiments.

3.3.8. Identification of potential interactors of N-terminally His_8-tagged substrate 4

According to the derived experimental conditions for the pull-down experiments, a biological triplicate assay series with 50 mM NEM at pH = 8 was performed. Eluates from these pull-downs were intended for analysis via mass spectrometry. The detection of the His_8-tag confirmed the success of the pull-down experiments (Figure 25).

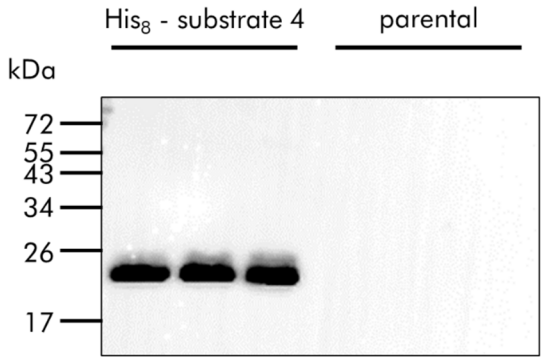


Figure 25: Confirmation of successful pull-downs for mass spectrometry via western blot. Three replicates each were performed for cells with N-terminally His_8-tagged substrate 4 and parental cells. For His_8-substrate 4 a band size of 23 kDa was expected. Eluates from pull-downs were separated by SDS-PAGE. After western blotting, the membranes were decorated with anti-His antibody. In the pull-down experiments pH = 8 and 50 mM NEM were used.

The expected bands at 23 kDa were present for the eluates from pull-down experiments with cells with N-terminally His_8-tagged substrate 4. Preparation of the samples for the mass spectrometry analysis was carried out under the guidance of Dr. Markus Räschle, TU Kaiserslautern. The analysis in the mass spectrometer was performed by Dr. Markus Räschle. Measurements were performed with all triplicates. The pull-down eluates from the parental strain were chosen as a control. Since no tag was present in this samples, they were suitable to determine proteins that bound non-specifically to the Ni-NTA beads.

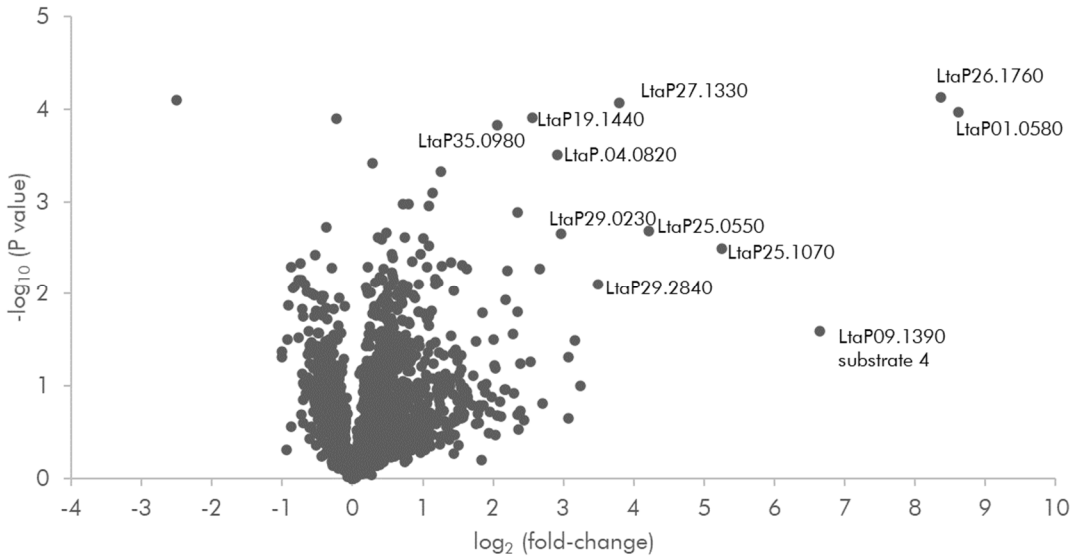


Figure 26: Volcano plot of proteins found in the mass spectrometry analysis. Proteins enriched by the presence of the N-terminal His_8-tag at substrate 4 were considered. Each protein was represented by one data point. The fold-change values correspond to the enrichment of the proteins between the experiments with cells with N-terminal His_8-tagged substrate 4 and parental cells. Labelled data points represent proteins that were significantly enriched. Data of the proteins discussed below are also listed in the supplements (S5).

Figure 26 shows the results of this analysis in a volcano plot. A total of 2041 proteins was identified. On the x-axis $\log_2$ (fold-change) and on the y-axis $-\log_{10}$ (P value) are given. Thus, data points around the origin corresponded to proteins that were not enriched by the presence of the His_8-tag in the pull-down experiments and were not of high significance. Proteins of high enrichment far from the origin were of special interest. Protein identification numbers of these were given in the plot (Figure 26). Substrate 4 was found to be enriched, as the data point for LtaP09.1390 shows. Therefore, a successful experiment could be assumed. To create a broader data base, more pull-down experiments with a subsequent analysis in mass spectrometer were performed.

3.3.9. Treatment of cells with azide

One approach to enrich short-lived intermediates between the substrates and an oxidoreductase resulted from inhibition of the respiratory chain. The electron transport in mitochondrial protein import involves a putative oxidoreductase as well as Erv. Entry to the respiratory chain occurs via the cytochrome *c* oxidase complex. Blocking the electron transport can possibly trap intermediates. Sodium azide was chosen to inhibit the cytochrome *c* oxidase complex.

Cells were treated with 5 mM azide, incubated overnight and one pull-down could be performed successfully (Figure 27).

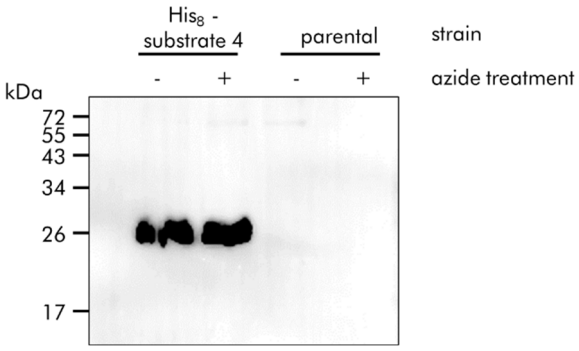


Figure 27: Image of western blot membrane with samples from azide treated cells. Cells with N-terminally His_8-tagged substrate 4 were used for the left samples and parental cells for the right samples. If indicated, 5 mM sodium azide were added to the cells and incubated overnight.. Cell lysates were separated on SDS-PAGE. The western blot membrane was decorated with anti-His antibody. The expected size for the His_8-substrate 4 was 23 kDa.

Cells with tagged substrate 4 were compared with parental cells, both with or without azide treatment. For tagged substrate 4, bands at 23 kDa were observed in both conditions. For further repetitions, no successful pull-down could be confirmed via western blot, even with varying azide concentrations and incubation times. A systematic search for reliably suitable conditions should be the starting point of future investigations on azide treatment of the cells.

3.3.10. Pull-down attempts of sTim1 and substrate 1

To expand the data set and to search for common interaction candidates, other substrates could also be used. Thus, pull-down experiments were performed for different substrates, according to the experimental conditions described in Chapter 3.3.7. Figure 28 shows the results for substrate 4, sTim1 and substrate 1.

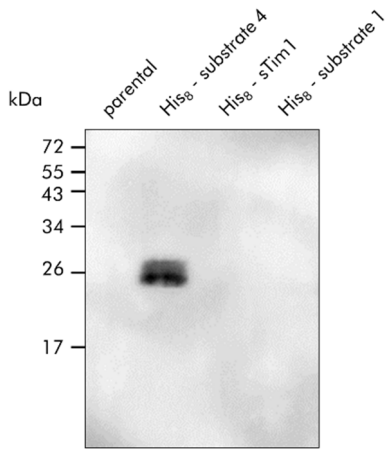


Figure 28: Image of a western blot membrane from pull-down experiments with homozygously inserted N-terminal His_8-tagging cassettes. Parental cells and cells from tagging attempts of *LtaP09.1390*, *LtaP25.1620* and *LtaP04.0660* were used. The expected sizes for the tagged proteins were: parental: none, substrate 4: 23 kDa, sTim1: 13 kDa, substrate 1: 17 kDa. The eluates from the pull-down experiments were separated by SDS-PAGE. The western blot membranes were decorated with anti-His antibody.

Only for the pull-down of substrate 4 a signal from the His_8-tag could be obtained. The expected size of the tagged substrate was 23 kDa and corresponded to the band observed in Figure 28. For none of the other substrates (data for substrate 3 not shown) could a band be detected. Thus, cell lines with tagged substrates other than 4 were assumed to be unsuitable for pull-down experiments under the specified conditions.

3.3.11. Treatment of cells with DTT or TMAD

Treatment of parental cells and cells with N-terminally His_8-tagged substrate 4 with DTT or TMAD was chosen to alter the redox conditions. Intermediates formed by an oxidoreductase with the tagged substrate 4 should be destabilised by reduction with DTT and stabilised by oxidising TMAD. After the treatment of cells for 30 min with DTT, TMAD or without additive, NEM was added to stabilize the current disulphides. A concentration of 50 mM NEM was also used during the subsequent pull-down. Pull-down experiments and mass spectrometry were performed as before. As shown in Figure 29, the expected band for substrate 4 with His_8-tag could be obtained.

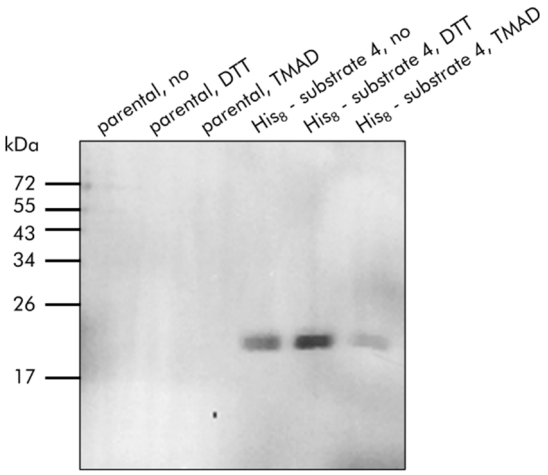


Figure 29: Confirmation of successful pull-downs for mass spectrometry via western blot (DTT or TMAD as additives in pull-down experiments). For the left samples parental cells were used, for the right samples cells with N-terminally His_8-tagged substrate 4 (size of 23 kDa expected). The use of additives is indicated: none, DTT or TMAD. A concentration of 1 mM was used for the additives. The eluates from the pull-downs were separated on SDS-PAGE. The western blot membrane was decorated with anti-His antibody. In the pull-down experiments was pH = 8 and the concentration of NEM was 50 mM.

In total, three biological replicates of the cell treatment were performed and the samples were prepared for the mass spectrometry as before. (Shown is a representative immunoblot from one replicate.)

3.3.12. Identification of potential interactors of N-terminally His_8-tagged substrate 4 with the additives DTT or TMAD

The analysis of the pull-down eluates from DTT or TMAD treated parental cells and cells with N-terminally His_8-tagged substrate 4 revealed three lists of proteins: 1269 obtained without additive, 1829 with DTT, 1290 with TMAD. Figure 30 show the volcano plots.

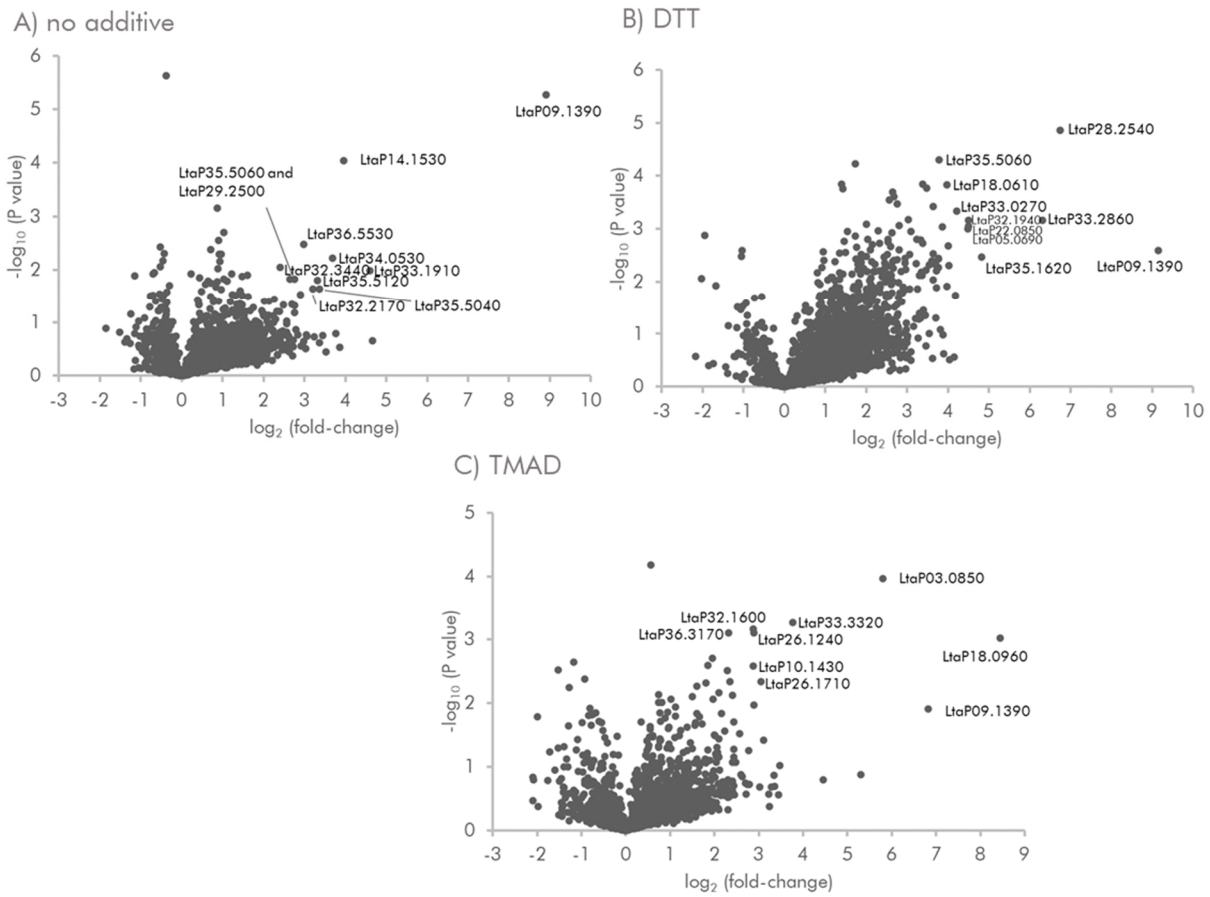


Figure 30: Volcano plot of proteins found in the mass spectrometry analysis (DTT or TMAD as additives in pull-down experiments). Proteins enriched by the presence of the N-terminal His_8-tag at substrate 4 were considered. Each protein was represented by one data point. The fold-change values correspond to the enrichment of the proteins between the experiments with cells with N-terminally His_8-tagged substrate 4 and parental cells. Panel A) shows the results obtained without additive, panel B) for cells treated with DTT before lysis and pull-down and panel C) for cells treated with TMAD before lysis and pull-down. Labelled data points represent proteins that were significantly enriched. Data of the proteins discussed below are also listed in the supplements (S5).

The volcano plots show data points for proteins from experiments without additive (A), with DTT (B) and with TMAD (C). Without additive 1270 proteins were considered, with DTT and TMAD 1274 each. Proteins that are significantly enriched in pull-downs with cells containing the N-terminally His_8-tagged substrate 4 compared to parental cells are assigned with their identification number. Again, substrate 4 (LtaP09.1390) was among the significantly enriched proteins in each experiment, confirming the success of the pull-downs and mass spectrometry.

The evaluation of the obtained protein lists was a major challenge to find candidates for Mia40 orthologs. Discussion of these follows in Chapter 4.3.

4. Discussion

4.1. Editing of genes encoding Mic20 and UF proteins

The first candidate ortholog of the oxidoreductase Mia40 in kinetoplastida studied in this work was Mic20, which was identified in *Trypanosomes*.[1–3] Several attempts for a chromosomal knock-out of the encoding gene failed. This may be an indication of the essentiality of *LtaPh_3313851*, especially since the mutation attempts did not lead to a homozygous introduction of the mutation cassettes either. The aneuploidy of *Leishmania tarentolae* could be a reason for more difficult genetic manipulation. The alteration of an essential gene in all chromosomes in the genome could lead to the creation of further copies of the original gene.[5,87,101] Therefore the obtaining of heterozygosity could indicate the essentiality of the cysteine residues. Possible changes in the protospacer adjacent motif sequence that would explain the preservation of a new copy could be investigated by sequencing. Efficiency of the transfections could also be impaired by the large size of the introduced cassettes. Rescue studies with episomal *LtaPh_3313851* could provide a clear statement on essentiality. Although latest findings exclude Mic20 as a Mia40 ortholog,[41] this protein may be of interest for understanding other mitochondrial processes. As a part of the MICOS complex, Mic20 plays undoubtedly an important role in mitochondria.

Further proteins of interest in this work are UF2 and UF3. These two candidates were identified in the work of Turra,[5] including an attempt to knock-out the corresponding genes. The successful genotyping was part of the present work. The genotyping of the knock-out attempt for the gene *LtaP32.0380* encoding for UF2, did not show amplicons specific for the unaltered gene. Thus, the knock-out could be considered complete and the gene dispensable, although an additional amplicon can not be explained. The insertion of the knock-out cassettes for *LtaP07.0980* could be shown to be heterozygous. In the context of the aneuploidy of *Leishmania tarentolae*, this could be an indication for the essentiality of the gene.[5,87,101] A search for motifs similar to the functional sites in Mia40 revealed sequences with CX_2C or CX_3C motifs in UF2 and a twin CX_8CC motif in UF3. Investigation of the cysteine motifs of the proteins of yet unknown function could be the subject of further studies. The identification of the two proteins in correlation with Erv

pull-downs could indicate a role in mitochondrial protein import. Further investigation of the proteins could help to understand correlations within these processes.

4.2. Editing of genes encoding potential substrates

Only for the gene *LtaP04.0660*, which encodes for substrate 1, homozygous insertion of the knock-out cassettes could be shown. This is a hint for the dispensability of the gene. For other potential substrates, information on gene essentiality can be derived from the tagging experiments. For none of the genes *LtaP25.1620*, *LtaP35.0210*, *LtaP19.1110* and *LtaP09.1390* (encoding for sTim1, substrate 2, substrate 3 and substrate 4) the homozygous insertion of the tagging cassette succeeded at both termini of the gene. This could be a hint for an impairment of the functionality of the proteins by the tagging. As discussed for the editing of the gene encoding Mic20, the aneuploidy of *Leishmania tarentolae* could lead to additional copies of essential genes in the case of manipulation and thus, to herterozygosity if the functions of the gene are affected.[5,87,101] Homozygous insertion of His_8-tagging cassettes was possible for *LtaP25.1620* at the N-terminus, for *LtaP35.0210* at the C-terminus and for *LtaP09.1390* at the N-terminus. These tagging variants may be less disruptive to the functions of the proteins.

4.3. Potential interactors of N-terminally His_8-tagged substrate 4

For the identification of further candidates for a Mia40 ortholog, potential substrates of this oxidoreductase are chosen as starting point. Tagging of these substrates is a central step before purification of possible intermediates formed with reaction partners. For most of the substrates, His_8-tagging cassettes could be introduced homozygously at the encoding genes at least at one terminus, and the N-terminally tagged substrate 4 could be observed in western blots. Thus, this construct is suitable for pull-down experiments, followed by mass spectrometry analysis to identify proteins in the eluates.

One limit of this method is the availability of complete protein sequences in data collections and their accessibility. Only completely and correctly assigned proteins can be found by comparison of peptides identified in mass spectrometry with data collections. The same applies for the assignment of properties to proteins. Screening for properties of proteins can only be successful if they are completely characterised.

4.3.1. Treatment of cells with azide, DTT or TMAD

Treatment of cells with azide did not lead to reproducible success in pull-down experiments. Finding optimal conditions such as duration of treatment or azide concentration could improve performance. For other additives, the treatment was repeatedly successful: cells treated with DTT and TMAD were used in pull-downs and the eluates obtained could be analysed by mass spectrometry. It should be noted, however, that the completeness of the reduction or oxidation has not been demonstrated. The exclusion of proteins found in cells reduced with DTT, for example, is therefore not justified, but the data can complement the results from experiments without additives and indicate the redox sensitivity of interactions. Data from different experiments could be combined to be more meaningful. A comparison of protein lists from different experiments can help to rank the significance of the proteins found. Promising candidates should be found reproducibly in independent experiments. For this purpose, not only the results of the present work were used, but also data from other sources. Turra performed pull-down experiments based on His_8-tagged Erv to find candidates for a Mia40 ortholog.[5] Candidates identified in these experiments were included in the evaluation. Two different data sets were considered: one derived from pull-downs of full-length His_8-tagged Erv and one from a truncated His_8-tagged Erv lacking the KISS domain.[42,43] The truncation of Erv could lead to altered kinetics of the protein's interactions, which is not yet fully understood.[5] This could also alter its interaction with a potential Mia40 ortholog. In *Trypanosomes*, a knock-down of Erv via ribonucleic acid interference (RNAi) was performed by Peikert et al.[7] Proteins affected by down regulation of Erv were also considered to be imported with involvement of Erv. It cannot be assumed that a Mia40 ortholog would also be imported via the Mia40/Erv pathway. Therefore, a Mia40 ortholog does not necessarily have to be included in the dataset of Peikert et al. In addition, the positions of the proteins were derived from tagging experiments in *Trypanosoma brucei*, which are available in the databases.[60,98] However, these data only provide an indication of the position of a protein in *Leishmania tarentolae*.

