Research Paper

A single-cysteine mutant and chimeras of essential *Leishmania* Erv can complement the loss of Erv1 but not of Mia40 in yeast

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Mia40/CHCHD4 and Erv1/ALR are essential for oxidative protein folding in the mitochondrial intermembrane space of yeast and mammals. In contrast, many protists, including important apicomplexan and kinetoplastid parasites, lack Mia40. Furthermore, the Erv homolog of the model parasite *Leishmania tarentolae* (*LtErv*) was shown to be incompatible with *Saccharomyces cerevisiae* Mia40 (*ScMia40*). Here we addressed structure-function relationships of *ScErv1* and *LtErv* as well as their compatibility with the oxidative protein folding system in yeast using chimeric, truncated, and mutant Erv constructs. Chimeras between the N-terminal arm of *ScErv1* and a variety of truncated *LtErv* constructs were able to rescue yeast cells that lack *ScErv1*. Yeast cells were also viable when only a single cysteine residue was replaced in *LtErv*. Thus, the presence and position of the C-terminal arm and the kinetoplastida-specific second (KISS) domain of *LtErv* did not interfere with its functionality in the yeast system, whereas a relatively conserved cysteine residue before the flavodomain rendered *LtErv* incompatible with *ScMia40*. The question whether parasite Erv homologs might also exert the function of Mia40 was addressed in another set of complementation assays. However, neither the KISS domain nor other truncated or mutant *LtErv* constructs were able to rescue yeast cells that lack *ScMia40*. The general relevance of Erv and its candidate substrate small Tim1 was analyzed for the related parasite *L. infantum*. Repeated unsuccessful knockout attempts suggest that both genes are essential in this human pathogen and underline the potential of mitochondrial protein import pathways for future intervention strategies.

1. Introduction

The vast majority of mitochondrial proteins are synthesized at cytosolic ribosomes and have to be imported into one of the four mitochondrial compartments [1]. One of these compartments, the intermembrane space (IMS) between the inner and outer mitochondrial membrane, harbors proteins that contribute to respiration, programmed cell death, the assembly of protein complexes, and the transfer of ions, metabolites and proteins [2–6]. In addition to a functional classification, IMS proteins can be also grouped according to their protein import pathway (reviewed in [2,7]): One group of proteins, hereinafter referred to as class I proteins, contains a positively charged N-terminal matrix-targeting signal that is followed by a hydrophobic transmembrane segment. This bipartite pre-sequence is recognized by the TIM23 complex, which inserts class I proteins into the inner mitochondrial membrane where they often undergo limited proteolysis. For example, yeast cytochrome b3 is imported via this pathway and its soluble domain is subsequently released into the IMS by the inner membrane protease Imp1 [8–10]. Another group of proteins from yeast and mammals, hereinafter referred to as class 2 proteins, is oxidatively trapped in the IMS by a system that consists of Mia40 and Erv1 in yeast or the respective mammalian homologs CHCHD4 and ALR.
2.1. L. infantum cell culture

L. infantum promastigotes (strain MHOM MA67/T1MAP263) were cultured at 25 °C in RPMI 1640 Glutamax supplemented with 10% (v/v) inactivated fetal bovine serum (FBS), 50 U/mL penicillin, 50 mg/mL streptomycin (Gibco), and 25 mM HEPES sodium salt, pH 7.4 (Sigma). Parasites were synchronized by three or four daily passages of 1 × 10^6 cells/mL and subsequently seeded at 10^6 cells/mL (day 0). Afterwards, growth curves for wild-type and mutant strains were recorded for up to 7 days by determining the cell density in a hemocytometer.