Figure 31 summarises different possible intersections of data from the present work and data from the Erv knock-down experiments of Peikert et al.[7]

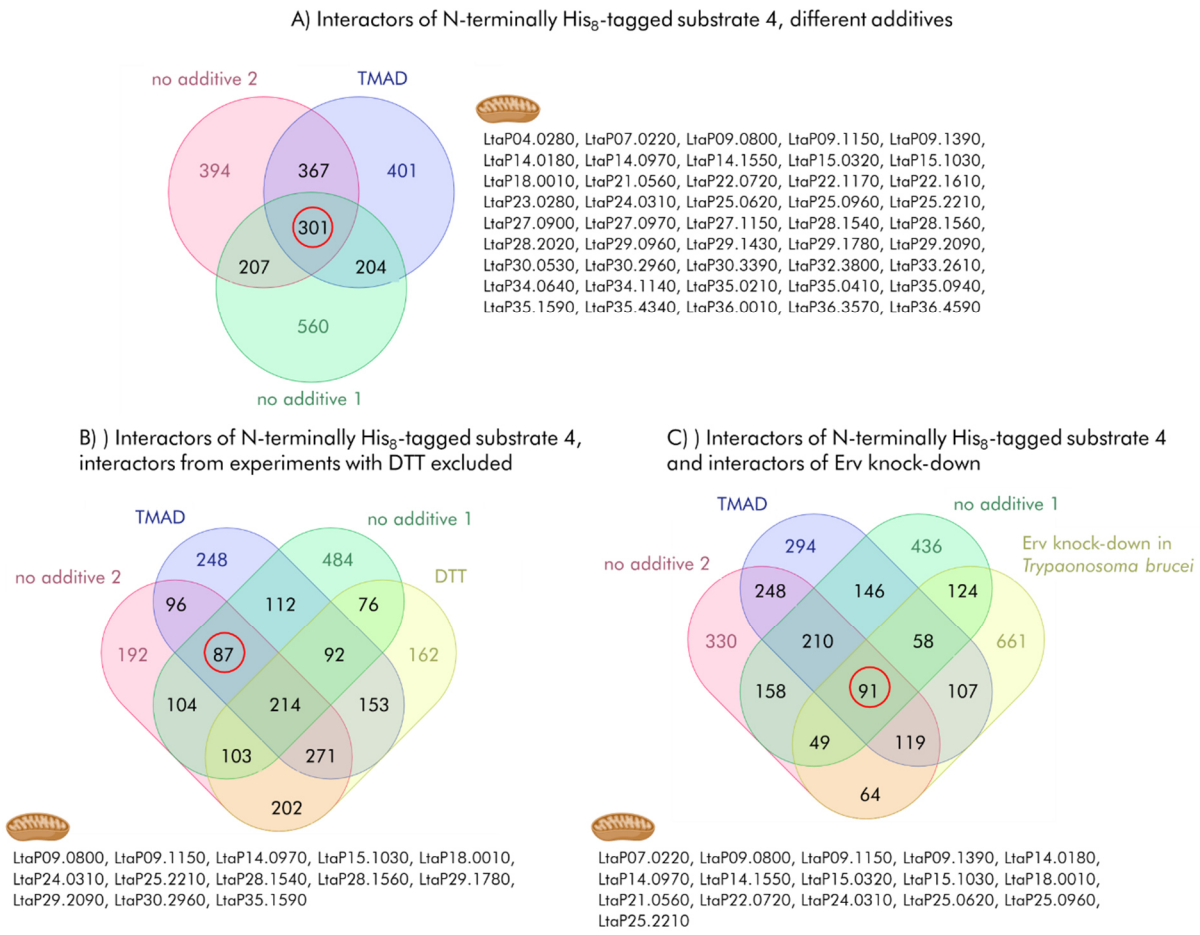


Figure 31: Candidates identified as potential interactors of N-terminally His8-tagged substrate 4 or of full-length Erv, considering import via Mia40/Erv pathway. The intersections of data from mass spectrometry of the present work or data from the literature were investigated.[7] Behind the mitochondrial symbol,[25] the proteins are listed that are potentially located in the mitochondrion and are assigned to the intersection with the red circle. The localisation data correspond to tagging experiments from orthologs in *Trypanosomes*.[60,98] The relative enrichment of the proteins comparing the parental strain and cells with His8-tagged substrate 4 are given in the supplements (S5). The venn diagrams were created with an online tool.[102]

Panel A) shows the intersection of proteins found as potential interactors of N-terminally His8-tagged substrate 4 in untreated cells and cells treated with oxidising TMAD. 301 proteins were identified in all three experiments. The localisation of these proteins was derived from tagging experiments of the corresponding ortholog proteins in *Trypanosoma brucei*.[60,98] Identification numbers of the 45 proteins found in all three experiments and potentially located in mitochondria are listed. In a second approach, proteins identified in experiments with reducing DTT were excluded (panel B). The list of the remaining proteins included only 13 proteins potentially located in the mitochondrion. It is assumed that the interactions of the excluded proteins with the N-terminally His8-tagged substrate 4 are very redox sensitive, whereas the interactions of the remaining proteins are less sensitive. In the third approach, proteins imported with involvement of Erv are highlighted, by considering

proteins affected by knock-down of Erv in *Trypanosoma brucei*. Orthologs of these proteins in *Leishmania tarentolae* are considered. Intersection of potential interactors of N-terminally His_8-tagged substrate 4 and proteins imported with involvement of Erv led to 91 proteins, 16 of them associated with the mitochondrion. Notably substrate 4 (LtaP09.1390) was found here because it was enriched in each pull-down, the import of its ortholog was related to Erv, and it is assumed to be localised in the mitochondrion. For an evaluation of the candidates, some properties of the selected proteins were considered. Among the proteins listed in panel A) (Figure 31) and thus interacting with His_8-tagged substrate 4 is one protein with CPC motif: LtaP35.1590, which is potentially located in the mitochondrion and thus a good candidate for the Mia40 ortholog. Function prediction is summarised in gene ontology terms, which are predicted from the sequences of the proteins and assign the same function to similar sequences (accessible via TriTrypDB[60]). For LtaP35.1590, function prediction yields oxidoreductase activity,[60] Figure 32 shows the amino acid sequence.

MLHRSFISAFQATRAARVSLVFKQLEGNAPLTKKNKPVNSWSDEFMKPPQSAEMTTKYGRYAKYSDPALCDVDTS
EEVVLNTYPDGAPQGRIEATAGVALKDYDASMWDEEFFRKHILKPKLADEVEDRARVTDYALNSAMLGFVILMARY
AVLPLWYVGQPAMSMVGQMNIEAEVGELDERQCTTVVWRGKPVFVYRRSARQMKEVTETPLSALKDPETDEARF
PDHRDKAVVIAICTHLGCVPIPNEGLFNGFFCPCHGSHYDPSGRIRQGPAPLNLEVPPYRWIDDNTIYMGKL

Figure 32: Amino acid sequence of protein LtaP35.1590.[60] Cysteines are highlighted in colour. Green: CPC, yellow: other cysteines. The protein was identified as potential interactor of N-terminally His_8-tagged substrate 4 (with or without addition of TMAD in the pull-down experiment). It exhibits a CPC motif, oxidoreductase activity is predicted and its ortholog in *Trypanosoma brucei* is possibly located in the mitochondrion.[60]

LtaP35.1590 exhibits a CPC motif and a CX_4C motif. Two further cysteine residues are present. In summary, LtaP35.1590 is a potential candidate for a Mia40 ortholog.

For two proteins that were identified as potential interactors of His_8-tagged substrate 4 (Figure 31), oxidoreductase activity is predicted: LtaP15.1030 and LtaP22.0720. Both exhibit single cysteines, but no motifs comparable to the functional sites of the Mia40 orthologs, such as a CPC, or also a CXXC motif as a redox active site or a twin CX_9C motif as part of the hydrophobic binding pocket. (A further discussion of possible motifs will follow in Chapter 4.3.5.) In general, for the discussion of the data, $CX_9CX_{3\text{-}100}CX_9C$ motifs were assumed to be possible twin CX_9C motifs. A comparison of candidate properties with other characteristics of Mia40 is given in the supplements (S5). Proteins that fulfil the same function could also have the same characteristics in terms of their structure or physical characteristics.

4.3.2. Potential interactors of Erv and ErvΔKISS

In the following, data from pull-down experiments of full-length Erv and Erv without KISS domain (ErvΔKISS) were considered. Intersections with potential interactors of N-terminally His_8-tagged substrate 4 were determined. Figure 33 shows candidates identified in the context of full-length Erv.

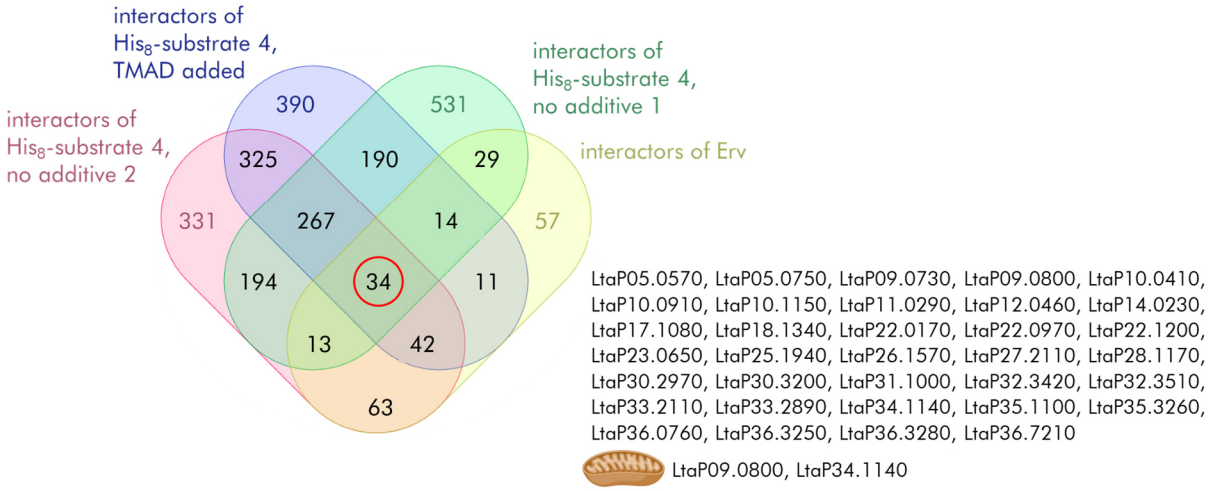


Figure 33: Candidates identified as potential interactors of N-terminally His_8-tagged substrate 4 or full-length Erv. The intersections of data from mass spectrometry from the present work or data from literature were investigated.[5] Behind the mitochondrial symbol,[25] the proteins are listed that are potentially located in the mitochondrion and are assigned to the intersection with the red circle. The localisation data correspond to tagging experiments from orthologs in *Trypanosomes*.[60,98] The amino acid sequences of potentially mitochondrial proteins are available in the supplements (S7). The relative enrichment of the proteins comparing the parental strain and cells with His_8-tagged substrate 4 are also given in the supplements (S5).The venn diagram was created with an online tool.[102]

34 proteins were identified, two of them were potentially found in the mitochondrion. None of these proteins exhibits a CPC motif. The only protein found with a twin CX_9C motif is LtaP36.3280. In Figure 34, additional proteins found in association with the truncated Erv[5] (lacking the KISS domain) and proteins whose orthologs are potentially imported involving Erv, as they were affected by an Erv knock-down in *Trypanosoma brucei*[7], were considered.

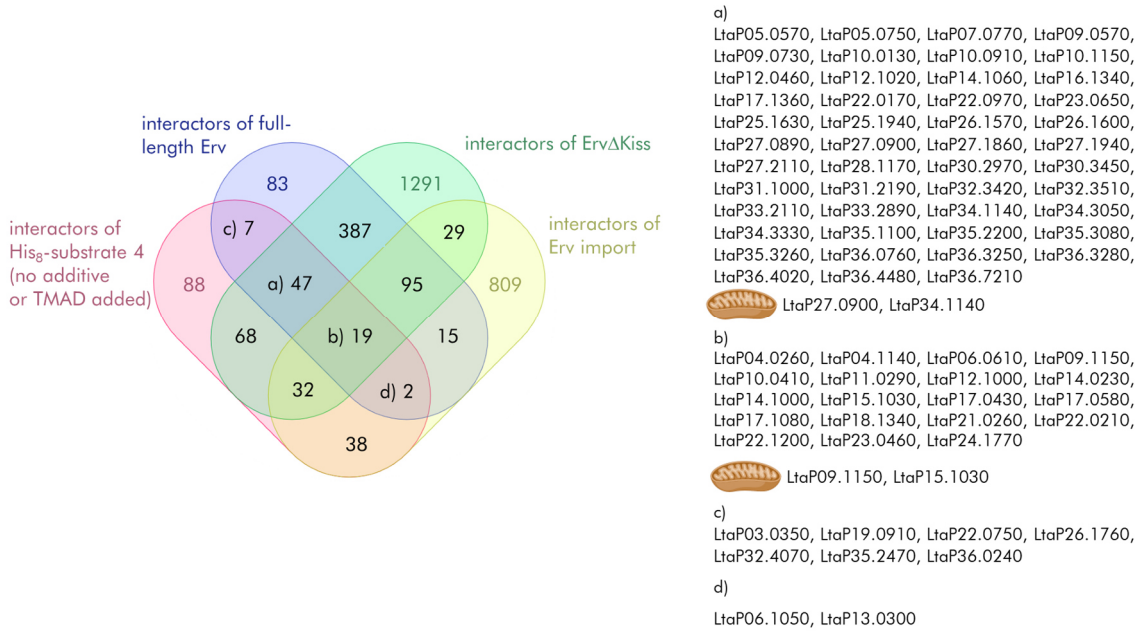


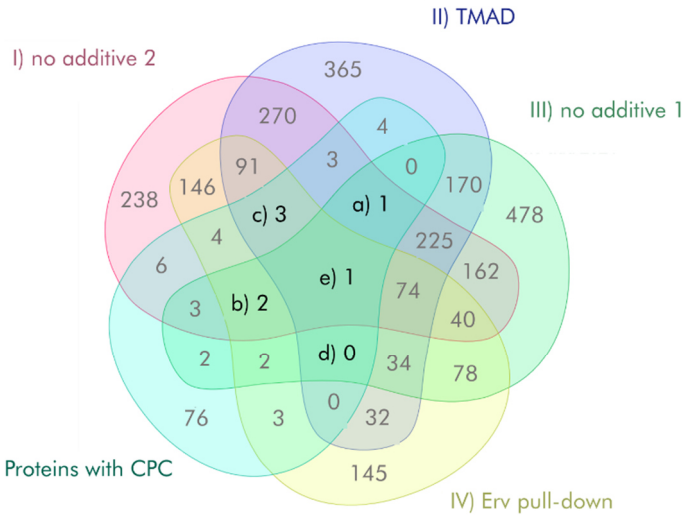
Figure 34: Candidates identified as potential interactors of N-terminally His_8-tagged substrate 4, full-length Erv, or truncated Erv.[5] Data were used from the present work (interactors of N-terminally His_8-tagged susbtrate 4), from Turra (full-length and truncated Erv)[5] and from Peikert et al. (Erv knock-down in *Trypanosoma brucei).*[7] The intersections of the data sets were investigated. Behind the mitochondrial symbols[25], proteins from the preceding lists are named, which are potentially located in the mitochondrion. The localisation data correspond to tagging experiments from orthologs in *Trypanosomes.*[60,98] The amino acid sequences of potentially mitochondrial proteins are available in the supplements (S7). The venn diagram was created with an online tool.[102]

The 47 proteins of list a) are potential interactors of N-terminally His_8-tagged substrate 4 and both versions of Erv, but their orthologs are not affected by Erv-knock down in *Trypanosomes*.[5,7] Thus, these proteins are not affected by the altered kinetics of Erv without KISS domain and are potentially not imported with the involvement of Erv. The proteins LtaP27.0900 and LtaP34.1140 are potentially located in the mitochondrion.[60] LtaP34.1140 has a CXXC motif, but no other motifs are present that are similar to the functional sites of known Mia40 orthologs. The protein LtaP30.2970 exhibits a CPC motif, but its sequence is not completely available (see the supplements, S7).[60] One protein in list a) exhibits a twin CX_9C motif: LtaP36.3280 (compare identification in Figure 33). The list b) contains 19 proteins that are potential interactors of N-terminally His_8-tagged substrate 4, and are affected by all experiments based on alteration of Erv.[5,7] In contrast to the proteins from a), these proteins are potentially imported with the involvement of Erv. Two proteins are potentially located in the mitochondrion. None of the proteins from b) exhibits a CPC motif. But LtaP23.0460 exhibits the motifs: CXC, CXXC and twin CX_9C. The amino acid sequences are available in the supplements (S7).[60] The seven proteins in list c) are not found in context of the truncated Erv[5] and are potentially not imported with the involvement of

Erv. None of them is potentially located in the mitochondrion or exhibits a CPC motif. In LtaP26.1760 and LtaP36.0240 a CX_9C motif was found.[60] List d) includes two proteins that could be imported with the involvement of Erv:[7] LtaP06.1050 (with a CXXC) and LtaP13.0300. These also show no potential position in the mitochondrion.[60]

4.3.3. Candidates with CPC motif

The next approach focuses even more on the cysteine motifs of the proteins. A specific search was conducted for proteins that have similar cysteine motifs to the known Mia40 orthologs. Since the active site of Mia40 is a CPC motif, the presence of one was chosen as the first criterion for this analysis. *Leishmania tarentolae* proteins were screened for CPC motifs. In the next step experiments based on His_8-tagged substrate 4 without additive or with TMAD and the experiment based on full-length Erv[42] were considered. CPC containing proteins were selected if they were also found in at least three other experiments (Figure 35).



a) proteins in I, II, and III	LtaP35.1590
b) proteins in I, III, and IV	LtaP14.1510, LtaP22.0060
c) proteins in I, II, and IV	LtaP05.0600, LtaP29.2460, LtaP30.2040
d) proteins in II, III, and IV	-
e) proteins in I, II, III and IV	LtaP30.2970

Figure 35: Candidates with CPC motif that are potential interactors of N-terminally His_8-tagged substrate 4 or full-length Erv. Proteins found in at least three experiments were considered. Potential interactors of N-terminally His_8-tagged substrate 4 (with or without addition of TMAD in pull-downs) identified in this work and potential interactors of full-length Erv from the work of Turra[5] were compared. The amino acid sequences of the proteins from a) – e) are available in the supplements (S7). The relative enrichment of the proteins comparing the parental strain and cells with His_8-tagged substrate 4 are also given in the supplements (S5).The venn diagram was created with an online tool.[102]

Proteins with CPC motif were identified using experiments with His_8-tagged substrate 4 and experiments from Turra[5]. Seven of them were found in at least three experiments. Selected properties of these proteins are listed in the supplements, S5, Table 9. Remarkably, the protein LtaP35.1590 was already discussed in Chapter 4.3.1. If the same data sets are considered with different focuses, it is possible to find the same proteins in several investigations. LtaP35.1590 is the only protein listed in Figure 35 whose *Trypanosoma* ortholog is potentially located in the mitochondrion.[60]

The next criterion that was chosen is the presence of a twin CX_9C motif known from Mia40 orthologs. Although the CPC motif of Mia40 contains the reactive cysteines, the twin CX_9C motif is also characteristic in Mia40. It could be vital for reactivity and functionality of the oxidoreductase. The protein encoded by *LtaP29.0620* with a CPC motif exhibits a twin CX_9C motif also and is found to be altered in Erv knock-down[7]. But it is not identified in the mass spectrometry analysis of the present work, or the work of Turra.[5] In both pull-downs without additives, the protein encoded by *LtaP22.0060* was found. The protein has two CPC motifs. However, many cysteine residues are present (Figure 36). This could be a contraindication for an efficient oxidoreductase.

MADGNGEDERSLRVADELRRETYECPSCLELVKLQQPIWSCQACFQIYHFACIRRWAQVDRDTV
VFSCPQCRHMQPKPLTDLCFCGKVSKPKYDPLVTPHSCGRLCGRVRPFCTHRCPVQCHPGPCPR
CQLMVGPQRCPCGTTTYTYPCGQPDPETTCDHPCRRPLACGTHMCLLSCHTGPCPPCSESTTLI
CYCGQTTKQHPCTKETSFACGSVCGKTLRCGEHKCTLLCHSGECPPCPTDPATVHTCPCGASE
LQTVRYACTDPIPKCGRICSKLLSCGQHHCQLQCHSGTCPPCEVRVDVSCRCRKVRKRLSCAES
QNFTCTYECGTKLSCGRHKCKVICCQDRNKTQTNSHMCFQVCGRPLPCGHTCEDLCHASTQ
CPPCVHVVTETLTCYCGAEVLRPPQQCGTQPPVCKRACRIPRPCGHPVGHNCHFGPCPPCGVP
VKRRCPRHNTLVMLSCGVTDLACEDECGMEMPCGHFCNRVCHSGPCLEEANPCRQQCDRLH
EECGHRCAKPCHSTTPCPPCSVYLRCTCNCGRVTRSLPCAMVGKRKAEGPNKFAVVVPCDNDCL
FTRRLDVLTSLSKTKNEKFLYSLMLWDAAQQKASGVKRVESQLMSFVEGNDSVISLPPANSETRALV
HALAKYFHVRSESVDNEPNRSCLLTKTGDTAAPPVLLSDAVRNSQMDPLQFLMQCAKPSVKKKLC
LVITGHHVTEIMLTSLLSDLAGRFVIAPPEVGKDGAQSFLIAFTTHKRAEEAVKKLEAGNTQHTFSVA
RPTV

Figure 36: Amino acid sequence of protein LtaP22.0060.[60] The cysteines are highlighted in yellow. Highlighted in red are those that determine the $CX_9CX_{12}CX_9C$ motif, in green the CPC motifs. The protein was identified as a CPC containing protein,[60] that is a potential interactor of N-terminally His_8-tagged substrate 4 (with or without addition of TMAD in pull-down; data from the present work) and of full-length Erv (data from Turra[5]).

The protein LtaP22.0060 shows similarities to cysteine motifs of Mia40. However, it is not known whether the ortholog oxidises proteins with a similar mechanism as Mia40 and whether cysteine motifs in a potential ortholog are similar. Function prediction for

LtaP22.0060 yields DNA-binding transcription factor activity and zinc ion binding, but no oxidoreductase activity or disulphide related activity.[60]

Besides the CPC motif known from Mia40 orthologs, other motifs can also be considered as redox-active sites. CXXC motifs, as found in thioredoxins, were also searched for. A list of the proteins found is available in the supplements (S8). For the proteins LtaP36.7210 and LtaP16.1340 disulphide isomerase activity or oxidoreductase activity are predicted respectively.

4.3.4. Potential interactors of N-terminally His_8-tagged substrate 4 with twin CX_9C motif

Proteins with twin CX_9C motif were also specifically searched for. As in the entire discussion, motifs with 3 to 100 amino acids between the two CX_9C motifs were selected. Figure 37 shows the number of proteins in the intersection of potential interactors of the N-terminally His_8-tagged substrate 4 with proteins containing such motifs.

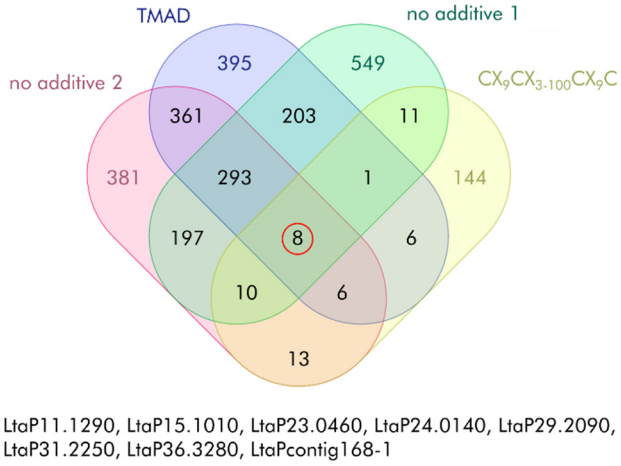


Figure 37: Candidates with twin CX_9C motif that are potential interactors of N-terminally His_8-tagged substrate 4. Potential interactors of N-terminally His_8-tagged substrate 4 (with or without addition of TMAD in pull-downs) were identified in this work. Proteins with twin CX_9C motifs were selected from the database.[60] The venn diagram was created with an online tool.[102]

Eight proteins are reproducibly present in all datasets, none of them exhibits a CPC motif. One protein is possibly located in the mitochondrion: LtaP29.2090. CXXC motifs are found in all proteins except LtaP24.0140 and LtaP31.2250. For none of the proteins, oxidoreductase activity is predicted. Two proteins were already discussed in Chapter 4.3.2: LtaP36.3280 and LtaP23.0460 are both potential interactors of the truncated Erv.[5]

LtaP36.3280 also potentially interacts with the full-length Erv[5] and LtaP23.0460 with the import via an involvement of Erv.[7] The amino acid sequences of the proteins are available in the supplements (S7). The data there also show that LtaP11.1290 is a very cysteine-rich protein. It is therefore assumed that it is less suitable as a candidate for a Mia40 ortholog.
To confirm any candidate it might be helpful to perform knock-out experiments as well as introduce point mutations at the CPC motif, if present. Also the redox potential or the redox state *in vivo* should be determined. Furthermore it would be essential to show disulphide exchange with substrate proteins.
In the preceding chapters, intersections of several experiments identified potential interactors of substrate 4 and Erv. Full-length Erv and Erv lacking the KISS domain were both considered. The import via an Erv related pathway was optionally considered. Candidates for a Mia40 ortholog were identified in all combinations. If a similar mechanism to the known Mia40/Erv pathway underlies, similar protein properties or amino acid motifs could possibly be found. Candidates with CPC motif, or with twin CX_9C motif were specifically identified. In the following chapter, the data of the present work will be compared in detail with the current literature.

4.3.5. Discussion of the Mia40 models from the current literature

As a candidate from *Trypanosoma brucei*, Mic20 was identified, but is contradicted by recent data.[1–4,41] The protein exhibits a CXXC motif, as known from thioredoxins, which are responsible for redox reactions of cysteines. Looking for thioredoxin domain-containing proteins in the data of the present work, several proteins were identified (see also Table 5). Whether thioredoxin-like proteins are good candidates as Mia40 orthologs also remains questionable because of the redox state of the proteins.[41]
The functional features of the known Mia40 orthologs include a C-terminal hydrophobic binding pocket (with a twin CX_9C motif-containing domain) responsible for the binding of the substrates and the N-terminal domain containing a CPC motif responsible for the oxidation of substrates.[52,103] Hydrophobic targeting signals in the substrate proteins interact with the binding pocket.[104,105]
The import mechanism of proteins using Mia40 was previously described as a folding-trap mechanism,[50,51] which is driven by the oxidation of the substrates. Later findings suggest

that the binding of the substrates is the driving force for the import.[52] This trans-site receptor model assumes that the CPC motif is dispensable for the import of substrates. However, to enable correct protein folding, the hydrophobic binding pocket needs to be in close proximity to the CPC motif.[52] Other data suggest that the C-terminal domain of Mia40 is essential for the import of Mia40 itself, but dispensable for its redox function.[103] By comparing the findings from the literature with the data of the present work, three models for the functional motifs of a Mia40 ortholog in *Leishmania tarentolae* were derived (Figure 38).

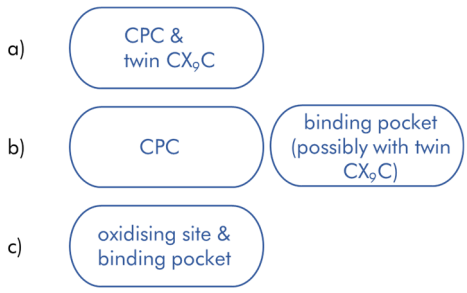


Figure 38: Models for the functional motifs of a Mia40 ortholog in *Leishmania tarentolae* that were derived from the findings of the present work and the literature.[42,50–52] Panel a) represents a protein with a CPC and a twin CX_9C motif, panel b) two proteins in which the functions are separated and panel c) a protein in which the oxidising site and the binding pocket do not match those of known Mia40 orthologs.

Scenario a) assumes that both critical motifs known from Mia40, the CPC motif and the twin CX_9C motif as part of a hydrophobic binding pocket, are present in the ortholog. The close resemblance to known orthologs of Mia40 would suggest a similar import mechanism, consistent with the presence of orthologs for Erv or the substrates in *Leishmania tarentolae*. On the other hand, no protein in *Leishmania tarentolae* has the otherwise highly conserved motifs of the functional sites (see the supplements, S1). The one protein found as a candidate in the present work that combines both motifs is LtaP22.0060. However, due to its high cysteine content (see Chapter 4.3.3), this protein is unlikely to be a good candidate. The specific and thus efficient reaction with the cysteine residues of the CPC motif could be impaired.

In scenario b) the main functions of Mia40 are assumed to be separated in *Leishmania tarentolae*: one protein carries the CPC motif, another protein a hydrophobic binding pocket, which could be present with a twin CX_9C motif. This is one possible explanation for the fact that no candidate has yet been identified that combines both functional motifs. In the literature the essentiality of the individual motifs[52,103–105] is also discussed, so that a division

of the functions would be conceivable. For example, both proteins could be the subunits of a non-covalently bound complex, so that both functional sites are in close proximity. In agreement with the results of Turra et al.,[42] a direct interaction between Erv and the substrates of the Mia40 orthologs would not take place. An imported protein would be bound non-covalently by the protein with the hydrophobic pocket, then a second protein with CPC motif would interact as oxidant to form the disulphide bridges in the substrate. The oxidant protein itself would be reoxidised by Erv. It remains questionable whether a protein whose binding pocket interacts non-covalently with a substrate can be co-purified with it in the experiments of the present work. Nevertheless, the data from the present work could also be screened for potential interactors of N-terminally His_8-tagged substrate 4 without CPC motif but with a hydrophobic pocket possibly containing a twin CX_9C motif. The only protein from *Leishmania tarentolae* with a $CX_9CX_{12}CX_9C$ motif that was identified as interactor of N-terminally His_8-tagged substrate 4 in the present work is LtaP22.0060 (see also the supplements, S7. For a more general list with potential interactors of N-terminally His_8-tagged substrate 4 with $CX_9CX_{3\text{-}100}CX_9C$ motifs see Chapter 4.3.4). Proteins with CPC motif that were identified are good candidates for the oxidising unit if they also interact with Erv (Chapter 4.3.3, summary in Chapter 5).

The third scenario (Figure 38 c) assumes that the oxidative centre and the binding pocket are present in the same protein. Neither the amino acid sequence of the oxidising site nor that of the binding pocket must necessarily show similarities with those from the known Mia40 orthologs. This could be another explanation for the lack of candidates with CPC and twin CX_9C motifs. For example CXXC motifs are known to function as thiol-disulphide oxidoreductases.[106] The only reproducibly found candidate with CXXC motif and twin CX_9C motif was LtaP29.2090 (see the supplements, S7, S5), which was found as potential interactor of N-terminally His_8-tagged substrate 4 (with and without TMAD in pull-downs) and as potential interactor of the truncated Erv.[42]

A table summarising all the candidates discussed and assigning them to a model for the functional motifs (from Figure 38) is available in Chapter 5 (Table 5). A more general screening of candidates including different possible functional motifs is also indicated for future studies.

5. Summary

It is known that oxidative folding of proteins after mitochondrial import follows the Mia40/Erv pathway in many organisms. In kinetoplastida, such as the parasite *Leishmania tarentolae*, the homolog of Erv is known, but no homolog for Mia40 could be identified yet. To approach this oxidoreductase from different starting points was the task of the current work. The first attempt was to investigate known candidates for a Mia40 ortholog in kinetoplastida. When the present work was started, Mic20 was considered a candidate,[1–3] but is currently excluded.[41] Nevertheless, Mic20 is a protein of interest in the context of the MICOS complex. Chromosomal gene editing was used to investigate the role of cysteine residues in the protein. Since knock-out of the gene *LtaPh_3313851*, which encodes Mic20, was not possible and insertion of the mutation cassettes to replace the codons for cysteines C82 and C85 resulted in heterozygous genes, it is assumed that *LtaPh_3313851* and the redox activity of the corresponding cysteines are essential.

Another approach to the unknown oxidoreductase was performed by Turra,[5] and is based on the reaction partner of Mia40, the sulfhydryl oxidase Erv. Two proteins of unknown function were identified by Turra and knock-out attempts were made. The knock-out of gene *LtaP32.0380* (encoding for UF2) was considered homozygous and the gene dispensable. UF2 exhibits motifs containing CX_2C sequences. Insertion of the knock-out cassettes for *LtaP07.0980* (encoding for UF3) was shown to be heterozygous. UF3 has a twin CX_8CC motif. Further studies on Mic20 or UF2 and UF3 could include knock-out attempts of the corresponding gene in combination with the introduction of episomal rescue plasmids. Existing heterozygous clones could be investigated for the presence of alterations of the protospacer adjacent motif sequences.

In the present work, the approach to find candidates for a Mia40 ortholog was based on its potential substrates. These were identified in the work of Liedgens.[6] Based on this selection, chromosomal tagging and knock-out attempts were performed. The gene *LtaP04.0660* (encoding for substrate 1) could be knocked-out and N-terminally edited homozygously. Thus, the gene is assumed to be dispensable. For the gene *LtaP35.0210* (encoding for substrate 2), no gene editing could be completed homozygously. This could be a hint for the essentiality of the gene. *LtaP19.1110* (encoding for substrate 3) could be edited at the

C-terminus to obtain a homozygous insertion, *LtaP09.1390* (encoding for substrate 4) at the N-terminus. The presence of the His_8-tag could be confirmed for substrate 4 and the correct position of the tagged protein was assumed. Eluates from pull-down experiments were investigated via mass spectrometry, comparing parental cells with cells exhibiting the N-terminal His_8-tag at substrate 4. For example, LtaP35.1590 was identified as one promising candidate for an ortholog of Mia40. The candidate was found as potential interactor of N-terminally His_8-tagged substrate 4 with a CPC motif. LtaP35.1590 is potentially located in the mitochondrion and oxidoreductase activity was predicted. The current work provided proteins potentially interacting with a substrate of a Mia40 ortholog, whereas data from Turra et al.[42] provided potential interactors of Erv and an Erv knock-down by Peikert et al.[7] showed proteins potentially imported with involvement of Erv. In addition, a targeted search for characteristic cysteine motifs of Mia40 was conducted. The intersection of the findings from all these data yielded further promising candidates for a Mia40 ortholog. For example, the cysteine-rich LtaP22.0060 was identified, because it exhibits a CPC and a twin CX_9C motif and is a potential interactor of N-terminally His_8-tagged substrate 4 and of full-length Erv. One candidate with the alternative active site CXXC and a twin CX_9C motif is LtaP29.2090. The protein was identified as potential interactor of N-terminally His_8-tagged substrate 4 and truncated Erv^5 and is potentially located in the mitochondrion. Many further candidates with different promising cysteine motifs have been identified (see Table 5).

Table 5: Summary of the candidates identified in the discussion (Chapter 4). The cysteine motifs that were assumed to be potential functional sites are indicated. Potential interactors correspond to the data from the present work (N-terminally His_8-tagged substrate 4), data from Turra et al. (full-length and truncated Erv)[5] and data from Peikert et al. (Erv knock-down in *Trypanosoma brucei*)[7]. Prediction of the mitochondrial position is derived from experimental data from *Trypanosoma brucei*.[60] Function prediction is derived from gene ontology terms.[60] The models refer to Figure 38: Candidates with CPC and twin CX_9C motif (a), Candidates with CPC or CXXC motif and candidates with twin CX_9C motif (b) and candidates with CXXC and twin CX_9C motif (c).

Protein ID	Motifs	Potential interactors	Predicted mitochondrial position of the ortholog in *Trypanosoma*	Comments	Model for which the protein is suitable (Figure 38)
LtaP05.0600	CPC	Erv full-length, N-terminally His_8-tagged substrate 4	no		b
LtaP06.1050	CXXC	Erv full-length, Erv knock-down, N-terminally His_8-tagged substrate 4	no		b
LtaP07.0980 (UF3)	twin CX_8CC	Erv full-length, Erv truncated	no	gene assumed to be essential	b

Protein ID	Motifs	Potential interactors	Predicted mitochondrial position of the ortholog in *Trypanosoma*	Comments	Model for which the protein is suitable (Figure 38)
LtaP11.1290	twin CX_9C, CXXC	N-terminally His_8-tagged substrate 4	no	cysteine-rich protein	c
LtaP14.1510	CPC	Erv full-length, N-terminally His_8-tagged substrate 4	no		b
LtaP15.1010	twin CX_9C, CXXC	N-terminally His_8-tagged substrate 4	no		c
LtaP16.1340	CXXC	Erv full-length, Erv truncated, N-terminally His_8-tagged substrate 4	no	oxidoreductase activity is predicted	b
LtaP22.0060	CPC, twin CX_9C	Erv full-length, N-terminally His_8-tagged substrate 4	no	cysteine-rich protein	a
LtaP23.0460	twin CX_9C, CXXC	Erv truncated, Erv knock-down, N-terminally His_8-tagged substrate 4	no		c
LtaP24.0140	twin CX_9C	N-terminally His_8-tagged substrate 4	no		b
LtaP29.0620	CPC, twin CX_9C	Erv knock-down	no		a
LtaP29.2090	twin CX_9C, CXXC	N-terminally His_8-tagged substrate 4	yes		c
LtaP29.2460	CPC	Erv full-length, N-terminally His_8-tagged substrate 4	no		b
LtaP30.2040	CPC	Erv full-length, N-terminally His_8-tagged substrate 4	no		b
LtaP30.2970	CPC	Erv full-length, N-terminally His_8-tagged substrate 4	no	sequence not completely available	b
LtaP31.2250	twin CX_9C	N-terminally His_8-tagged substrate 4	no		b
LtaP32.0380 (UF2)	CXXC	Erv full-length, Erv truncated	no	gene assumed to be dispensable	b
LtaP34.1140	CXXC	Erv full-length, Erv truncated, N-terminally His_8-tagged substrate 4	yes		b
LtaP35.1590	CPC	N-terminally His_8-tagged substrate 4	yes	oxidoreductase activity predicted	b
LtaP35.3530	CPC	N-terminally His_8-tagged substrate 4	no		b
LtaP36.3280	twin CX_9C, CXXC	Erv full-length, Erv truncated, N-terminally His_8-tagged substrate 4	no		c
LtaP36.7210	CXXC	Erv full-length, Erv truncated, N-terminally His_8-tagged substrate 4	no	disulphide isomerase activity predicted	b
LtaPcontig168-1	twin CX_9C, CXXC	N-terminally His_8-tagged substrate 4	no		c

Confirmation of all the candidates is now pending. Gene editing such as knock-outs or mutations at sequences coding for the CPC or twin CX_9C motifs or other potentially reactive or vital sites could provide information about their essentiality. Showing disulphide exchange reactions with substrate proteins, for example by *in vitro* reconstitution assays,

would be a possible confirmation. In addition, it could be useful to search not for specific amino acid motifs such as CPC or twin CX_9C, but for functional units such as an oxidising site and a hydrophobic binding pocket.

6. References

(1) Schneider, A. Evolution of Mitochondrial Protein Import – Lessons from Trypanosomes. *Biol. Chem.* **2020**, *401* (6–7), 663–676. https://doi.org/10.1515/hsz-2019-0444.

(2) Kaurov, I.; Vancová, M.; Schimanski, B.; Cadena, L. R.; Heller, J.; Bílý, T.; Potěšil, D.; Eichenberger, C.; Bruce, H.; Oeljeklaus, S.; Warscheid, B.; Zdráhal, Z.; Schneider, A.; Lukeš, J.; Hashimi, H. The Diverged Trypanosome MICOS Complex as a Hub for Mitochondrial Cristae Shaping and Protein Import. *Curr. Biol.* **2018**, *28* (21), 3393-3407.e5. https://doi.org/10.1016/j.cub.2018.09.008.

(3) Eichenberger, C.; Oeljeklaus, S.; Bruggisser, J.; Mani, J.; Haenni, B.; Kaurov, I.; Niemann, M.; Zuber, B.; Lukeš, J.; Hashimi, H.; Warscheid, B.; Schimanski, B.; Schneider, A. The Highly Diverged Trypanosomal MICOS Complex Is Organized in a Nonessential Integral Membrane and an Essential Peripheral Module. *Mol. Microbiol.* **2019**, *112* (6), 1731–1743. https://doi.org/10.1111/mmi.14389.

(4) Hammond, M. J.; Nenarokova, A.; Butenko, A.; Zoltner, M.; Dobáková, E. L.; Field, M. C.; Lukeš, J. A Uniquely Complex Mitochondrial Proteome from Euglena Gracilis. *Mol. Biol. Evol.* **2020**, *37* (8), 2173–2191. https://doi.org/10.1093/molbev/msaa061.

(5) Turra, Gino Lucas, G. L. Expanding the Genetic Toolbox of Leishmania Tarentolae — Novel Techniques for the Analysis of Mitochondrial Protein Import in Kinetoplastid Parasites, University of Kaiserslautern, Kaiserslautern, 2020.

(6) Liedgens, L. Investigation of Oxidative Protein Folding in Protist Mitochondria and Elucidation of the Catalytic Mechanism of Glutaredoxins. **2018**. https://doi.org/10.11588/HEIDOK.00025426.

(7) Peikert, C. D.; Mani, J.; Morgenstern, M.; Käser, S.; Knapp, B.; Wenger, C.; Harsman, A.; Oeljeklaus, S.; Schneider, A.; Warscheid, B. Charting Organellar Importomes by Quantitative Mass Spectrometry. *Nat. Commun.* **2017**, *8* (1), 15272. https://doi.org/10.1038/ncomms15272.

(8) Nomenclature and Symbolism for Amino Acids and Peptides (Recommendations 1983). *Pure Appl. Chem.* **1984**, *56* (5), 595–624. https://doi.org/10.1351/pac198456050595.

(9) IUPAC-IUB Comm. on Biochem. Nomenclature (CBN). Abbreviations and Symbols for Nucleic Acids, Polynucleotides, and Their Constituents. *Biochemistry* **1970**, *9* (20), 4022–4027. https://doi.org/10.1021/bi00822a023.

(10) Campbell, N. A.; Urry, L. A.; Cain, M. L.; Wasserman, S. A.; Minorsky, P. V.; Reece, J. B. In *Campbell Biologie, Chapter 6*; Paululat, A., Heinisch, J. J., Eds.; bio - Biologie; Pearson: Hallbergmoos, 2019.

(11) Hjort, K.; Goldberg, A. V.; Tsaousis, A. D.; Hirt, R. P.; Embley, T. M. Diversity and Reductive Evolution of Mitochondria among Microbial Eukaryotes. *Philos. Trans. R. Soc. B Biol. Sci.* **2010**, *365* (1541), 713–727. https://doi.org/10.1098/rstb.2009.0224.

(12) Henze, K.; Martin, W. Essence of Mitochondria. *Nature* **2003**, *426* (6963), 127–128. https://doi.org/10.1038/426127a.