2.2. Generation of L. infantum knockout and rescue constructs

Fragments of the 5’ and 3’ UTRs of L. infantum ERV (LiERV) for homologous recombination were PCR-amplified from L. infantum genomic DNA using primer pairs P1/P2 and P3/P4 (Table S1). Both PCR products were cloned into the HindIII/Spel and BamHI/XhoI restriction sites of plasmids pGL345 and pGL726, which carry coding sequences for hygromycin B phosphotransferase (Hygr) or phosphomycin amidase (PhleoR), respectively. Deletion constructs for L. infantum small TIM (LiTIM) were generated likewise. Fragments of the 5’ and 3’ UTRs of LiTIM1 were PCR-amplified with primers P5/P6 and P7/P8 (Table S1) and cloned into the HindIII/Spel and BamHI/XhoI restriction sites of pGL345 and pGL726. Before transfection of L. infantum, the replacement cassettes were excised from the plasmids by digestion with HindIII/BglII and purified from agarose gels. Rescue plasmid pTEX-NEO-LiERV was assembled by cloning the LiERV ORF, which was PCR-amplified with primers P9/P10 (Table S1), into the BamHI/HindIII restriction sites of pTEX-NEO. This trypanosomatid expression vector carries a coding sequence for the neomycin phosphotransferase (NeoR) [46]. To generate the pTEX-NEO-LiTIM rescue plasmid, the LiTIM ORF was PCR-amplified with primer pair P11/P12 (Table S1) and cloned into the BamHI/XhoI sites of pTEX-NEO.

2.3. L. infantum transfection and selection

Transfections were performed in an Amaxa Nucleofector (Lonza) using program U-033 as described previously [47]. Briefly, 5 × 10^7 logarithmic phase promastigotes were suspended in Human T Cell Nucleofector (Lonza) and transfected with 2–10 μg DNA. Parasites were allowed to recover in 10 mL of culture medium without selection drug for 24 h before centrifugation and plating on medium M199 agar plates containing 10% (v/v) iPBS, 50 U/mL penicillin, 50 mg/mL streptomycin, 0.1 mM adenine, 0.023 mM hemin, 25 mM HEPES sodium salt, pH 7.4 (all from Sigma), and the selective drug(s). Hygromycin (Invitrogen) was used at 15 μg/mL, G418 (Sigma) at 15 μg/mL, and phleomycin (Sigma) at 17.5 μg/mL. Colonies were picked after 1–2 weeks and transferred to liquid medium.

2.4. Generation and site-directed mutagenesis of yeast constructs

Full length LTERV, LTERV\(^{\text{17S}}\), LTERV\(^{\text{C89S}}\), LTERV\(^{\text{C300S}}\) and LTERV\(^{\text{C300S}/\text{C304D}}\) in plasmid pQE30 as well as SCERV1 and PFERV in pXY232 were cloned previously [43]. Additional mutant and chimeric pXY232-LTERV constructs were generated as follows. LTERV\(^{\text{C302/C1095}}\) in pQE30 was generated by two rounds of site-directed mutagenesis using primer pairs P26/P27 and P28/P29 (Table S2) and pQE30-LTERV as a template. LTERV, LTERV\(^{\text{C17S}}\), LTERV\(^{\text{C89S}}\), LTERV\(^{\text{C300S}/\text{C304D}}\) and LTERV\(^{\text{C302/C1095}}\) were PCR-amplified using primer pair P30/P31 (Table S2) with the according pQE30 constructs as templates. The truncated constructs LTERV\(^{\text{C-arm}}\) and LTERV\(^{\text{N-arm}}\) were PCR-amplified using primer pairs P32/P31 and P30/P33 (Table S2) with pQE30-LTERV as a template. Chimera 1–5 were amplified by overlap extension PCR according to a standard protocol [48] using sense primer P34, overlap primer pair P35/P36 or P37/P38, and one of the antisense primers P39-P41 (Table S1) with pXY232-SCERV1 and pXY232-LTERV as templates. Fusion constructs encoding the N-terminal mitochondrial targeting signal and transmembrane segment of ScMia40 (Mia40N) MIA40N-PFERV, MIA40N-LTERV, MIA40N-LTERV\(^{\text{C-arm}}\), MIA40N-LTERV\(^{\text{N-arm}}\), MIA40N-LTERV\(^{\text{C-arm}}\) and MIA40N-Chimera 1–6 were also amplified by overlap extension PCR using the sense primer P48, one of the overlap primers P51-P54, and one of the antisense primers P31/P33/P34/P40/P41/P50. All PCR products were subsequently cloned into the EcoRI/XhoI restriction sites of the yeast expression vector pYX232. Chimera 6 was generated by site-directed mutagenesis using primer pair P42/P43 (Table S2). The truncated Mia40 variant SCMA40\(^{\text{Carm}}\) (encoding residues 1–70 and 284–403) was previously cloned into the
single-copy plasmid pRS314 [49] and was a kind gift by Johannes Herrmann. All constructs were confirmed by commercial sequencing (GATC Biotech).