(13) Lill, R.; Freibert, S.-A. Mechanisms of Mitochondrial Iron-Sulfur Protein Biogenesis. *Annu. Rev. Biochem.* **2020**, *89* (1), 471–499. https://doi.org/10.1146/annurev-biochem-013118-111540.
(14) Rouault, T. A.; Tong, W.-H. Iron–Sulphur Cluster Biogenesis and Mitochondrial Iron Homeostasis. *Nat. Rev. Mol. Cell Biol.* **2005**, *6* (4), 345–351. https://doi.org/10.1038/nrm1620.
(15) Bonora, M.; Patergnani, S.; Rimessi, A.; De Marchi, E.; Suski, J. M.; Bononi, A.; Giorgi, C.; Marchi, S.; Missiroli, S.; Poletti, F.; Wieckowski, M. R.; Pinton, P. ATP Synthesis and Storage. *Purinergic Signal.* **2012**, *8* (3), 343–357. https://doi.org/10.1007/s11302-012-9305-8.
(16) de Souza, W.; Attias, M.; Rodrigues, J. C. F. Particularities of Mitochondrial Structure in Parasitic Protists (Apicomplexa and Kinetoplastida). *Int. J. Biochem. Cell Biol.* **2009**, *41* (10), 2069–2080. https://doi.org/10.1016/j.biocel.2009.04.007.
(17) Cavalier-Smith, T. Symbiogenesis: Mechanisms, Evolutionary Consequences, and Systematic Implications. *Annu. Rev. Ecol. Evol. Syst.* **2013**, *44* (1), 145–172. https://doi.org/10.1146/annurev-ecolsys-110411-160320.
(18) Speijer, D. Birth of the Eukaryotes by a Set of Reactive Innovations: New Insights Force Us to Relinquish Gradual Models. *BioEssays* **2015**, *37* (12), 1268–1276. https://doi.org/10.1002/bies.201500107.
(19) Mereschkowsky, C. Theorie Der Zwei Plasmaarten Als Grundlage Der Symbiogenesis, Einer Neuen Lehre von Der Entstehung Der Organismen. *Biol Cent.* **1910**, *30*, 278–303.
(20) Gray, M. W. Mitochondrial Evolution. *Cold Spring Harb. Perspect. Biol.* **2012**, *4* (9), a011403–a011403. https://doi.org/10.1101/cshperspect.a011403.
(21) Karp, G. In *Molekulare Zellbiologie, Chapter 5*; Beginnen, K., Vogel, S., Kuhlmann-Krieg, S., Translators; Springer-Lehrbuch; Springer: Berlin Heidelberg, 2005.
(22) Watt, I. N.; Montgomery, M. G.; Runswick, M. J.; Leslie, A. G. W.; Walker, J. E. Bioenergetic Cost of Making an Adenosine Triphosphate Molecule in Animal Mitochondria. *Proc. Natl. Acad. Sci.* **2010**, *107* (39), 16823–16827. https://doi.org/10.1073/pnas.1011099107.
(23) Klingenberg, M. The ADP and ATP Transport in Mitochondria and Its Carrier. *Biochim. Biophys. Acta BBA - Biomembr.* **2008**, *1778* (10), 1978–2021. https://doi.org/10.1016/j.bbamem.2008.04.011.
(24) Morelli, A. M.; Ravera, S.; Panfoli, I. The Aerobic Mitochondrial ATP Synthesis from a Comprehensive Point of View. *Open Biol.* **2020**, *10* (10), 200224. https://doi.org/10.1098/rsob.200224.
(25) *Biorender*. https://biorender.com/.
(26) Campbell, N. A.; Urry, L. A.; Cain, M. L.; Wasserman, S. A.; Minorsky, P. V.; Reece, J. B. *Campbell Biologie*, 11., aktualisierte Auflage.; Paululat, A., Heinisch, J. J., Eds.; bio - Biologie; Pearson: Hallbergmoos, 2019.
(27) Gordaliza-Alaguero, I.; Cantó, C.; Zorzano, A. Metabolic Implications of Organelle–Mitochondria Communication. *EMBO Rep.* **2019**, *20* (9). https://doi.org/10.15252/embr.201947928.
(28) Bereiter-Hahn, J.; Vöth, M. Dynamics of Mitochondria in Living Cells: Shape Changes, Dislocations, Fusion, and Fission of Mitochondria: DYNAMICS OF MITOCHONDRIA. *Microsc. Res. Tech.* **1994**, *27* (3), 198–219. https://doi.org/10.1002/jemt.1070270303.

(29) Calvo, S. E.; Mootha, V. K. The Mitochondrial Proteome and Human Disease. *Annu. Rev. Genomics Hum. Genet.* **2010**, *11*, 25–44. https://doi.org/10.1146/annurev-genom-082509-141720.
(30) Dickson-Murray, E.; Nedara, K.; Modjtahedi, N.; Tokatlidis, K. The Mia40/CHCHD4 Oxidative Folding System: Redox Regulation and Signaling in the Mitochondrial Intermembrane Space. *Antioxidants* **2021**, *10* (4), 592. https://doi.org/10.3390/antiox10040592.
(31) Hauser, R.; Pypaert, M.; Hausler, T.; Horn, E. K.; Schneider, A. In Vitro Import of Proteins into Mitochondria of Trypanosoma Brucei and Leishmania Tarentolae. *J. Cell Sci.* **1996**, *109* (2), 517–523. https://doi.org/10.1242/jcs.109.2.517.
(32) Chacinska, A.; Koehler, C. M.; Milenkovic, D.; Lithgow, T.; Pfanner, N. Importing Mitochondrial Proteins: Machineries and Mechanisms. *Cell* **2009**, *138* (4), 628–644. https://doi.org/10.1016/j.cell.2009.08.005.
(33) Rissler, M.; Wiedemann, N.; Pfannschmidt, S.; Gabriel, K.; Guiard, B.; Pfanner, N.; Chacinska, A. The Essential Mitochondrial Protein Erv1 Cooperates with Mia40 in Biogenesis of Intermembrane Space Proteins. *J. Mol. Biol.* **2005**, *353* (3), 485–492. https://doi.org/10.1016/j.jmb.2005.08.051.
(34) Wrobel, L.; Trojanowska, A.; Sztolsztener, M. E.; Chacinska, A. Mitochondrial Protein Import: Mia40 Facilitates Tim22 Translocation into the Inner Membrane of Mitochondria. *Mol. Biol. Cell* **2013**, *24* (5), 543–554. https://doi.org/10.1091/mbc.e12-09-0649.
(35) Neupert, W.; Herrmann, J. M. Translocation of Proteins into Mitochondria. *Annu. Rev. Biochem.* **2007**, *76* (1), 723–749. https://doi.org/10.1146/annurev.biochem.76.052705.163409.
(36) Specht, S.; Liedgens, L.; Duarte, M.; Stiegler, A.; Wirth, U.; Eberhardt, M.; Tomás, A.; Hell, K.; Deponte, M. A Single-Cysteine Mutant and Chimeras of Essential Leishmania Erv Can Complement the Loss of Erv1 but Not of Mia40 in Yeast. *Redox Biol.* **2018**, *15*, 363–374. https://doi.org/10.1016/j.redox.2017.12.010.
(37) Eckers, E.; Cyrklaff, M.; Simpson, L.; Deponte, M. Mitochondrial Protein Import Pathways Are Functionally Conserved among Eukaryotes despite Compositional Diversity of the Import Machineries. *Biol. Chem.* **2012**, *393* (6), 513–524. https://doi.org/10.1515/hsz-2011-0255.
(38) Banci, L.; Bertini, I.; Cefaro, C.; Ciofi-Baffoni, S.; Gallo, A.; Martinelli, M.; Sideris, D. P.; Katrakili, N.; Tokatlidis, K. MIA40 Is an Oxidoreductase That Catalyzes Oxidative Protein Folding in Mitochondria. *Nat. Struct. Mol. Biol.* **2009**, *16* (2), 198–206. https://doi.org/10.1038/nsmb.1553.
(39) Herrmann, J. M.; Riemer, J. Mitochondrial Disulfide Relay: Redox-Regulated Protein Import into the Intermembrane Space. *J. Biol. Chem.* **2012**, *287* (7), 4426–4433. https://doi.org/10.1074/jbc.R111.270678.
(40) Chacinska, A.; Pfannschmidt, S.; Wiedemann, N.; Kozjak, V.; Sanjuán Szklarz, L. K.; Schulze-Specking, A.; Truscott, K. N.; Guiard, B.; Meisinger, C.; Pfanner, N. Essential Role of Mia40 in Import and Assembly of Mitochondrial Intermembrane Space Proteins. *EMBO J.* **2004**, *23* (19), 3735–3746. https://doi.org/10.1038/sj.emboj.7600389.
(41) Kaurov, I.; Heller, J.; Deisenhammer, S.; Potěšil, D.; Zdráhal, Z.; Hashimi, H. The Essential Cysteines in the CIPC Motif of the Thioredoxin-like Trypanosoma Brucei MICOS Subunit TbMic20 Do Not Form an Intramolecular Disulfide Bridge in Vivo.

Mol. Biochem. Parasitol. **2022**, *248*, 111463. https://doi.org/10.1016/j.molbiopara.2022.111463.

(42) Turra, G. L.; Liedgens, L.; Sommer, F.; Schneider, L.; Zimmer, D.; Vilurbina Perez, J.; Koncarevic, S.; Schroda, M.; Mühlhaus, T.; Deponte, M. *In Vivo* Structure-Function Analysis and Redox Interactomes of Leishmania Tarentolae Erv. *Microbiol. Spectr.* **2021**, *9* (2), e00809-21. https://doi.org/10.1128/Spectrum.00809-21.

(43) Eckers, E.; Petrungaro, C.; Gross, D.; Riemer, J.; Hell, K.; Deponte, M. Divergent Molecular Evolution of the Mitochondrial Sulfhydryl:Cytochrome c Oxidoreductase Erv in Opisthokonts and Parasitic Protists. *J. Biol. Chem.* **2013**, *288* (4), 2676–2688. https://doi.org/10.1074/jbc.M112.420745.

(44) Basu, S.; Leonard, J. C.; Desai, N.; Mavridou, D. A. I.; Tang, K. H.; Goddard, A. D.; Ginger, M. L.; Lukeš, J.; Allen, J. W. A. Divergence of Erv1-Associated Mitochondrial Import and Export Pathways in Trypanosomes and Anaerobic Protists. *Eukaryot. Cell* **2013**, *12* (2), 343–355. https://doi.org/10.1128/EC.00304-12.

(45) Ceh-Pavia, E.; Tang, X.; Liu, Y.; Heyes, D. J.; Zhao, B.; Xiao, P.; Lu, H. Redox Characterisation of Erv1, a Key Component for Protein Import and Folding in Yeast Mitochondria. *FEBS J.* **2020**, *287* (11), 2281–2291. https://doi.org/10.1111/febs.15136.

(46) Tang, X.; Ang, S. K.; Ceh-Pavia, E.; Heyes, D. J.; Lu, H. Kinetic Characterisation of Erv1, a Key Component for Protein Import and Folding in Yeast Mitochondria. *FEBS J.* **2020**, *287* (6), 1220–1231. https://doi.org/10.1111/febs.15077.

(47) Peleh, V.; Zannini, F.; Backes, S.; Rouhier, N.; Herrmann, J. M. Erv1 of Arabidopsis Thaliana Can Directly Oxidize Mitochondrial Intermembrane Space Proteins in the Absence of Redox-Active Mia40. *BMC Biol.* **2017**, *15* (1), 106. https://doi.org/10.1186/s12915-017-0445-8.

(48) Carrie, C.; Giraud, E.; Duncan, O.; Xu, L.; Wang, Y.; Huang, S.; Clifton, R.; Murcha, M.; Filipovska, A.; Rackham, O.; Vrielink, A.; Whelan, J. Conserved and Novel Functions for Arabidopsis Thaliana MIA40 in Assembly of Proteins in Mitochondria and Peroxisomes. *J. Biol. Chem.* **2010**, *285* (46), 36138–36148. https://doi.org/10.1074/jbc.M110.121202.

(49) Allen, J. W. A.; Ferguson, S. J.; Ginger, M. L. Distinctive Biochemistry in the Trypanosome Mitochondrial Intermembrane Space Suggests a Model for Stepwise Evolution of the MIA Pathway for Import of Cysteine-Rich Proteins. *FEBS Lett.* **2008**, *582* (19), 2817–2825. https://doi.org/10.1016/j.febslet.2008.07.015.

(50) Lutz, T. Import of Small Tim Proteins into the Mitochondrial Intermembrane Space. *EMBO J.* **2003**, *22* (17), 4400–4408. https://doi.org/10.1093/emboj/cdg421.

(51) Koehler, C. The Small Tim Proteins and the Twin Cx3C Motif. *Trends Biochem. Sci.* **2004**, *29* (1), 1–4. https://doi.org/10.1016/j.tibs.2003.11.003.

(52) Peleh, V.; Cordat, E.; Herrmann, J. M. Mia40 Is a Trans-Site Receptor That Drives Protein Import into the Mitochondrial Intermembrane Space by Hydrophobic Substrate Binding. *eLife* **2016**, *5*, e16177. https://doi.org/10.7554/eLife.16177.

(53) Kawano, S.; Yamano, K.; Naoé, M.; Momose, T.; Terao, K.; Nishikawa, S.; Watanabe, N.; Endo, T. Structural Basis of Yeast Tim40/Mia40 as an Oxidative Translocator in the Mitochondrial Intermembrane Space. *Proc. Natl. Acad. Sci.* **2009**, *106* (34), 14403–14407. https://doi.org/10.1073/pnas.0901793106.

(54) von der Malsburg, K.; Müller, J. M.; Bohnert, M.; Oeljeklaus, S.; Kwiatkowska, P.; Becker, T.; Loniewska-Lwowska, A.; Wiese, S.; Rao, S.; Milenkovic, D.; Hutu, D. P.; Zerbes, R. M.; Schulze-Specking, A.; Meyer, H. E.; Martinou, J.-C.; Rospert, S.;

Rehling, P.; Meisinger, C.; Veenhuis, M.; Warscheid, B.; van der Klei, I. J.; Pfanner, N.; Chacinska, A.; van der Laan, M. Dual Role of Mitofilin in Mitochondrial Membrane Organization and Protein Biogenesis. *Dev. Cell* **2011**, *21* (4), 694–707. https://doi.org/10.1016/j.devcel.2011.08.026.

(55) Hell, K. The Erv1–Mia40 Disulfide Relay System in the Intermembrane Space of Mitochondria. *Biochim. Biophys. Acta BBA - Mol. Cell Res.* **2008**, *1783* (4), 601–609. https://doi.org/10.1016/j.bbamcr.2007.12.005.

(56) Mordas, A.; Tokatlidis, K. The MIA Pathway: A Key Regulator of Mitochondrial Oxidative Protein Folding and Biogenesis. *Acc. Chem. Res.* **2015**, *48* (8), 2191–2199. https://doi.org/10.1021/acs.accounts.5b00150.

(57) Deponte, M.; Hell, K. Disulphide Bond Formation in the Intermembrane Space of Mitochondria. *J. Biochem. (Tokyo)* **2009**, *146* (5), 599–608. https://doi.org/10.1093/jb/mvp133.

(58) Cavallaro, G. Genome-Wide Analysis of Eukaryotic Twin CX9C Proteins. *Mol. Biosyst.* **2010**, *6* (12), 2459. https://doi.org/10.1039/c0mb00058b.

(59) Koch, J. R.; Schmid, F. X. Mia40 Targets Cysteines in a Hydrophobic Environment to Direct Oxidative Protein Folding in the Mitochondria. *Nat. Commun.* **2014**, *5* (1), 3041. https://doi.org/10.1038/ncomms4041.

(60) *TriTrypDB*. https://tritrypdb.org/tritrypdb/app.

(61) Specht, S. Structure-Function Analysis and Membrane Association of Leishmania Tarentolae and Plasmodium Falciparum Erv. **2019**. https://doi.org/10.11588/HEIDOK.00026102.

(62) Klatt, S.; Simpson, L.; Maslov, D. A.; Konthur, Z. Leishmania Tarentolae: Taxonomic Classification and Its Application as a Promising Biotechnological Expression Host. *PLoS Negl. Trop. Dis.* **2019**, *13* (7), e0007424. https://doi.org/10.1371/journal.pntd.0007424.

(63) Beverley, S. M. GENE AMPLIFICATION IN *LEISHMANIA*. *Annu. Rev. Microbiol.* **1991**, *45* (1), 417–444. https://doi.org/10.1146/annurev.mi.45.100191.002221.

(64) Taylor, V. M.; Muñoz, D. L.; Cedeño, D. L.; Vélez, I. D.; Jones, M. A.; Robledo, S. M. Leishmania Tarentolae: Utility as an in Vitro Model for Screening of Antileishmanial Agents. *Exp. Parasitol.* **2010**, *126* (4), 471–475. https://doi.org/10.1016/j.exppara.2010.05.016.

(65) Simpson, L. Behavior of the Kinetoplast of *Leishmania Tarentolae* upon Cell Rupture*. *J. Protozool.* **1968**, *15* (1), 132–136. https://doi.org/10.1111/j.1550-7408.1968.tb02097.x.

(66) Habibzadeh, S.; Doroud, D.; Taheri, T.; Seyed, N.; Rafati, S. Leishmania Parasite: The Impact of New Serum-Free Medium as an Alternative for Fetal Bovine Serum. *Iran. Biomed. J.* **2021**, *25* (5), 349–358. https://doi.org/10.52547/ibj.25.5.349.

(67) Ticha, L.; Kykalova, B.; Sadlova, J.; Gramiccia, M.; Gradoni, L.; Volf, P. Development of Various Leishmania (Sauroleishmania) Tarentolae Strains in Three Phlebotomus Species. *Microorganisms* **2021**, *9* (11), 2256. https://doi.org/10.3390/microorganisms9112256.

(68) Mendoza-Roldan, J. A.; Latrofa, M. S.; Iatta, R.; R. S. Manoj, R.; Panarese, R.; Annoscia, G.; Pombi, M.; Zatelli, A.; Beugnet, F.; Otranto, D. Detection of Leishmania Tarentolae in Lizards, Sand Flies and Dogs in Southern Italy, Where Leishmania Infantum Is Endemic: Hindrances and Opportunities. *Parasit. Vectors* **2021**, *14* (1), 461. https://doi.org/10.1186/s13071-021-04973-2.

(69) Bates, P. A. Leishmania Sand Fly Interaction: Progress and Challenges. *Curr. Opin. Microbiol.* **2008**, *11* (4), 340–344. https://doi.org/10.1016/j.mib.2008.06.003.
(70) Akhoundi, M.; Kuhls, K.; Cannet, A.; Votýpka, J.; Marty, P.; Delaunay, P.; Sereno, D. A Historical Overview of the Classification, Evolution, and Dispersion of Leishmania Parasites and Sandflies. *PLoS Negl. Trop. Dis.* **2016**, *10* (3), e0004349. https://doi.org/10.1371/journal.pntd.0004349.
(71) *msf leishmaniosis*. https://www.msf.ch/de/unsere-arbeit/krankheiten/viszerale-leishmaniose.
(72) World Health Organization. *Thirteenth Meeting of the WHO Vector Control Advisory Group: Meeting Report, Virtual Meeting, 7–10 December 2020*; World Health Organization: Geneva, 2021.
(73) *Kigali declaration*. https://unitingtocombatntds.org/.
(74) Basile, G.; Peticca, M. Recombinant Protein Expression in Leishmania Tarentolae. *Mol. Biotechnol.* **2009**, *43* (3), 273–278. https://doi.org/10.1007/s12033-009-9213-5.
(75) Fritsche, C.; Sitz, M.; Weiland, N.; Breitling, R.; Pohl, H.-D. Characterization of the Growth Behavior OfLeishmania Tarentolae – a New Expression System for Recombinant Proteins. *J. Basic Microbiol.* **2007**, *47* (5), 384–393. https://doi.org/10.1002/jobm.200710111.
(76) Kushnir, S.; Gase, K.; Breitling, R.; Alexandrov, K. Development of an Inducible Protein Expression System Based on the Protozoan Host Leishmania Tarentolae. *Protein Expr. Purif.* **2005**, *42* (1), 37–46. https://doi.org/10.1016/j.pep.2005.03.004.
(77) Beneke, T.; Madden, R.; Makin, L.; Valli, J.; Sunter, J.; Gluenz, E. A CRISPR Cas9 High-Throughput Genome Editing Toolkit for Kinetoplastids. *R. Soc. Open Sci.* **2017**, *4* (5), 170095. https://doi.org/10.1098/rsos.170095.
(78) Turra, G. L.; Schneider, L.; Liedgens, L.; Deponte, M. Testing the CRISPR-Cas9 and GlmS Ribozyme Systems in Leishmania Tarentolae. *Mol. Biochem. Parasitol.* **2021**, *241*, 111336. https://doi.org/10.1016/j.molbiopara.2020.111336.
(79) Sharma, R.; Avendaño Rangel, F.; Reis-Cunha, J. L.; Marques, L. P.; Figueira, C. P.; Borba, P. B.; Viana, S. M.; Beneke, T.; Bartholomeu, D. C.; de Oliveira, C. I. Targeted Deletion of Centrin in Leishmania Braziliensis Using CRISPR-Cas9-Based Editing. *Front. Cell. Infect. Microbiol.* **2022**, *11*, 790418. https://doi.org/10.3389/fcimb.2021.790418.
(80) Martel, D.; Beneke, T.; Gluenz, E.; Späth, G. F.; Rachidi, N. Characterisation of Casein Kinase 1.1 in *Leishmania Donovani* Using the CRISPR Cas9 Toolkit. *BioMed Res. Int.* **2017**, *2017*, 1–11. https://doi.org/10.1155/2017/4635605.
(81) Dean, S.; Sunter, J.; Wheeler, R. J.; Hodkinson, I.; Gluenz, E.; Gull, K. A Toolkit Enabling Efficient, Scalable and Reproducible Gene Tagging in Trypanosomatids. *Open Biol.* **2015**, *5* (1), 140197. https://doi.org/10.1098/rsob.140197.
(82) *LeishGEdit*. http://www.leishgedit.net/.
(83) Simpson, L.; Shaw, J. RNA Editing and the Mitochondrial Cryptogenes of Kinetoplastid Protozoa. *Cell* **1989**, *57* (3), 355–366. https://doi.org/10.1016/0092-8674(89)90911-2.
(84) Camacho, E.; Rastrojo, A.; Sanchiz, Á.; González-de la Fuente, S.; Aguado, B.; Requena, J. M. Leishmania Mitochondrial Genomes: Maxicircle Structure and Heterogeneity of Minicircles. *Genes* **2019**, *10* (10), 758. https://doi.org/10.3390/genes10100758.
(85) Simpson, L. Kinetoplast RNA of Leishmania Tarentolae. *Cell* **1978**, *14* (1), 169–178. https://doi.org/10.1016/0092-8674(78)90311-2.