2.5. Yeast cell and complementation assays

S. cerevisiae cells were grown at 30 °C in 1% yeast extract, 2% peptone (YP) medium or in synthetic (S) minimal medium supplemented with the bases adenine and uracil [50]. For growth analysis, the medium contained either 2% glucose (YPD, SD), 3% galactose (SGal) or 3% glycerol (SG). Plasmid shuffling experiments were described as previously [43] with a YPH501-derived Δerv1 strain (genotype ura3-52, lys2-801::mbr, ade2-101::trp1-Δ63, his3-Δ200, leu2-Δ1, erf1::His3) that harbors a SCERV1 copy under control of the MET25 promoter on the URA3-containing plasmid pRS426 [51]. Briefly, cells were transformed with the TRP1-containing plasmids pYX232-SCERV1, pYX232-LTERV, pYX232-Chimera 1–6, pYX232-LTERVcer2×, pYX232-LTERVcerN, pYX232-LTERVcer2×N, pYX232-LTERVcer2×Nter, pYX232-LTERVcer2×NterΔ63, pYX232-LTERVcer2×NterΔ63ΔC109S, and the respective MIA40N constructs according to a standard protocol [52]. After transfection and selection on SD agar plates without histidine, uracil and tryptophan, yeast strains were grown overnight in uracil-containing liquid medium. Overnight cultures were diluted in sterile water to an optical density of 600 nm (OD600) of 0.1 and further diluted 1:10, 1:100 and 1:1000. Drops (10 µL) of the overnight cultures and each serial dilution were spotted on SD agar plates without histidine and tryptophan containing 50 mg/L uracil and 1 g/L 5-fluoroorotic acid (FOA) for negative selection and plasmid shuffling [53]. The same dilutions were spotted on SD agar plates without histidine, uracil and tryptophan as a control. Analogous complementation assays for plasmids pYX232-FFERV, pYX232-LTERV, pYX232-FFERVcer2×, pYX232-LTERVcer2×Nter, and the respective MIA40N constructs and pRS314±SCMIA400 constructs were performed with a Δamio40 strain, which has the same genetic background with a Δamio40:S.pombeHis5 instead of the erf1::His5 replacement. This strain harbors a copy of a short functional version of SCMIA40 under the control of its endogenous promotor on the URA3-containing plasmid pRS316 [49]. All plates were incubated at 30 °C or the indicated temperature for up to one week. Single colonies or, in the absence of colonies, swabs of background cells from FOA plates were subsequently grown at 30 °C for 48 h. Plates were shaken at 183 rpm for 550 s before each hourly measurement.

2.6. Cell fractionation and western blot analysis

Clones from plasmid shuffling experiments that only grew on uracil-containing medium were incubated in 50 mL SD liquid medium without histidine and tryptophan until they had reached an OD600 of 1.0–1.8. Ten OD volumes of cells were harvested by centrifugation (1750 × g, 10 min, 4 °C) and disrupted as described previously [54]. Briefly, yeast cells were resuspended in 300 µL ice-cold buffer containing 0.6 M sorbitol, 80 mM KCl, 20 mM MOPS, pH 7.4 that was freshly supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) before 200 µL glass beads (Ø 0.5 mm, Sigma-Aldrich) were added. The mixture was vortexed six times for 30 s before glass beads and cell debris were removed by centrifugation (1000 × g, 3 min, 4 °C). The supernatant was centrifuged again (20,000 × g, 15 min, 4 °C) to separate the mitochondria-containing pellet fraction from the cytosol-containing supernatant fraction. The pellet fraction was directly boiled in 2 × SDS-PAGE Laemmli-buffer. The supernatant fraction was precipitated by the addition of 5–6 volumes of ice-cold acetone to 150 µL supernatant and incubated overnight at −20 °C. The mixture was subsequently centrifuged (15,000 × g, 15 min, 4 °C) and the precipitate was air dried for 15 min at room temperature before boiling in 2 × SDS-PAGE Laemmli-buffer. For western blot analysis, 20 µL of the pellet sample and 10 µL of the supernatant sample were separated by SDS-PAGE on a 15% gel followed by wet blotting and immunodecoration with antibodies against L. infantum promastigotes were prepared as follows. Parasites were harvested by centrifugation (3000 × g, 10 min, 4 °C) and washed twice in ice-cold PBS. Parasites were suspended at 6.25 × 10⁸ cells/mL in 4% (w/v) SDS, 50 mM Tris-HCl, pH 7.4 and stored at −70 °C until further use. Before separation by SDS-PAGE, 8 µL extracts were mixed with 12 µL PBS and 5 µL 5 × SDS-PAGE Laemmli-buffer and heated for 10 min at 65 °C.