(86) Laffitte, M.-C. N.; Leprohon, P.; Papadopoulou, B.; Ouellette, M. Plasticity of the Leishmania Genome Leading to Gene Copy Number Variations and Drug Resistance. *F1000Research* **2016**, *5*, 2350. https://doi.org/10.12688/f1000research.9218.1.

(87) Mannaert, A.; Downing, T.; Imamura, H.; Dujardin, J.-C. Adaptive Mechanisms in Pathogens: Universal Aneuploidy in Leishmania. *Trends Parasitol.* **2012**, *28* (9), 370–376. https://doi.org/10.1016/j.pt.2012.06.003.

(88) Rovai, L.; Tripp, C.; Stuart, K.; Simpson, L. Recurrent Polymorphisms in Small Chromosomes of Leishmania Tarentolae after Nutrient Stress or Subcloning. *Mol. Biochem. Parasitol.* **1992**, *50* (1), 115–125. https://doi.org/10.1016/0166-6851(92)90249-J.

(89) Sterkers, Y.; Lachaud, L.; Bourgeois, N.; Crobu, L.; Bastien, P.; Pagès, M. Novel Insights into Genome Plasticity in Eukaryotes: Mosaic Aneuploidy in *Leishmania*: Mosaic Aneuploidy in *Leishmania*. *Mol. Microbiol.* **2012**, *86* (1), 15–23. https://doi.org/10.1111/j.1365-2958.2012.08185.x.

(90) Database Resources of the National Center for Biotechnology Information. *Nucleic Acids Res.* **2016**, *44* (D1), D7–D19. https://doi.org/10.1093/nar/gkv1290.

(91) Bae, S.; Park, J.; Kim, J.-S. Cas-OFFinder: A Fast and Versatile Algorithm That Searches for Potential off-Target Sites of Cas9 RNA-Guided Endonucleases. *Bioinformatics* **2014**, *30* (10), 1473–1475. https://doi.org/10.1093/bioinformatics/btu048.

(92) Schneider, C. A.; Rasband, W. S.; Eliceiri, K. W. NIH Image to ImageJ: 25 Years of Image Analysis. *Nat. Methods* **2012**, *9* (7), 671–675. https://doi.org/10.1038/nmeth.2089.

(93) Rudolph, J.; Jürgen, C. *A Network Module for the Perseus Software for Computational Proteomics Facilitates Proteome Interaction Graph Analysis*; preprint; Bioinformatics, 2018. https://doi.org/10.1101/447268.

(94) Krogh, A.; Larsson, B.; von Heijne, G.; Sonnhammer, E. L. L. Predicting Transmembrane Protein Topology with a Hidden Markov Model: Application to Complete Genomes11Edited by F. Cohen. *J. Mol. Biol.* **2001**, *305* (3), 567–580. https://doi.org/10.1006/jmbi.2000.4315.

(95) Amos, B.; Aurrecoechea, C.; Barba, M.; Barreto, A.; Basenko, E. Y.; Bażant, W.; Belnap, R.; Blevins, A. S.; Böhme, U.; Brestelli, J.; Brunk, B. P.; Caddick, M.; Callan, D.; Campbell, L.; Christensen, M. B.; Christophides, G. K.; Crouch, K.; Davis, K.; DeBarry, J.; Doherty, R.; Duan, Y.; Dunn, M.; Falke, D.; Fisher, S.; Flicek, P.; Fox, B.; Gajria, B.; Giraldo-Calderón, G. I.; Harb, O. S.; Harper, E.; Hertz-Fowler, C.; Hickman, M. J.; Howington, C.; Hu, S.; Humphrey, J.; Iodice, J.; Jones, A.; Judkins, J.; Kelly, S. A.; Kissinger, J. C.; Kwon, D. K.; Lamoureux, K.; Lawson, D.; Li, W.; Lies, K.; Lodha, D.; Long, J.; MacCallum, R. M.; Maslen, G.; McDowell, M. A.; Nabrzyski, J.; Roos, D. S.; Rund, S. S. C.; Schulman, S. W.; Shanmugasundram, A.; Sitnik, V.; Spruill, D.; Starns, D.; Stoeckert, C. J.; Tomko, S. S.; Wang, H.; Warrenfeltz, S.; Wieck, R.; Wilkinson, P. A.; Xu, L.; Zheng, J. VEuPathDB: The Eukaryotic Pathogen, Vector and Host Bioinformatics Resource Center. *Nucleic Acids Res.* **2022**, *50* (D1), D898–D911. https://doi.org/10.1093/nar/gkab929.

(96) Dean, S.; Sunter, J. D.; Wheeler, R. J. TrypTag.Org: A Trypanosome Genome-Wide Protein Localisation Resource. *Trends Parasitol.* **2017**, *33* (2), 80–82. https://doi.org/10.1016/j.pt.2016.10.009.

(97) Rappsilber, J.; Mann, M.; Ishihama, Y. Protocol for Micro-Purification, Enrichment, Pre-Fractionation and Storage of Peptides for Proteomics Using StageTips. *Nat. Protoc.* **2007**, *2* (8), 1896–1906. https://doi.org/10.1038/nprot.2007.261.
(98) *TrypTag*. http://tryptag.org/.
(99) *TrypTag substrate 4 orthologue*. http://tryptag.org/?query=Tb927.11.13510.
(100) Smyth, D. G.; Nagamatsu, A.; Fruton, J. S. Some Reactions of N-Ethylmaleimide [1]. *J. Am. Chem. Soc.* **1960**, *82* (17), 4600–4604. https://doi.org/10.1021/ja01502a039.
(101) Lachaud, L.; Bourgeois, N.; Kuk, N.; Morelle, C.; Crobu, L.; Merlin, G.; Bastien, P.; Pagès, M.; Sterkers, Y. Constitutive Mosaic Aneuploidy Is a Unique Genetic Feature Widespread in the Leishmania Genus. *Microbes Infect.* **2014**, *16* (1), 61–66. https://doi.org/10.1016/j.micinf.2013.09.005.
(102) *molbiotools*. https://molbiotools.com/listcompare.php.
(103) Murschall, L. M.; Gerhards, A.; MacVicar, T.; Peker, E.; Hasberg, L.; Wawra, S.; Langer, T.; Riemer, J. The C-Terminal Region of the Oxidoreductase MIA40 Stabilizes Its Cytosolic Precursor during Mitochondrial Import. *BMC Biol.* **2020**, *18* (1), 96. https://doi.org/10.1186/s12915-020-00824-1.
(104) Sideris, D. P.; Petrakis, N.; Katrakili, N.; Mikropoulou, D.; Gallo, A.; Ciofi-Baffoni, S.; Banci, L.; Bertini, I.; Tokatlidis, K. A Novel Intermembrane Space–Targeting Signal Docks Cysteines onto Mia40 during Mitochondrial Oxidative Folding. *J. Cell Biol.* **2009**, *187* (7), 1007–1022. https://doi.org/10.1083/jcb.200905134.
(105) Milenkovic, D.; Ramming, T.; Müller, J. M.; Wenz, L.-S.; Gebert, N.; Schulze-Specking, A.; Stojanovski, D.; Rospert, S.; Chacinska, A. Identification of the Signal Directing Tim9 and Tim10 into the Intermembrane Space of Mitochondria. *Mol. Biol. Cell* **2009**, *20* (10), 2530–2539. https://doi.org/10.1091/mbc.e08-11-1108.
(106) Li, S.; Shen, G.; Li, W. Intramembrane Thiol Oxidoreductases: Evolutionary Convergence and Structural Controversy. *Biochemistry* **2018**, *57* (3), 258–266. https://doi.org/10.1021/acs.biochem.7b00876.
(107) *VeuPathDB*. https://veupathdb.org/veupathdb/app.
(108) *prediction transmembrane domains*. https://services.healthtech.dtu.dk/service.php?TMHMM-2.0.
(109) *HostDB*. https://hostdb.org/hostdb/app/.
(110) Mohammadzadeh, T.; Sadjjadi, S.; Habibi, P.; Sarkari, B. Comparison of Agar Dilution, Broth Dilution, Cylinder Plate and Disk Diffusion Methods for Evaluation of Anti-Leishmanial Drugs on Leishmania Promastigotes. *Iran. J. Parasitol.* **2012**, *7* (3), 43–47.

Supplements

S1 Cysteine motifs of Mia40 orthologs

S. cerevisiae …CPCLGGMAHGPCGEEFKSAFSCFVYSEAEPKGIDCVEKFQHMQDC…

N. crassa …CPCLGGMAHGPCGEEFKAAFSCFVYSTEEPKGMDCIEKFSHMQDC…

H. sapiens …CPCLGGMASGPCGEQFKSAFSCFHYSTEEIKGSDCVDQFRAMQEC…

Figure 39: Cysteine motifs of Mia40 orthologs in *S. cerevisiae*, *N. crassa* and *H. sapiens*.[95,107]

S2 Recodonised sequence of the gene encoding Mic20

Recodonised sequence encoding Mic20 with linkers, tags and restriction sites (only N- or C-termial tag are amplified)

…CATATGATGTACCCTTACGATGTGCCTGATTACGCGTACCCATACGACGTGCCAGACTACGCATACCCGTACG
ATGTGCCCGATTACGCAggttctggtagtggttccggttccggttctTCGATGACACAGAGCATCGTACGCCGGATTTTCGGGG
ACCGGAAGCTACCTGAGAATTTGTCGAACGAAGAATACGACAAGTATATGCAGGATAACTTCCCTAAGTGGATGA
AGGAGTTCGAGGACGGAGGCTTCCTAGAGAAGACAAAGTTGCCCCCTATTAAAAGCGAGGAGGAGTTCATCA
CCAAGCTCCAAGAGCATAAGGACGAGTTGATGGTGATTAAGTACTGGAAGCACGGCTGCATCCCGTGCCTCAC
CTTCGCCGAGATGTATAAGGAGGCGGCGGAGAGGTGCTGTAAGGAGAAGAAGAAAATCGTCTGGTACAGCGT
GGACACGAAGGCCTTGAACACACGCCAACTAATCGACTACCAACTAATCTCGGGCACTCCAACGATCCAGACG
TTTACGGGCCAGAAGCAGGTGGGGAACGAGATTAGGGCCGTGAGTACCGAGGAGTTGCTCAGCGAGCTGG
ACAAGCGCGTGCCCAAATCCACCTCTggttctggtagtggttccggttccggttctTACCCTTACGATGTGCCTGATTACGCGTA
CCCATACGACGTGCCAGACTACGCATACCCGTACGATGTGCCCGATTACGCATAGTAGGGATCC…

Translation

…MYPYDVPDYAYPYDVPDYAYPYDVPDYAGSGSGSGSGSSMTQSIVRRIFGDRKLPENLSNEEYDKYMQDNFPKWMKEFE
DGGFLEKTKLPPIKSEEEFITKLQEHKDELMVIKYWKHGCIPCLTFAEMYKEAAERCCKEKKKIVWYSVDTKALNTRQLIDYQL
ISGTPTIQTFTGQKQVGNEIRAVSTEELLSELDKRVPKSTSGSGSGSGSGSYPYDVPDYAYPYDVPDYAYPYDVPDYA-…

Figure 40: Recodonised sequence of the gene encoding Mic20(top panel). The translation to the amino acid sequence is also given (bottom panel). The sequences for the 3x-HA-tag are highlighted in green, start- and stop codons in yellow, restriction sites in blue or grey. The sequences for the linker are written in blue.

S3 Chromosomal editing of the gene encoding substrate 2 to produce a C-terminal His_8-tag

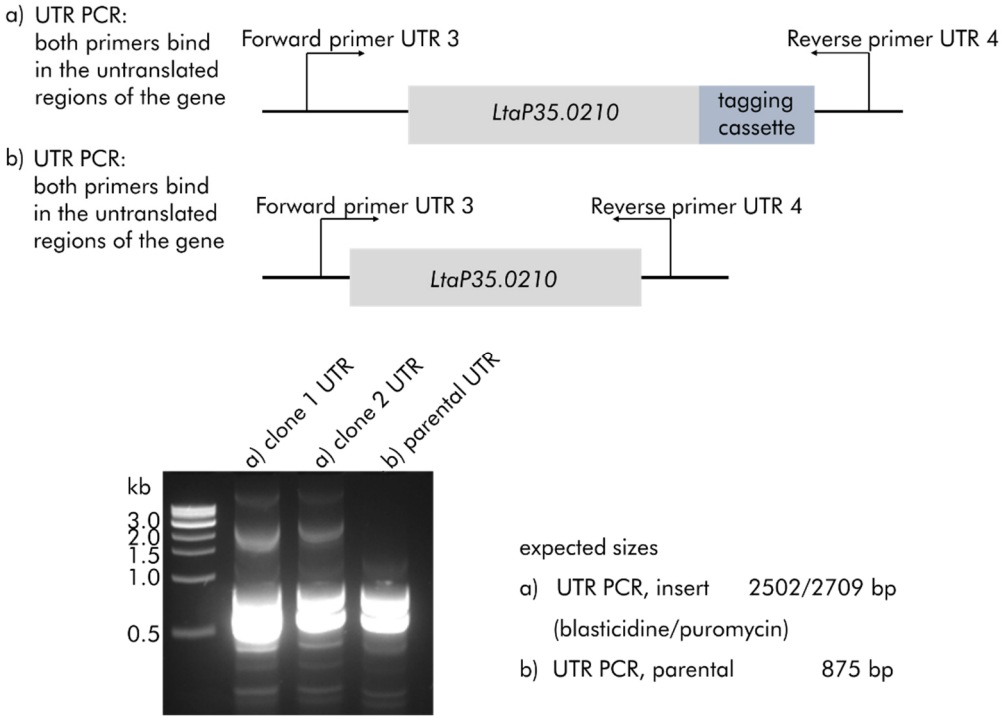


Figure 41: Genotyping of two clones from the C-terminal tagging attempt of *LtaP35.0210* encoding substrate 2. A His_8-tag was encoded by the tagging cassettes. The top panel shows schematically the position of the primers used to verify the presence of the C-terminal tagging cassette. The bottom panel shows an image of the agarose gel separating the amplicons for two clones with C-terminal tagging cassettes. The numbers of the primers used for the genotyping are: 3, 4 (Table 3). Heterozygous insertion of the tagging cassette was assumed for both clones.

S4 Transmembrane domains in substrate 4

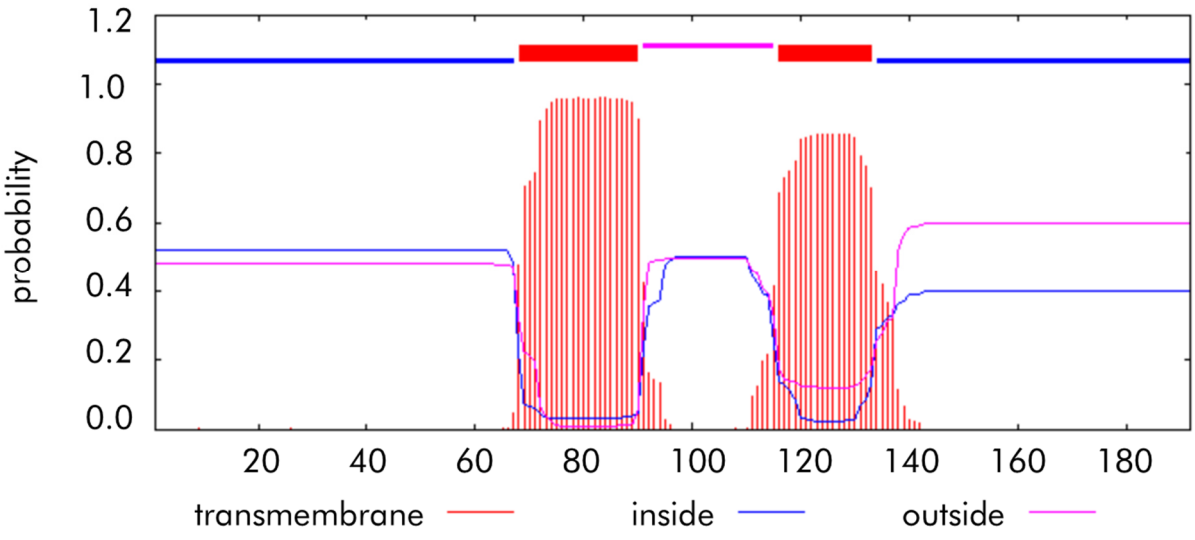


Figure 42: Prediction of the transmembrane domains in LtaP09.1390 (substrate 4).[94,108] Two transmembrane domains are predicted.

Amino acid sequence of substrate 4

MQAPKTSTSSATGEVAPASLQWVSETPFVTDPAQVAVFDIPVEYVRRIEEVQRVYGNHYMEHRMYTNA
WNHAAMTLGFQCCWLGVGAWIVSRGLRYSDPMNSIVARWVQHSRVRRLTTPISCFGLFIFAATCMQ
MPYDFRLLREASVGMEAHGAVMKKLMEERHLLYREGMQAKEKFEAKEAEAFANVMKAQ

Figure 43: Amino acid sequence of substrate 4 (LtaP09.1390).[60] Cysteines are highlighted in yellow.

S5 Enrichments of the discussed candidates

Table 6: Enrichments of the discussed candidates. The data from the mass spectrometry analysis are used. The differences of Student's T-tests for proteins identified as potential interactors of N-terminally His_8-tagged substrate 4 and of the proteins identified in experiments with the parental strain are given. The columns show the logarithm to base two for the enrichment factor of a protein. The indicated additives were used as described in the pull-down experiments that preceded the mass spectrometry analysis.

Protein ID	no additive 1	no additive 2	DTT	TMAD
LtaP09.1390	6.65	8.90	9.15	6.82
LtaP03.0350	0.58	2.62	-0.23	0.68
LtaP04.0260	0.20	1.83	1.16	0.43
LtaP04.0280	0.02	1.29	1.41	1.09
LtaP04.1140	0.03	0.75	1.33	0.90
LtaP05.0570	1.11	0.05	-0.07	1.57
LtaP05.0600	-	1.76	1.50	0.54
LtaP05.0750	0.02	0.08	0.61	0.35
LtaP06.0610	0.13	0.21	0.42	0.66
LtaP06.1050	0.21	1.09	1.11	0.42
LtaP07.0220	0.52	0.64	2.53	0.61
LtaP09.0570	0.04	1.22	2.21	1.02
LtaP09.0730	0.06	0.07	1.01	2.08
LtaP09.0800	0.14	0.07	0.63	0.80
LtaP09.1150	0.31	0.63	0.53	1.16
LtaP10.0130	0.35	1.01	2.65	0.60
LtaP10.0410	0.89	1.02	0.68	0.66
LtaP10.0910	0.38	0.01	0.50	1.37
LtaP10.1150	0.92	0.43	0.40	0.23
LtaP11.0290	0.94	2.03	0.67	1.79
LtaP11.1290	0.64	0.88	0.41	0.55
LtaP12.0460	0.20	1.84	0.82	0.50
LtaP12.1000	0.46	1.57	-0.11	0.86
LtaP12.1020	0.50	0.34	1.65	0.25
LtaP13.0300	0.12	0.39	1.32	1.53
LtaP14.0180	0.14	0.34	1.11	0.92
LtaP14.0230	0.36	0.38	0.38	0.57
LtaP14.0970	0.16	0.86	0.30	0.51

Protein ID	no additive 1	no additive 2	DTT	TMAD
LtaP14.1000	1.54	1.68	1.73	2.01
LtaP14.1060	0.37	0.79	0.99	0.15
LtaP14.1510	0.82	0.06	0.00	-0.25
LtaP14.1550	0.20	0.98	2.01	1.15
LtaP15.0320	0.18	0.43	1.35	0.45
LtaP15.1010	0.32	1.14	1.98	1.63
LtaP15.1030	0.30	0.14	0.43	0.65
LtaP16.1340	2.29	1.24	0.66	0.11
LtaP17.1080	0.78	0.04	0.19	0.04
LtaP17.1360	0.95	0.35	1.55	1.24
LtaP18.0010	0.10	0.87	0.52	0.47
LtaP18.1340	0.17	0.05	0.76	0.82
LtaP19.0910	0.51	1.55	3.1	2.38
LtaP21.0560	0.21	1.14	2.59	2.11
LtaP22.0060	0.28	0.78	0.94	-0.24
LtaP22.0170	0.10	1.19	1.31	0.62
LtaP22.0210	0.02	1.94	1.06	1.25
LtaP22.0720	0.03	1.37	3.54	1.95
LtaP22.0750	0.82	2.49	3.28	1.19
LtaP22.1170	0.72	0.83	1.42	0.64
LtaP22.1200	0.01	2.01	3.30	1.70
LtaP22.1610	0.45	1.34	1.02	1.39
LtaP23.0280	1.14	0.13	0.86	0.26
LtaP23.0460	0.39	0.37	0.15	0.57
LtaP23.0650	0.05	0.17	0.23	0.48
LtaP24.0140	0.90	0.98	3.10	1.51
LtaP24.0310	0.06	0.37	0.54	1.00
LtaP25.0620	0.63	0.05	2.07	0.12
LtaP25.0960	0.26	0.07	0.82	0.88
LtaP25.1940	0.31	0.26	0.71	0.81
LtaP25.2210	0.32	0.33	0.64	0.46
LtaP26.1570	1.65	1.67	2.06	0.18
LtaP26.1600	0.03	0.93	1.57	0.52
LtaP26.1760	8.36	0.42	0.10	0.24
LtaP27.0890	0.14	0.15	0.61	0.06
LtaP27.0900	0.06	0.58	1.24	1.13
LtaP27.0970	0.09	0.37	2.26	2.27
LtaP27.1150	0.05	0.16	1.00	0.92
LtaP27.1860	0.07	0.25	1.29	0.84
LtaP27.1940	0.08	0.14	0.55	0.34
LtaP27.2110	0.30	1.01	0.81	0.79
LtaP28.1170	0.25	0.03	-0.19	0.32
LtaP28.1540	0.59	0.33	-0.1	1.96
LtaP28.2020	0.98	2.14	2.53	1.15
LtaP29.0960	0.01	0.30	1.82	1.42

Protein ID	no additive 1	no additive 2	DTT	TMAD
LtaP29.1430	0.25	0.55	1.57	1.37
LtaP29.1780	0.17	1.32	0.04	0.99
LtaP29.2090	0.07	0.50	1.72	1.33
LtaP29.2460	-0.12	0.73	1.25	1.28
LtaP30.0530	0.05	0.11	1.17	0.81
LtaP30.2040	-	1.65	-0.02	0.04
LtaP30.2970	0.06	0.11	1.18	1.11
LtaP30.3200	0.14	0.29	0.41	0.42
LtaP30.3390	0.42	1.10	1.06	1.42
LtaP31.1000	0.33	0.05	1.13	0.20
LtaP31.2190	0.10	0.33	0.86	0.68
LtaP31.2250	1.05	0.93	-0.41	0.94
LtaP32.3420	0.13	0.47	0.54	0.44
LtaP32.3510	0.54	1.30	2.15	1.65
LtaP32.3800	0.10	0.01	1.98	1.24
LtaP32.4070	0.52	2.22	3.01	3.44
LtaP33.2110	0.10	0.76	1.07	0.35
LtaP33.2610	0.23	0.40	1.25	0.52
LtaP33.2890	0.33	1.61	0.85	1.58
LtaP34.0640	0.84	0.36	0.83	0.81
LtaP34.1140	0.57	0.34	1.00	0.74
LtaP34.3330	0.13	1.51	0.78	0.16
LtaP35.0210	0.48	0.12	0.98	0.47
LtaP35.0410	0.07	0.34	1.03	1.15
LtaP35.0940	0.26	0.15	1.23	1.42
LtaP35.1100	0.27	0.96	1.28	0.86
LtaP35.1590	0.09	0.13	0.58	1.33
LtaP35.2200	0.55	0.88	-0.56	0.86
LtaP35.2470	0.52	0.50	3.90	0.07
LtaP35.3080	0.05	0.70	0.71	0.43
LtaP35.3260	0.18	0.95	0.67	0.65
LtaP35.3530	-	2.40	1.98	1.18
LtaP35.4340	0.56	1.91	0.83	1.91
LtaP36.0010	1.03	0.82	0.88	0.21
LtaP36.0240	0.42	1.86	0.08	3.24
LtaP36.0760	0.01	0.04	0.32	0.07
LtaP36.3250	1.31	0.75	0.8	0.18
LtaP36.3280	1.03	0.23	0.6	0.15
LtaP36.3570	0.17	0.37	1.95	1.25
LtaP36.4020	0.09	0.71	0.97	0.64
LtaP36.4590	0.18	0.19	2.63	0.96
LtaP36.7210	0.10	0.26	1.13	0.53
LtaPcontig168-1	0.93	2.30	0.00	0.70

S6 Physical properties of known Mia40 orthologs and candidates

Table 7 shows selected properties of yeast Mia40 and the human ortholog CHCHD4.

Table 7: Properties of yeast Mia40 and human CHCHD4.[95,107,109] Gene onthology terms were used for the prediction of the functions.

Protein ID	Organism	Product description	# TM domains	Molecular weight / [kDa]	Isoelectric point	Function prediction
YKL195w	*Saccharomyces cerevisiae*	Mia40	1	45	4.19	oxidoreductase activity; protein-disulphide reductase activity
ENSG00000163528	*Homo sapiens*	coiled-coil-helix-coiled-coil-helix domain	0	17	4.0	oxidoreductase activity; protein-disulphide reductase activity

In the following, the properties of known Mia40 orthologs are compared with those of selected candidates. The protein LtaP35.1590, for example was found as potential interactor of N-terminally His_8-tagged substrate 4 with a CPC motif. The protein does not exhibit a transmembrane domain, its molecular weight is 34 kDa and the isoelectric point is 6.26.
Table 8 lists selected properties of potential interactors of N-terminally His_8-tagged substrate 4 imported with the involvement of Erv (Figure 31, panel C) and allows a comparison with characteristics known from Mia40 orthologs (Table 7).

Table 8: Properties of potential interactors of N-terminal His_8-tagged substrate 4 and of Erv import. The candidates were found by intersection of data from RNAi Erv knock-down experiments from the literature,[7] compared with proteins found in the present work as potential interactors of N-terminally His_8-tagged substrate 4 (with or without TMAD in the pull-down experiments). Proteins that are potentially located in mitochondria were selected.[60,98]. The following cysteine motifs were searched for: CC, CPC, CXC, CXXC, CX_3CCX_9C, $CX_9CX_{3\text{-}100}CX_9C$.[60]

Protein ID	Product description	Molecular weight / [kDa]	IEP	# TM domains	Cysteine motifs	Function prediction
LtaP07.0220	hypothetical protein, conserved	66	7.21	0	CX_3C	-
LtaP09.0800	oligopeptidase b	83	6.12	0	-	serine-type endopeptidase activity; serine-type exopeptidase activity; serine-type peptidase activity
LtaP09.1150	mitochondrial RNA binding protein 2	35	10.37	0	-	DNA binding
LtaP09.1390	hypothetical protein, conserved, Substrate 4	22	8.33	2	CX_9C, CC	-
LtaP14.0180	hypothetical protein, conserved	27	4.9	0	-	-
LtaP14.0970	ADP/ATP mitochondrial carrier-like protein	40	8.34	1	CXXC	-
LtaP14.1550	glutathione-S-transferase/glutaredoxin, putative	35	9.75	1	CXXC	electron transfer activity; protein binding; protein-disulphide reductase activity
LtaP15.0320	ribonucleoprotein p18, mitochondrial precursor, putative	21	7.21	0	-	-
LtaP15.1030	glutamate dehydrogenase	11	7.55	0	-	glutamate dehydrogenase (NAD+) activity; oxidoreductase activity
LtaP18.0010	hypothetical protein, conserved	114	6.77	0	CXC	-
LtaP21.0560	dihydrolipoamide acetyltransferase precursorlike protein	26	9.94	1	-	-
LtaP22.0720	NADH-cytochrome b_5 reductase, putative, cytochrome-b_5 reductase, putative	32	8.21	0	-	oxidoreductase activity
LtaP24.0310	fumarate hydratase, putative	61	6.9	0	-	fumarate hydratase activity; hydro-lyase activity; lyase activity
LtaP25.0620	hypothetical protein, conserved	16	8.99	0	-	-
LtaP25.0960	cyclophilin, putative, cyclophilin a	28	8.98	1	CX_9C, CXXC, CXC	peptidyl-prolyl cis-trans isomerase activity
LtaP25.2210	hypothetical protein, conserved	18	9.39	1	-	-

In Table 9, the proteins that were identified based on their CPC motif are listed.

Table 9: Candidates with CPC motif that are potential interactors of N-terminally His_8-tagged substrate 4 or full-length Erv. The proteins were identified in at least three experiments. The properties were assigned from TriTrypDB.[60]

Gene ID	Product description	Molecular weight / [kDa]	Isoelectric point	Function prediction
LtaP05.0600	Hypothetical protein, conserved	158	8.06	RNA binding
LtaP14.1510	Hypothetical protein, conserved	105	4.27	-
LtaP22.0060	Hypothetical protein, conserved,transcription factor-like protein	85	8.07	DNA-binding transcription factor activity; nucleic acid binding; zinc ion binding
LtaP29.2460	Ubiquitin hydrolase, putative	95	6.35	thiol-dependent deubiquitinase; zinc ion binding
LtaP30.2970	Glyceraldehyde 3-phosphate dehydrogenase, cytosolic, putative, glyceraldehyde 3-phosphate dehydrogenase, glycosomal	55	9.09	calcium-dependent cysteine-type endopeptidase activity
LtaP35.3530	Hypothetical protein, conserved	78	9.18	NAD binding; NADP binding; oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor
LtaP30.2040	Hypothetical protein, conserved	46	7.14	-

Since the charge of a protein can correlate with the redox properties, the IEP can be a hint for reactivity of a protein, but can also vary for proteins of the same function as comparison of IEPs from Erv in different species shows: *Neurospora crassa*: 7.51, *Saccharomyces cerevisiae*: 8.23, *Plasmodium falciparum*: 8.68, *Leishmania tarentolae*: 6.99.[43,95,107]

S7 Amino acid sequences of selected candidates

LtaP03.0350 | Hypothetical protein, conserved

MSGKVGIFEEAGVADSCEPAVHTGTTVAASAATVGKGAKMVQHRRGRPRKVVVYYFDGDTENDEGVEEAVENDLSNTESEEL
KMFSHRRYSNAPEHLCCSHARVPAHKPISPENPRIGSPTPLGFFTFGITTLLYSVHKANICPLNTATMSLVLLFGGLTQFLCGFLELI
NMNTLGCTISTTYGAFWGATAVLVVWPEGNGVGKSDDTYMGIFFLLWFVFAGSLFASSFRSPFACMILFFLVPLNFLLQSIGFFT
RSDTVAHVAGYVGVIAGVVATYLGIAFTLRDVYGRSLLPLFFQKNMRYVEW

LtaP05.0600 | Hypothetical protein, conserved

MYAHVRGLFFVCRHACSDPLTLPVSVVGAIHLCEVSLHACAPVCGLELITVTIRDDHEMEGWERRRRTGGILLKIEECVCVCACA
DARACPCFYFGCCSAHCLHLHVYTSTAPPHLTPAAACLSFPLSVTSPPYLHLLHASVGGAHATHARSITVGSRRRLGSVLSLRGGS

LPTSRHTQTRTHTHARTHAHRDRERERETCVQRVVLLPLTCLPCHTPSLSFVGFFLFFVLSACAHPTALSNSAPPLXXXXXXXXXXXXX
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXXXXXXXXSLPPVSSNGERHHWQHNGRLMKRFTPPSMPTCPSRQPRGASAAPALPHKVDDREDLADVDDNNG
GVGFDSTTVLLYDGAHQSASKPVDGPSGSSGSGGGSTAALPYIMYEPAYVQQPQQQQGVLGSVLPDEKAVTAASWRSSSV
FPAPDAPYSQPQPRMLPPPYITPASPRTPFTAGGVGILGPGTTEAVSAPPSTSAYAPLSATAESATRAPVKVATSTDVLEVPLHAPRLR
VPTNLPLEQTILLDLLATAVVQGGPTTEEEIVKREMGRGNPAFAFLGEKFNHPCMLYYRWRLYSLLQGDTLVSWRTQPFQIEEAR
RAYVWVPPPPLKVGPECLVGLHQPELLDPAFATGNGGDAVATVAGVQEEDGTDVIVRSARSRRRHHHRRHHRDHSADSSTNR
KRRRCEGGNERGHGNRSRSGSSSTTTTTTTSESDGEQDESKGSGTTVGERGDGDEEKTNAKNHSLGQSPAGTSAMAPPSEA
TADVARVRLPPPSAQWISRQCTAHRHVFAILQPHLLETWTALLNPYAIADPATMREGDLGTLCRRWLRRDEISRRMMFAIRHADAI
HHLLSVLLDAVVKVAYVATARSRQQPTVSVDPIDSNVYCVEALWYLFLLHDIAMNASNTPEASAASATSVNKNVSSRQPQVLSTA
SGMSGATSTGPPSVDEEFFASEGSLEALEALYEIFSRQHTATHAGASTPPSPAVAAGATPVTATAVSPSSAPGSTPRPRRRQRHPQ
RRSPYERCGDALEIILPTLMEACTATALAVSMDKERQRSQAAATTPTAAARVRHFKVLTPHQQQQQSKAKGKPKAAEPESAMAS
SSTTLSVGGGPSLSLRLDAGSDAAALTKTPSSAGVGADTAASSTSSAPAVLLLHWLKAFLIVWMNAEQPRQPPPSPPAGAATAA
GVVVPPGTVWPDGGADAADMPDVSLTYHVNPQHASLLGLDAASQQHRNVCHQSPLLSARACAILKDRYSFLF

LtaP06.1050 | Deoxyribose-phosphate aldolase, putative

MSLDMLSRPGRKGELNPQILERVRFLAAELGFPELEQKFAHLKDGTRAWCTGSASDGADVSAYIDHTLLKADASRAAISQLCEE
AKTYHFKAVCVNGCHVAQCVQLLAGSEVRVGSSCGFPLGQMTSAMKAAEAAEAIASGAHEVDMVINVGALKSKCYADVFK
DIKGVCDVCANAGAVSKVILETCLLSEEEIMDVCIMSVAAGATFVKTSTGFSTKGATPEAVDIMLAVVGNAALVKVSGGVRDRST
AMQYIEAGVRRIGTSSGIAIVSP

LtaP07.0980 | Hypothetical protein, conserved | UF3

MKGASLRRQQRHSHRPSFSQGSDAPVSLVFRTHGDSLSKAAFLFCPKNFNLANPVSATDSAAAAASASSSASILPVSSLPAQSAR
HGVTPSPQSAGPHDGKRFTSAASSSSAAESVQTLVEAIVELRPSSQSVHHGHGACGGGMGSSHNLSGLTPGNLLTSGTGDG
TTGLLSLSAHAPSNSLAGAGPYVDLMAASPFVSTPSSASFAGASGGEGLSMAITPPPGASRNGESLRTPTMSRRQGGVVGVYA
GTLGIANVPSIPSSSGVPNTTAGGGSGPAGGTSAASGGGAIGAGGSCFLMHPSGGWICRAMAFNEKEVSHANGAISVNQS
LMSKCALINEVAKGKIMARILSSYQDVQKRYGLSHVEVTVDNQYLGRSDMFLIAEALRERILYEGEELHVCGFRMRVREMLQAVV
SSPNTTPSNAATSLVPPAFSAALPGVSASSAVVSSATVTPAGVGTSGGTASTAASGAGASGSAMSMGAGAVGTTGSTLASAAD
AITYVRVGNGIVLPTTRINFQSLSPVHYILLELSREMWETTMDGRIALTLAIRKFLSELLLKQLPKHKASPLICVVFTGRVRSEFQFSRH
SDVFHMLELPRDLRSNADIVEEIRMHCEALVDRVLREVQDSAWRQLEAEVRAYREATAASHSRASGSKSAGYRLANDGDDQDT
DPHLLRREREVSNATATASGINQFPSAGETLKRGRTSVAFSAATAATEASFDAYVRSRQQYIESITVERLFVHAKQSNTLEALSLLLER
CTHSYADRNLTLCGHAVTVVSAGKGFYQVTNNLVQVLCARLHDTGLEKVHIVCVGRPPLHVTPLLEYTSDDDTLRSQYHLHSC
VLVRAGVGARDMGGGAPTSLRAGEVERYYETPEWMSVLFFHASLQHKCGGETVFELLPKECWLREHSVMAALKTLWTTAPEPR
QHALQPHSRLHSTPTLLPGGATGDGGEEGGTTSADAGATGGLNGRGEAAVDAAMMGSCGGPDVAAGELSLPGGSPPLLIP
GLGLRSYPVPEVTLPVNSHSANLLLYGDICDAETPTSQHVALPTGVTTDFAITAGGIGGRPSQLPVPLHLPPFVGEPFGASWTGS
MSVEHVASPTSPLCSSSGKAAAVSWLGAAAAATAAAAAPLSTISKSSSPLLVRSATNNSSLSAFPTAPTQGSMPQGMTGTVTQA
VQAVAWQQSQLQQGLQPMRASYTLPHSSAGSALPQHARCGHPMQTFNGMPFIAAAANSGGVWGMEVAPAPTVAWSSL
QPRRSRTGATGAAGTAYHHLAAPNGNGGHRTTMMTAATSAFGGGRASHSNSLPSYYTTSGKPALHISHSFDHKTTTNPEPRS
LGGLEYSMRSAGSSLRGPYANAPRLHNRSLLWRDRSTSKSSMTSIVTSVPAATTAAGADLSLSCNNAAGGLRSVQLRLEDGHM
YRDVMGVAVSFAFCAWYCLHTSKPHHDCGGGTMVVTSTGATNATVSHAMAARQGGGGAAASAMAVAGCSSPFAPTVIAR
RPLQRDGERSALADSATGAAAEFSYNDASLQLSGKRWHKASSGTRSDAVFCGCNVVDSDLRNGYLILEITINAAVLPQSFWSP
RLLLDDNCWAHLMRCFTGRDDRLTLDDGLGFTSEDGTDDDVLTIDGSSVDGPLSPLHGSGGRRCREEEGGGNAAGGKGA
AGGTGGAPDSNGTGDDYCFQSDFRPTEVFCRSHSGITPDGRTLILPTHNNSVYFHSSEEILLRFTPQLFVSTVDEVDEAALKQA
SSPTSTVRSHHDPSAADGEAAERRPTASCALPSASQNEGVETLSDSAAVELLGRQDIFKGLEKEGEGSCPPLTTSGAAAATTADA
TEDVGGGGGPTPVPPNPPGADVMQSATQSGSINSGDSVTRLRAKTGKDVTTATSPLSNSACPSGCVNIDSEAMEPAADVPLQ
RLAPPETDSAMTSTELPPTPKGSTGRSTKSTTPASRPCPPRLRPTATLANSPVVAEGMPSPLPFRAHALPIIATNRRRSSSIAGPPSSSLT
PTTATHLGGQNSVYMFHHDEVPLMPGTTVTVGFVQLRNSLLFAIKPINPLRHGSEEEHREDLTALSQSAESVSTATVLRRRWQW
KHPDVASAPPHLASWTRLCTCRLLPLYGSKPAYPRESFYTVPPHQYTVTGHGSNHADPADDKRRLLEFVLQRLQQQYQIVVDSP
GLAAGSQWPRADPSPNARLEMSLGHQTHTLKVGETAKTISVTRMLHRGMYSNGQVTHMLRYRYLLWNYLADEFTTCEVHME
AQVGERSAFRWEPLDCWLQERPKAFIPRGPDLWLRAHEVAVVLLPEDAAHPPSYEEFCRFINSRFSVHLSTYGKVAWKSHEGDV
SFDIPETMPLMLPATPPLMEVVLVHDNPLTLDTRNVVDRVTGRTVGFEVPVKERLTSVDINLPQEYDSHCCYFVKVSWLACVASIV

VDWMGSFIANAARFHFHAVPLGCYYSTRKAESLTVHYDVEAASPDQEPALRRALMELLMTPAYKYYPDTPVLTRNCRLVHLSGLC
CVIVPPSATTVAQWYQNAFVRQGSAEQLELLTQFKEAVAKARQRVAEVAGGSASAARVPHPATKEV

LtaP09.0800 | Oligopeptidase *b*

MPLESSIAAGAQPPIAAKKPHRVTFGYVSDEDRGPNPMNPPRYHDDPYFWMRDDDRKDLAVIQHLKEEKAYFEARTADIASLRD
VIYTEHISHIKEDDMSAPYVNGQYRYYTREVKGKSYKIYCRVARDKEPGDTAAEEVILDVNQVAEGKPFCDVKAVESAPPKHDLV
AFSVDMSGNEVYTIEFKSMSNPTTQVIADKVSGTNGKIVWGFDHTSIFYVTKDDTLRDNKVWRHVMGKPQSDDVCLYEEKNP
LFSAFMYKAADANTLIVGSESSETTEVHLLDLRKGNTHNTLEIVRPREKGVRYDVDMHGTSHLLILTNEGGAVNHKLLMVPREQL
SDWSRVLVEHSEDIFMESVAVRSRYLVVAGRRAGLTRIWTMTVDPQDGVFKVGTELREVEMKEPIFTVHLVESQMVEYEESMFRM
EYSSLATPNTWFDVNPHDHSRTAVKVREVGGGFDAANYKVERRFATAPDQAKIPISIVYHKDLDMSQPQPCMLYGYGSYGIS
MDPQFSIQHLPYCDRGMIFAIAHIRGGSEMGRAWYEIGAKYLTKRNTFSDFISAAECLIDAKLTTPSQLACEGRSAGGLLVGAV
LNMRPDLFKVALAGVPFVDVMTTMCDPSIPLTTGEWEEWGNPNEYKYYDYMLSYSPVDNVRAQEYPNIMVQCGLHDPRVAY
WEPAKWVSKLRAYKTDGNEILLNMDMESGHFSAKDRYKFWKESAIQQAFVCKHLKSTVRLLARR

LtaP09.1390 | Hypothetical protein, conserved | substrate 4

MQAPKTSTSSATGEVAPASLQWVSETPFVTDPAQVAVFDIPVEYVRRIEEVQRVYGNHYMEHRMYTNAWNHAAMTLGFQCC
WLGVGAWIVSRGLRYSDPMNSIVARWVQHSRVRRLTTPISCFGLFIFAATCMQMPYDFRLLREASVGMEAHGAVMKKLMEERH
LLYREGMQAKEKFEAKEAEAFANVMKAQ

LtaP11.1290 | Surface antigen-like protein

MNAQGMSPPPENRAAKLLVALTLLLIDAILARAELTCGTINCLTCVPGHPLACAVCEEGFGVVNNGCAECSQKHCKNCSLNV
YKCVECTDGYEFDGAVCVRKCADPNCGECEAHAERCDQCKENYRFVGTMCVQGCSDRNCRKCGVHVDKCQECYADY
MFDGAVCVRKCADPHCKKCVARADRCQECKSDYMLDGAVCVRKCADVNCKKCVAQIHSCQECNSDYRLDGGVCVRKC
ADVNCKKCDAKGDRCEECALGQVLTNTGMCQRSGDSASAVTPVAIATTVLTASLLAVAA

LtaP13.0300 | Flagellar radial spoke protein, putative

MASTMLSEEELERQFANAKAYLMQADKDGVSAYDQLTRLMELLLDENPENVAGDLSKLQEILSFIQTHSFACGESTSACYEAT
QVPAEDLRRFEDNKKLFDLPAPEVRTVIEQPDPYTTITTTTVVPLKAPSFESIVGQNKYWTAAGCGLSDDEAFLLDRSITNLAMEKN
LQDIKFFGKIFGTHSDYFVLRTRRYLEVGETVYVETNTMGKPPRKKGMVPVQAEPGYVGVNRYTFWVTASPSDKWEKLPDVTPQ
QINAARETKRFFTGDLSAAVAETFPWGEATYLRAQLARIISSTTIAPQGALEEPEPDQETEEDEDEDAEDDAPQKPKEAKYKPLVV
VSPDYGVEEMDVAQLTTDRWVHAEGYIYKNGRQTKVPEKPEPEEGEGEGEGDEETAVEGDAQSEDEEEEEPEEEEEVELFTPIQ
NDYLYGVVDIPQPPPVMNDEDEEEEEPEEEPGEPDNTPLKPLRDEDVPDDDPTKVKIACWTMRVENNVSKAHRIAVVQSLHWP
GATAYAAEGGKRWSCVYFGNGMKKTDAAFTPPPAPPVQSECADITEVDDPTATVEKLVRRGEEIPEADSEAEMDELEEEEDS

LtaP14.1510 | Hypothetical protein, conserved

MSLFGEPLPFEDDTATSLIRRAGFPPDKMYAQMPEVYLCPCCSEFSKQEERDRLLREAREAKEAERRRLMQLDVFEERARPLPSGD
ARRKAYRRVVEEEPTEMVSNVPPPPQTIDDLRRAKRDQKVPKLVVTVHCGRQVSAEPDDRISVIVRCGAFEGQTEKVLRGREVMV
PWEELFEFPYINSNEPLEVLVVNDALPEKNDQVVGGVVVPCTALHDRNHGDQEPLPIMPEDSMQNGYGSSQEGPLGTIVVSW
YLRRADDKMDEEAIEQQTLSVPLGCTFVVHHLYQYTDHGAEPYTGGVLCVLRDTDDNCSESVLYNPGTKPEPSVAPYYPKEGY
YYLPGNPSQVMRLNTERKLGHILVCVPKQEESSAEEEELLVIGAVPLEFERLYTKGSAVLLIESKSKEDALWGEITVEWANKPLDDV
DPHPTSLEAEKESLFFTIVRGVNLVRRDGEPVAEGYVSVGTGELEGTTVEAPATQDSEGNNIISWNQEVRFIEVDPAEKTVEVQVY
EKERLICVGTCELDDNDEGVVIVQMYHAANLNEEAGEIVGSYKRLHAPRTVEDGIYRQHRAAEKGPYKGADEEDEEQEELHQ
ATEHDESNVHGDEEEEEEQVHVSQVPPTDERAMSINEDLSEEEKGAEQTAPYETQQRQRSEPQEQESKEDQQDVPHQQESR
EEEQLGQTQREKELQEGAEQQSQPERTEEEXXXXXXXXXXXXXXXXXXXXXXXXVQQSYAAQEEAPEPKGEEELIETQKPEEKDLQ
RSYAAQEEAAEPEEEDLQRSEAVPEEEEYAEELQHEQYEVESAHAAEAGEFDPREYHTEAQSAGRPSGEEAAAYTPTSGKPQHR
RRSWAPPAHKGKDYKQPWYPCGSPETDQHVPFSKTNLSKKNLESIRQLETYKSRIEEESVRRGSTPRRSSARGQSPSFQ

LtaP15.1010 | Hypothetical protein, conserved, succinate dehydrogenase, putative

MFITHHALLTPLFYLHQPLSPPGTHTHTRRCCHHLQNDSKHTGNANMPSAPLPGQLANYSSPLYMYSHLIKNSTAKTPQLYTAK
DNSKTAMHLLTRRGATANYTVNRWYLKEKVDSSNRMRLEGLYECVLCASCTGSCPQYWWNREHFLGPAVLLQSYRWLVEPLD
RDFDERVRMFETGTLVNMCHNIFNCSITCPKFLNPGLASKEIKRLSSPAAKRVGPAIEMEPSKN

LtaP15.1030 | Glutamate dehydrogenase

MMRHTVSRAVAQLGGRAFAVHHAVAAKTQLGVSSLSTQRCFASSNATSPPVSLDAIVQAVTAKAVFDKSITEPLTKTFVSGLEKS
SYSRNFSQTQMIDHVQGLLTAEARFRTGDSFEYVHENSNTAFYICHNDPQKKLRMLRKMARFVSLNQDPNLGSNTHHYVSED
RKFAIYIASIEPFVESGSGEEHVNPKSPALPTLSAARQMTEEQKKIIRGLLRRQLNSVFPVFHVEEVEKGHVSFTMATMVERTNYVAS
LLSIFQEIEGAEVMMSVSYSFSNGVQVYGFEIRGAEVGQIEERASLVGLLPNRPFNAITRLHEAGALSCEETVFIDAAVIFAMYFTPS
PTTDDYRHLKAILSKEPNGVNRLNNLRSSLTQEVMSERYTGSVIALYPEFVKLIYEDFRLGSTPERRAAIAEKITQRLREDDRAEYDRT
LFMSFLKFNEVIVKHNFCKTEKAALAFRLDPAFLKELEYPLVPHGIFLFAGGQWRGFHIRFTDIARGGVRMILCKDRDYRKNKRSV
FQENYNLAHTQLLKNKDIPEGGSKGTILVSSRYLNKFDEVRCQHIFLQYVDALLDVIIPGEEGVVDGLKSEEIIFLGPDENTAGTF
PAAGALYSKGRGYKSWKSFTTGKDPELGGIPHDVYGMTTHSVRAYVTSIYEKLGLKESEMRKFQTGGPDGDLGSNEVLRSKEK
MVGMVDISASLHDPNGIDREELARLAHHRLPLREFSRSKLSPEGFLVLTEDHNVKLPDGTLVEDGSRLRNEFHFLKYSDADVFVPC
GGRPRSVTLENVGRFLKVPDADGASMMEGKYSNLSPEQLKFKIIVEGANLFISQDARVALEKCGVTLIKDASANKGGVTSSSLEV
FAGLCLSDEEHRKYMSAKSATDAPEFYKKYVKEILDRIEENAKLEFNAIWREWEKNPQQPKTFIADALSRKNVQIRANMLSSDIFQ
NRDLIRYVMDQYAPKTLKEVVPVDLMLKRVPENYQHAICAMWLASRYVYQTGVDSNEFDFFRYMTEVYATVAKSAQ

LtaP16.1340 | Hypothetical protein, conserved

MNTFQILWWLAITAGAVLAYHLTYFLGLIAYGIAVCGLCNWWMSAFMRAGPRTSRQVPHQHIAYCQQLSALELSKAYREGRL
SCVEVVRTFIEHIKAVNPYINALVFECFDEAMEAAAEADRVWAAWREHKDPKRMPSWLLGVPCTVKECMECRGCPNTSGNPN
RSRIISETDSPVVKNFRDAGAIILGVTNTSELCMWCESSNYMYGITSNPYDTRCLVGGSSGGEGAAAGAVFSTFSLGSDIGGSIR
MPAFFNGVYGYKASPHYIANVGQHPAAKTSANHYMATGPISRFPEDLIPLSQVAARGGFRLDPVLYPPCSPLKKVVDLNHHPLR
VYALEDYGIPWIHVSESQIEAVHAAAEALRERYNAKITYVNLRTPSRSTGGTVPPEFLPFSRTLPMWMAVLSKDPAEAKFSAFMSQ
GHVGGVNWFAEAVRWMLGRSKHTLPAIALCLLETVDLVMPRCLKFSHDDELGPFKMGLESLLGGDGIIITPTFPSAAPRHHFPL
WSPFQFQYTAAFNVLQLPVTACPVWPGASMANRYKSISIHEERSMRLPHDFHLPKGVQVVSAMHQDELCISVAIALKHALGGGY
RYPSWALLEGKDY

LtaP19.0910 | 4-coumarate:coa ligase-like protein

MLRNTIRWASVLSAQHFGKYDVCRSNMRLSRRSIPVDVPRALTTLSSSQVVTPFLRAAATAMTVPQQVRFASTTSASSATATATSD
AAGAGARVYKSKLPSVMDKVNQERTLYEYLMKRIKEKDPKKIATVQTENGKELTYAKVLQATDWCARALYHHANVRKGDVVCI
CMLNTIIYGPVVYGALRLGALVSLVNAIAEPPLLAYFLRESDSKVILGMRYFRKQLEEAVAIVEKETGKKVTILYPEDFFKKWYIWPVP
RSYDGLKGATLDDTIVIPFSSGTGGLSKGVKLSNRALIANAEQLGTAFQLSPEDAGIAVLPFFHIYGFTASLNAGYVHGVTQIVM
LKYTVEDYLKASEKYKPTINLVAPPILISLLKNADKLKQHDMSSLKRFCCGAAPLGSETVDAIEKVLPNVSVTQGYGMTEMAPAVT
VPDGLKHKVPGSCGVLVADTELRIVKVDDSQQSGADKSAGIDAEPGAEGEVWVRGPQLMNGYLRDEDTAMCMQDGWYR
TGDIGKFDVESGELVITDRLKELIKYKGFQVSPASLEALLLTHPWVRDCVVIGVPDPRDVSFENPRALVSLQPSVLPKDAVRASDVL
YRFVMSRMPPHKRLHGGVRIVSEVPRNLSGKLLRRQARKDEAELIKAQAEKVLATKATSPKGDTNASASA

LtaP22.0060 | Hypothetical protein, conserved, transcription factor-like protein

MADGNGEDERSLRVADELRRETYECPSCLELVKLQQPIWSCQACFQIYHFACIRRWAQVDRDTVVFSCPQCRHMQPKPLTDL
CFCGKVSKPKYDPLVTPHSCGRLCGRVRPFCTHRCPVQCHPGPCPRCQLMVGPQRCPCGTTTYTYPCGQPDPETTCDHPC
RRPLACGTHMCLLSCHTGPCPPCSESTTLICYCGQTTKQHPCTKETSFACGSVCGKTLRCGEHKCTLLCHSGECPPCPTDPAT
VHTCPCGASELQTVRYACTDPIPKCGRICSKLLSCGQHHCQLQCHSGTCPPCEVRVDVSCRCRKVRKRLSCAESQNFTCTYE
CGTKLSCGRHKCKVICCQDRNKTQTNSHMCFQVCGRPLPCGHTCEDLCHASTQCPPCVHVVTETLTCYCGAEVLRPPQQ
CGTQPPVCKRACRIPRPCGHPVGHNCHFGPCPPCGVPVKRRCPRHNTLVMLSCGVTDLACEDECGMEMPCGHFCNRVCH
SGPCLEEANPCRQQCDRLHEECGHRCAKPCHSTTPCPPCSVYLRCTCNCGRVTRSLPCAMVGKRKAEGPNKFAVVVPCDND
CLFTRRLDVLTSLSKTKNEKFLYSLMLWDAAQQKASGVKRVESQLMSFVEGNDSVISLPPANSETRALVHALAKYFHVRSESVDN

EPNRSCLLTKTGDTAAPPVLLSDAVRNSQMDPLQFLMQCAKPSVKKKLCLVITGHHVTEIMLTSLLSDLAGRFVIAPPEVGKDGA
QSFLIAFTTHKRAEEAVKKLEAGNTQHTFSVARPTV

LtaP22.0720 | Nadh-cytochrome *b5* reductase, putative

MRAFIASVAGFAGASMYQSGSEVEAWGSKPAFSQEKFQSYKLIQVENETHNTKRLRFALASNKTHLDLPVASCITLRYTDAQG
HEVMRPYTPINLVEDEGHFDLVVKCYPDSKMGSHLFSLKVGDSIEVKGPWHTFDVKPGQYSTIGMIAGGTGLTPMFQIMNNV
LHAPENQTRISLLYANKTEGDILLGKELDTLAKEYPGKFATYHCLATPPKRWTGYSGHINKAIIQETMPGPKHQGDSCVLVSGPP
SFMKAICGSKDYSSNPPKQGPLEGYLKELGYSASGVFKF

LtaP22.0750 | Hypothetical protein, conserved

MDRTKTDSELQQEQSDELFARQLHEQLCNSDSVEHAVPPQRGTSKAVQEAADADFARQLQEEIYRQEAAVSTDNPRQTEDN
RITVECPTCTFINYMKPSSSSKHWRCAQCHGPLKENASHPGHISHRDLVECRVCHCLNKLPSKKSDAILCGACYQELGSTPNT
ASTVEREESQRTVQLRCGQCSVINAVTVGVDVKKLEFLCGGCGVLNTMTLE

LtaP23.0460 | ATP-binding cassette protein, putative, ABC transporter-like protein

MPPHQKEARNTVPRKLHASVLRAATAVVTVLLLLVARVPVTQALSMIPASAEAVMWSGVDPVHASYWESEPITCLNGGIPDGDH
CVCPLYYTSPNCSQRQCPNSIGGQGTHRVLPVNPTNKFCEHCDEKEFTGLNCQLCQSNAACRQYVGSSAECNNSTSTRG
DNKQFQCTLKNEFFIRLMSGNRNISVEVVLNCSTSDGTALRKSDQAVCNMALYRIEPNHSYVDPFFRCEAQGCTLKYGQKDP
DQTNTNNNTMRQRFFRAARTSGQVVLIAECALLALLGVFTTLLGPSRTKTITVTLASAITGTVLVYIFLMILSMQPISAPEQTVIYEC
QKTHCACAEDPPPQYDPICSEKEELSKVILPAIQNSIRFVCGQNTSVCELTLEDLSLVFQVDCRASECVDPVRFPDDSPDDSHD
GGGVAVLAKQNLGILLVMLVLLVMSGVGTHYYYTTSVSYKRDKDFLITFHMRTPYGDDGGGDETGGPAVLDERHVHLNGNE
DDSAGLHEQEPLLYNSPAPRAERRAFRRRSVSSSRHSEGNVSVRASVYDEASTLTPAECARIEELRRLSAAPLELQVSELQYTLNAPL
LGAVEEGSQRTLLQRINFTVHSGDVLAIMGPSGAGKTTLLDLLSARAKSGKVSGTIALNGTPITTSGSCAAQYRNIIGYVSQEDT
LLPSLTVEQTILYAARLKLPRALSDNTVRRIVGRVIETLKLQHCAHTFIGGETARGISGGEKRRVSIAVELLANPRILYLDEPTSGLDTV
SAKRVVEAVVALAKDSTMRMYAKHYFAFQPIVIFSIHQPSQEIYELFDKVLLLSRGVSIYCGAAASAAATLEQRVTTAFGHTREVPR
KDAHNNQAEYLMKVEEILDDGVRAELQEEDALESINTTGAPYGSASTGRSPLATSRTQVPPCRSPESPPPRAPSQSDGCPEFND
EGEQVSGADILVTTFGFRVYYANVYEQLQLLVSRSITCLFGSFHLVICHSAVVACLATLMCVLYHEQALDLPGSLNRAGSVSFLLL
VTSFLSLSCLEQLIVERKLFHVERENGFYTTCPYLISKIVVDIVPLRIIPAMVLASVIYFPMGFRVDAGLHFFYFILIIVLFSICMTMTILCI
GIVSGSFGAAALLSSVFILWNFLFGGALVQAETIPPSLHPFQSISPFFLAFESLMVNELNGQHCVFSPTDETGKKSSASIPIMCVQY
LANIGLKPERFNADVMQLAVYCLLLVGLSWMLLSSCSKLIR

LtaP24.0140 | Glycosomal membrane like protein

MYLLLLHFSIFTFFRGRLPELLHSPDLRCRSLVASLFSAAPASQANAQSERLFPAQNYSSPHRVLYLFSSTPPHQTFKMSLAVTLNTY
MAATDSRDKMIKGAGCFCKMMGALYGNSDFIKAGSAMSDARCLMRMLSWLTNVQKISDAMEKRIVQPRDVLFVLRVLFDGI
FSLLDNIVFAGRFFNPNSPHLTQMSYISRASLFYGYVVAVVLDLYDLVRCPNMPKRGDRCLVLMRNTCDLVSAIGNVCPADIGA
SNVAGLGLISAVIATREQLLAAAAKSGGNTIGAIPGKMSVQEMKK

LtaP26.1760 | Hypothetical protein, conserved

MASEKAVESVRGFCGPAMYADSAVHARVTLADVPRSADGLRLLASNGNWSEVLTLAEQLETEIEAKAQRHPLKYASVPWVTSSS
ESTPRVSPENPKGAPMTTHSPPEMDVTAADNSILRLSTDAAVLSARLPYVLVQVTANLKMRRVVAAKKVVDALGDIEGEGFRHPVT
RESFAPFSLRLVAAFLPLYVGAPMEAQKKLYALLEECLQRERQCGASNSDARIPAVVDADAVKQPATSVALQRTWSRQVLRVQR
ALLHVHIHMNQPSLAHSLVEQVLRSEEIWRHKFHDLSDELHQLRHSLHLQQLLCLALHIGDASRAQAMRAAIQKIAAPTAAESS
TEAASSNAVSNSNLCKLVVLSCDAFIAVFNGEYKRAVQLFREVMDAAAQMKQALRAAADGPGTDVHSSPGSDGFVSEDTLRR
WVLQDICANAQVSHTTCQAYCSDSDPTRLMSSLCTTLEDYAKAEPQVLCNSDAFVESLVRFYTLSGDRTANLNRLADLLEVFRC
DRMSLPSLEALV

LtaP27.0900 | 2-oxoglutarate dehydrogenase E1 component, putative

MMRRALSGVVAVRASAMRSYTDARTIRKPNPYDQLVNAENQHYVEDLMRQYEADSALVDASWVPVLEAIRSGKDESPVVATFS
RPTDAKSLSEKQRHDNMRLSWMIREYERFGHHMANVEPLKGYHADNCSLGSRTLAPEEFGFTREDLTQVFNVNFGASHDATF
VSGGTSMTLQQILDHLRRLYCGPIGFEFMSSGFFDLRNWFRQEVTDALQPLSAGERKLYYNDVVRACGFEKFLQLKYATKHRF
GLDGGEVLIPALKAAILTSSDLGVQSAIIGMAHRGRLNVLANVLHKSLRTILNEFEGHVSIEDAHLSGDVEYHLGKRKHVKLPNN
KSIELDLLPNPSHLEAVNPLVLGKAHARQIYMNDVECTSVLPILIHGDAAFAGQGSCYETMGFCELENFHVGGTLHLVINNQIG
FTTNPNDSRASAYCTDLSKVNNAPVMHVNGDDVDACVKAAKIAARFRQQFHHDIIIDLVCYRRNGHNETDLPDFTQPQLYD
QIRQHPRLVDIYTETLIKDGVLTAEEAKAKDKEWDVILRQAYDRMSSAQNFVKVMPVFDPESENTSADLSSAKIAATRVPPPVLPV
DTGVETETLRAAGVRLATIPKEMQKAHPVVERTYAARKKGTEQGDAIEWCQAELMALATLSMQGVPIRLTGEDVERGTFTQRH
AGITDMKTNLKYFPVKMLSPSQALITISNSSLSELGVCGFEMGYNMENTRSITMWEAQFGDFANGAQVIFDQFLCCCEEKWN
EHSSLILSLPHGYSGAGPEHSSARVERFLQLSDDSDCVPSDFRHFPNDQALEIRIRRHNWQVTYPSTPANYFHLLRRQGLREFAKP
LINLFSKARLRAPNLSKLSDMTQGSSFKAVIDTARAPDTVARKVVFCSGQIESIVNDAKASMQKETPGVHDDVVLVTVEQLAPFP
WEQVADVMEKYAQRNPKTEFVWLQEEPRNMGMWMHMRPRMNNLMHHLGLKQKRIGVVSRASAASPSTGYGSVHVEEEKK
LIAETLA

LtaP29.2090 | Fumarate hydratase, putative

MKARLSVFDLRQLHLFGVPFRHCSSLLFELPRSRTHTPHVSTLSLSLWSTRHTGVAAARRCPLLDVLCSTYATVHLSQPQRQRYRTS
SLPFSPESCVGWSSPTPVTYPMSLCDQCEIGCRRAGIKDIEDTSAVNADFHFSAIFQPTDPHHQEVQFTKIEGSEKYVEELEVFGR
NVLKVNPEALTILAHRAFSDVHHFFRKDHLEGWRRIIEDPEASDNDRYVATTLLKNACIAAGRVLPSCQDTGTAIVLGKRGELCW
TGGEDEKYLSKGVWNAYKYHNLRYSQTAALDMFKECNTGDNLPAQLDLLAVPGSDYEFLFIAKGGGSANKAYLYQETKALL
NPKSLRAFIEEKLKTLGTAACPPYHLALVIGGTSAEMTMKTVKLASCRYYDSLPTTGDKYGRAFRDLEWEKIVMEVAQKSGIGAQ
FGGKYFAHQARVIRLPRHGASCPVGLAVSCSADRQILAHINKSGIYVEQLEQNPAQYLPDIPAAHLSTTSVNVDLKRPIDEVRQ
QLSQYPVGTRVMLNGTLMVARDIAHAKIKEMMDNGEPLPTYMKTSPIYYAGPAKTPEGYASGSFGPTTAGRMDSYVDLFQSH
GGSYITLAKGNRSKQVTDACKKHGGFYLGSIGGPAAILAKNSIKEVVCLAFPELGMEAVWKIGWKTSPHLLSSMTRATTCTRRR
FRKSTMSPCRDSAPAPSIPPPPPPPPPPSLFGCPSLPLTSPLSLVCVCVGGWVGGCVCVCHSQKDEYPHCSIYLFRLLLCCPFSRVP
PTLLTVVSSCTPMTGGIVARGCVWCAFGHEAVKSIAQCLE

LtaP29.2460 | Ubiquitin hydrolase, putative

MLAPPTVVQDVFHYFPGSPHSSCHLRRFGTVNRSATLSRRVCRTWLHADFTFFISSRVHPCGRNTSSRYFPHVHAFPFTPFSHLFS
QVSFHFCLRLSLFFCPCALPAELLACTCTMPFPVSQEACPHAWSDSAIVTPFPGKTVHRDECAYCCRTCRQGDGVYVCMACH
AGLCCEHLEKHMAVHGAHAMYTTVKKLPAKETAEVKDVNYLGVVAPAEYETAVCCADCQVQFAQVPDLAVEAYSSIINAPEP
GAQDSAVDGGLGRLQKPQCPHLVCLEQLASPFALCPPTSDDVCSVEMCECHVNNWMCVTCGAIGCPRPEAGGQGHAL
QHYELTGHPACVKLGTITARGADYYCYACDDDVDDVHFAAHMAHFGIDVQTAKKTAKTMGELEYDYSSQFDFNKITEAGVS
LTSSYGPGHTGIENIGNSCYLASVVQCLMSLSPFQHSFFPGKSTPHQAQCTADPYNCHHCQTERVAAGLLSGEFSREGHDLT
NSICPRLFKKVYAQQHPDFSTGAQQDAQEYLLYLLEKMKRHVHLSSSSATAGNVQQQTHPSDIMEMVMEQRMQCSKCQR
VRYTYEMDKCLSLPIPIDPVSASLVAATANEAEIDAIRPRCSLEDCLQAATLPSSIECRCEACLEPAVYHTVTHLASFPDVLPVYIRRTY
FDMTTHSTKKIDAYVNVPQEIVLEAYRGTGLQPGEVLMPASSAFPERQTRADSYAAAPPQEVDEVALVSVVSMGIDMDIARYALL
QTNMNVERAIDYIFSRPNIAEEIKATSLLRAAEQKQVTQAHNTTSVSVPKPATDGAASYELTAMISHMGGSARTGHYVCHIKDA
ETGKWLLFNDEKVGESQSPPFSLASLYFFQRKV

LtaP30.2040 | Calpain-like cysteine peptidase, putative

MQATYATHKEKHRSVFSTLIESGGLFLCFVSLASCPCVSKQKMSYYIGYSQREIYRKRCDELGCACNSAVIQLLSDVPGEVTSLTS
LDLSRNFLGRKGVIPLLDVIESAAQLRSLDLRDQQLGNDTVEVICARLRRHPSLLTLNLSNNPITLAISSALLELASQNTVLQNIYLE
NTLVRPSMVTAIEVQLGKNRALANAASAVSTVDGGSSRRICGITSSATGPCAVGTKAVSPLAEQSALPTAAGGGKSAAAMLRRD
DSTAPIAAGSARPRQCIPAHELQHVLRSYNHSTADCFFYSDPTKDVWAWCEDRHYVFEDDQFSSHNDHLHRTARHTYGIAG
WRRVGELYPDATLFGCEGARTNSNDTAQQATSHTNGAGFPDTCQDTVKEVMGVERAAVGVTPCGSNAAYVQPTSVFWQL
PTDVPEGFTWIFTVLMASLKEVESLYSLFCASSTEYPDGGMRVQRGTACPGIYTMRVFVDGQWRYLLVDDFLPVDKYGQLIFTK
PSMDNKAFWPCLFEKALAKLHGGYHFLDSQFDKHHDGIDGPNIRKHISWLFLEKENSQRANSAAGDGAGGAAVYDNGSD
TDWHTVPDDSLICEEVAKNCGRIMSRLTKGLYDSYRLHPVEEVPLTLFEELHKVLGVPLSSANVRGGNANPLGGRQTESLKRSFL
ASCNVSVDVPASVCGVPSYMIAAIAFSRDASRTCRGIHPRCAYEIVRVCHAGGVRLLEMRNPWCGTEKWTGDWADGSPLWK
MNPEVAELLLTRGHIGRHIQRGNSNLSQLSNSALTVERMSFKRSLFGTLSEMGGSMTEASLTTRADSVISLSRGTQAAAAPAQKS
TFWMSYTDFLQNFEWVHTCRVFGDEFHRQDVHGAWTRDSAGGNAREPSWYRNPHYRFSLPYRATVYMQLTRRDRRLHQTR

GPVMEREEGGGGIALELLCDTHYPLHCPTISRRVAETNTCGPLHISAPEDFVTGNEENERGIQETMSGRGFGDIDGDFHGAFS
SLLFSEERQIGNCLSLGALLDAGTQYWIVPSTCTPRVLDEFDLVVISTSPFILQAAQESQYWDRRTIMSELLFSTSARTQQAGPNE
GEVAIVFDSRTRVSTDANLVLQNQKRRCSRRFAGSSVKPSLETESATFCSAACRVVVAVSMDLVVAGEETINSSVEDAYDHVSIQ
AEERHATPTLQLAIVPGELGAEGRPTRVVGKLEPLTARTYVLDRHTLLETVIMPANPNTVEHYTAICSLHPEGSRARVEYRVWCSAPL
LEVMDLPMLSKQDVTLMWDDENGSGNYYEGTGHPQVELSKLRPFQRFAISMRMLDYDTIKPAIMFSVICNDQQQGEPVDGR
LGNSDVLLQSAYVHDTYVQDGFHLDEQPPESLIIIPCLQPTGTKGRCLLTISSDSEDYCVYPLRRAAAYPL

LtaP30.2970 | Glyceraldehyde 3-phosphate dehydrogenase, cytosolic, putative, glycosomal

MAPIKVGINGFGRIGRMVFQAICDQGLIGTEIDVVAVVDMSTNAEYFAYQMKHDTVHGRPKYTVEAVKSSPSVETADVLVVNG
HRIKCVKAQRNPADLPWGKLGVDYVIESTGLFTDKLKAEGHIKGGAKKVVISAPASGGAKTIVMGVNQHEYSPASHHVVSNAS
CTTNCLAPIVHVLTKENFGIETGLMTTIHSYTATQKTVDGVSLKDWRGGRAAAVNIIPSTTGAAKAVGMVIPSTKGKLTGMSFRV
PTPDVSVVDLTFRATRDTSIQEIDKAIKKAAQTYMKGILGFTDEELVSADFINDNRSSVYDSKATLQNNLPGEKRFFKVVSWYDNE
WAYSHRVVDLVRYMAAKDAASSKMXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXGGLCPCTIGAHVAGIH

LtaP31.2250 | Hypothetical protein, conserved

MALQYSTETMRVQGIGSPYASLAELYISDPINASSRTLDSARTTLNWSEMPGAFLNIKAYQDELGVGCSGWGMDVNLLWALYP
SVYWFLGIMLARYLCQYSFARFGLYVGVVTQNSGCQRHGIASDKHGLASLSPRNRKKIMKFQNQMWLSLFYTASSCFGYYV
QRSQPWFKLPLDDEASLHLLLPHPYNPPEELIMYYHCGLAFYFSELCSLYIIERHVKRSDFLEYVVHHITTLLLILCSHIGLEHRFGAY
VLFIHDASDIMLSVSKSLNYMWEGVARQHRHNNKRTEKSSRAHRRSFLHRCIVSAVNLNCCFAIFVFCFFLSALLLAFYGQGNH
THVPQSAPRQF

LtaP32.0380 | Hypothetical protein, conserved | UF2

MIRDQCHAYCRCSYCAQEPQKSCNTGASMWATMEALDRPGMSKDELYMKSLRQSLRAKAFLDSSKNAGKFIPDEQLLSSIKR
RDAKDLALTAELVPVRRRDRLQELVLLENKHRLREEAALRAERRALGLPEEEEEEEPVSTAHSDSPASTRPSSKESMQQRLSSGAATP
ASRCSAEHDLHAMLKGIKNIVDEDPAPSSHPLTREQVYKLRQLMHTQKKKTREMVDNCENQPHVCGHCLDVDVQARHLSAL
PNCSVAYGLTGKVINPEEGIKFGTHTQREPNGMFNTSTADAAEVSGRRSRLTSYNAHPYGTGVVFLPVSSLNGKSQNAVGSQ
STRFRASFNNNIGKGEPVEEELQFPGSTYNVYDSGFAKATTPQTETKANKAERTVKIDTASSNESSQHGVNELKVAAIAAPEQPT
YNSSAAMWMTTNQDRELDMKRFLEFQKKLKTNSKRVYEETASRSAKALPA

LtaP32.4070 | unspecified product

MGKTVLSCRKGNGSVYQVHGHKRLGPAKLRILDYAERHGXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXAARRTGRIRGGKAVKGAWHPEE

LtaP34.1140 | P-glycoprotein-like protein

MPKDSERDASANGGSVFELDDSQELFSRNQPFEEGVAEHKDCLDNNDHGYSSTSQSREANNTLHTHDVHAEEGYLVHENK
LSSQNDNTHGGRKPLFSAQEESERVVVRKTVGPIAIFRYADTIDRALMIIGTVCAVCAGSGVPLFSFIFGRIATDLMSDVGSAEAN
AAKTSLIMVYVGIGMFVASGAHVLCWTVAASRQEARIRVNFFRAVLRQDIGWHDEHSPGELTARMTGDIRVLRNGINDKLSQG
VMSAAMGIIGYIAGFIFSWELTLVMIGMMPLIIVMASIIGSIVAKMTESSRKYFSKAGSVATEVMENIRTVQVFGREDYELQRFAEE
VVRAQEHGIRKEFAGNLSAAVVMALVYISYTIAFFFGSYLVEWGRRDMADIISTFLAVLIGSFGLGAISTSATAFTESRAAAFEIFKVI
DRVPPVDIDAGGVPVTGFKQSIEFRNVQFSYPSRPDMILFRNLNLTIKCGQKIAFSGQSGCGKSSVMGLIQRFYDPVGGAVLC
DGMDLRELCLHDWRDQIGIVSQEPNLFAGTMMENVRVGKPNATEEEVIEACRQANIHDTIMSLPDQYNTPVGAVGSQLSG
GQKQRIAIARALVKKPPILLLDEATSALDRKSEMEVQRSLDQLMQKGKMTVIVIAHRLETIRNVDCIYYVKYDGAEGSKIAESGTF
DELMELGGEFAAMARVQGVPTGDAGNIAQEQSDSKLNDFPNSILDEATLAQLDEEAPRTERQKVPIEDLAKWEVNGVNVGFS
RLMGMNKGEAWAVALGVLGSAVRGASTPTNSILMGHMLRVLGEYNVDQNIERLRSGTNLYAPLFLVFAFSNFMGWILHGFY
GYAGEHLTTKIRVLLFRQIMRQDMSFFDIPGRDAGSLAGMLSGDCEAVRQLWGPSIGLKVQTACIIAAGIVVSFIYQWKLAFVA
LACMPLLIAGSVAERMMMNRYTKSKEGDAGDTIATEALSSVRTVTSFNMKADRVEAFEATLRLETPRNVKKGIIAGGIYGSTHFIS

FGAYALCFWYGGKLIDKGEAQFMDVMIASMSILFGAQSVGEAGAFATKLADAERSAKRIFSVIDRVPDVDIEQSGDKDLGKG
CEIDFRRVQFIYAARPKQVVLASVNMHFGEGTSNGLVGQTGCGKSTIIQMLARFYDRRSGLICVNGKDLSSLDIAEWRRNISSV
FQEPNLFSGTVRENIRYSRQDATDEEVEEAARLAHIHWEIMKWPEGYNTELGYKGSALSGGQKQRVAIARGLLRRPKLLLLDEAT
SALDNVTEAKVQEGINAYLEKYRVTSVSIAHRLTIIRHCDHIILLDSGHIIEEGSHEELMALGGEYKMRYDLYTGASS

LtaP35.2470 | Kinetoplastid membrane protein-11

MQEQNAKFFADKPDESTLSPEMKEHYEKFERMIKEHTDKFNKKMHEHSEHFKQKFAELLEQQKAAQYPAK

LtaP36.0240 | Histone H4, putative, pseudogene

MAKGKRSADAKGSQKRQKKVLRDNIRGITRGCVRRMARRGGVKRISSEIYEEVRRVLKAYVEDIVRCSTAXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXRRTASHQAMGPVRRCRCGVEPRSLCMAENGHAAEWKVISMTM

LtaP36.3280 | Hypothetical protein, conserved

MTSPSSSMPPAPPPALLPVEEPPIGTQPAAHLALGNTSQNTSSNSTAGAIVCDNCKTLGHLRRNCPKIKCNLCKRLGHYRRDCP
QDASKRVRSVEGAPHEEVNLDEEYRWSVCRNCGSSRHIQANCPVRYQALECYQCHQLGHMMTTCPQTRCYNCGTFGHSS
QICHSKPHCFHCSHSGHRSSECPMRSKGRVCYQCNEPGHEAANCPQGQLCRMCHRPGHLVAHCPEVVCNLCHVKGHTA
GVCDNVHCDNCGRNHETVNCHQPYMAPVPQDRVSEERSASLSSDHGCRSTGQSNNSATPHKSDDSASNRSDETRPATTIS
STPVVPPYVPLDEPKKDGRVAIVVDGSYFERCVSISRNRSDPAYYRHIANALLYTIDYIGDIFQREPFAFWFDTDTAAFTEFVEMSMP
LAHREKAFRETAMRKRYLTDEMNSSRSLPNVVAKLVGGMKRQRGFTEDGPGHVWVQTGVDVASATCVIELFQDRRQFQQVV
LLCGDADIYPAVQYCNTLRRSSHQHADSNPVRVCGTSDSMSKLYGQQQDLTDFLPRLLLDRPSHTEGGRTLEFPTSKVFLTAH

LtaP36.7210 | Protein disulphide isomerase

MKRLILAFVLCALLFCVASAEVQVATKDNFDKVVSGDLTLVKFYAPWCGHCKTLAPEFEKAADALAGVATLAEVDCTKEEDLSSK
YEIKGFPTLYIFRNGEKVKDYDGPRTAAGIVSYMKAQSGPSMKTIAKAEELEELKKEAFPVCVVKTASGDSKLASMMTKLAGSLRT
QMNFALVTDASISADDAMESVTVYRQGKEREAYSGITPMTEESVEKFLGIAVLNFFGELNQQTFQLYMNANKEKPLGWLFVDK
NTDPKLKESLVAVAEKYRSKVLLTHIDGEQYRPVSRQLGFPEDVKFPAFVMDLERRHHVMDTDIPVTPESIAEFVEKYLKGETKESM
MSEAVPEKETVNGLTTVVGHTIEKYTDGTQNVMLLFYAPWCGHCKKLHPEYEKVAKNLESENVIIAQLDATTNDFDRKKFEVSG
FPTMYFIPAGESPMVYDGGRTAEDIEAFLKPYLRASAASSDKPSGNSDDGDL

LtaPcontig168-1 | Universal minicircle sequence binding protein

MLTTQSLPHTLSFSTSLRLLLRYRILPFAFNQRLPFLPSHSRFLHSPLAEHTYTAFRFPESFSALFLSRSIFFSRSPLSIIMSAVTCYKCGEA
GHMSRSCPRVAATRSCYNCGETGHMSRDCPSERKPKSCYNCGSTEHLSRDCTNEAKAGADTRSCYNCGETGHMSRDCPN
ERKPKSCYNCGSTEHLSRECPDRH

S8 Candidates with CXXC motif

The following proteins with CXXC motif were identified as potential interactors of N-terminally His_8-tagged substrate 4 (with and without addition of TMAD in pull-down experiments), of full-length Erv and of truncated Erv^5: LtaP05.0570, LtaP10.0910, LtaP12.1000, LtaP14.0230, LtaP16.1340, LtaP22.0210, LtaP22.1200, LtaP23.0460, LtaP27.2110, LtaP28.1170, LtaP31.2190, LtaP32.3510, LtaP33.2110, LtaP33.2890, LtaP34.1140, LtaP34.3050, LtaP35.2200, LtaP36.3250, LtaP36.3280, LtaP36.4020, LtaP36.7210.

Among these LtaP12.1000, LtaP14.0230, LtaP16.1340, LtaP22.0210, LtaP22.1200 and LtaP23.0460 were additionally found to be potential interactors of the Erv knock-down in Trypanosoma brucei.[7] Proteins with CXXC motif identified as potential interactors of N-terminally His_8-tagged substrate 4 (with and without addition of TMAD in pull-down experiments) and of full-length Erv^5 are: LtaP06.1050, LtaP22.0750.

S9 Proteins in *Leishmania tarentolae* with a $CX_9CX_{12}CX_9C$ motif

All proteins from *Leishmania tarentolae* that contain a $CX_9CX_{12}CX_9C$ motif are listed below:[60] LtaP04.0900, LtaP08.0830, LtaP15.0660, LtaP22.0060, LtaP29.0620, LtaP34.0050, LtaP34.0490, LtaP35.3010, LtaP36.1080, LtaP36.6860.

S10 Drop dilution assays

Drop dilution assays are used in yeast to compare growth behaviour of different cell lines or to different additives. Drop dilution assays with *Leishmania* are rarely used.[110] Nevertheless, the use of this kind of assays for *Leishmania tarentolae* is conceivable, but the mobility of the cells on the surface must be overcome. Thus, Miro Halaczkiewicz and Tarik Begic developed suitable protocols as part of their internships. The following protocols are based on the work of Tarik Begic.

Stamp

BHI agar was mixed with the additives, poured into petri dishes and solidified with open lid for 10 min. After further cooling for 1 h at room temperature, a stamp was used to form

even depressions. The stamp was applied for 5 s. The cells were applied to the resulting wells in different concentrations according to the dilution series.

BHI agar: 3.7% (w/v) BHI, 0.8% (w/v) agar, 0.08% (w/v) folic acid, 10% (v/v) heat-inactivated FBS, 20 µg/mL hemin.

24-well plates

BHI agar was mixed with the additives, poured into 24-well plates and solidified with open lid for 10 min. The cells were added to the different wells in different concentrations according to the dilution series.

BHI agar: 3.7% (w/v) BHI, 0.8% (w/v) agar, 0.08% (w/v) folic acid, 10% (v/v) heat-inactivated FBS, 20 µg/mL hemin.

Proof of principle

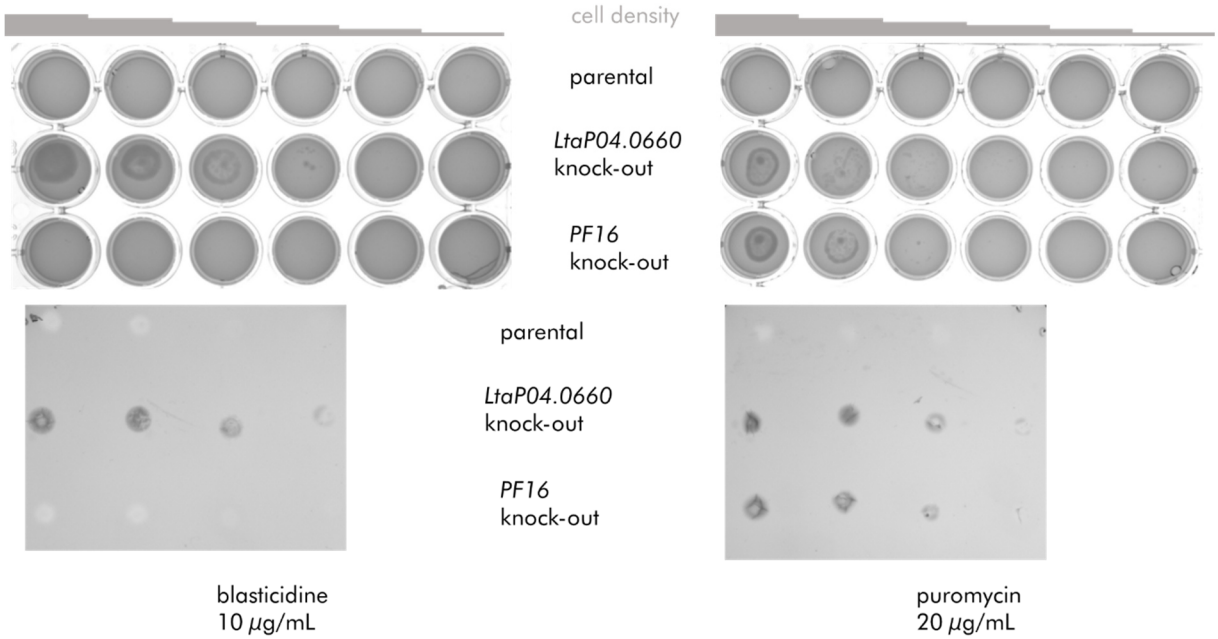


Figure 44: Images of drop dilution assays in 24-well plates (top panels) or carried out with a stamp (bottom panels). The experiments were performed and documented by Tarik Begic. The dilution series of the cells started with 2 x 10^7 cells/mL. The dilution factor between the wells or spots was 1:5. 2 µL of the cell suspensions were applied for each dilution. The parental strain from Turra[5] has no resistance. Cells with homozygously inserted knock-out cassettes for *LtaP04.0660* (encoding substrate 1) were available from the present work and exhibited resistance against blasticidine and puromycin. Cells with knock-out cassettes for *PF16* (encoding flagellar motility factor) were available from the work of Turra et al.[78] and exhibited resistance against puromycin. The images were taken after 6 days of incubation.

Curriculum vitae

Luzia Schneider

Higher education

April 2019 – June 2022

PhD studies at the TU Kaiserslautern

PhD thesis in the group of Prof. Marcel Deponte

Title of the thesis: „Oxidative folding of proteins in the mitochondrial intermembrane space of *Leishmania tarentolae*“

April 2016 – December 2018

Master of science at the University of Konstanz

Master thesis in the group of Prof. Helmut Cölfen, in cooperation with BASF SE

Title of the thesis is confidential

October 2012 – March 2016

Bachelor of science at the University of Konstanz

Bachelor thesis in the group of Prof. Sebastian Polarz

Title of the thesis: „Synthese und Untersuchung anisotroper Zinkoxidnanopartikel in ionischen Flüssigkeiten“ (synthesis and investigation of anisotropic zinc oxide nanoparticles in ionic liquids)