2.7. Yeast DNA extraction and PCR analysis

Plasmid DNA was extracted from yeast as described previously [57]. For PCR analysis, 1 µL of extracted DNA was mixed with 1 µL of primer pairs P44/P45, P44/P46, and P47/P45, 400 µM dNTP mix (Thermo Scientific) and 2.5 U Taq DNA polymerase (NEB) in a final volume of 50 µL to amplify SCERV1, Chimera 3, and Chimera 6, respectively. Initial denaturation was carried out at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 60 °C for 45 s, and elongation at 68 °C for 45 s. The last cycle was followed by a final elongation step at 68 °C for 90 s.

2.8. Yeast growth assays on agar plates and in liquid medium

For growth assays on agar plates, yeast strains were incubated in 1 mL liquid SD medium without histidine and tryptophan and cultured in the logarithmic growth phase for up to five days. Drop dilutions assays in sterile water were subsequently performed as described above. Dilutions were spotted on SD, SG or SGal agar plates without histidine and tryptophan. All plates were incubated at 30 °C for up to 12 days. Growth assays in liquid medium were performed in a Tecan Infinite F200 Pro plate reader using transparent flat bottom 96-well-plates (Greiner). Briefly, 200 µL of liquid cultures in SD, SG or SGal medium were diluted to an absorbance of 0.1 in the according medium and were grown at 30 °C for 48 h. Plates were shaken at 183 rpm for 550 s before each hourly measurement.

2.9. Protein import into isolated mitochondria

Radiolabeled Tim13 precursor protein was synthesized in vitro in a TNT-coupled reticulocyte lysate system according to the manufacturer’s standard protocol (Promega). Mitochondria were isolated as described [13] and quantified based on their protein content using a Bradford protein assay (BioRad). The import of Tim13 precursor protein into isolated mitochondria was performed in import buffer [56] containing 0.1 mg/mL BSA, 5 mM NADH, 2.5 mM ATP and 2 mM 2-mercaptoethanol. The import reaction contained 15% of lysate and 100 µg of mitochondria in 100 µL import buffer. The import reaction was incubated for 10 min at 25 °C and then stopped by diluting the reaction tenfold with ice-cold SH-buffer (0.6 M sorbitol, 20 mL HEPES-KOH, pH 7.4) with 50 µg/mL proteinase K. Proteinase K treatment was performed for 20 min and then stopped by addition of 2 mM PMSF. Following isolation of mitochondria, the import of precursor protein was analyzed by SDS-PAGE and autoradiography.

2.10. Mia40 redox mobility shift assays

Mitochondria (1 mg/mL) were incubated for 20 min at 25 °C in SH-
buffer with or without 5 mM reduced glutathione (GSH). Following incubation, the samples were treated for 20 min at 25 °C with 40 mM iodoacetamide in 50 mM Tris (pH 8.0), 0.6 M sorbitol to prevent artificial oxidation. The redox state of re-isolated mitochondria was analyzed by non-reducing SDS-PAGE and western blotting with antibodies against ScMia40. The buffers used were first degassed for 10 min using a Vario vacuum pump (Vacuumbrand) and then flushed with nitrogen. All sample cups were also flushed with nitrogen.

2.11. Ni-NTA pull-down of His-tagged ScMia40

Pull-down assays were performed with a yeast strain that encodes a His-tagged version of ScMia40 on the plasmid pYX142 replacing wild type ScMia40 [27]. This strain was transformed with the plasmid pYX232-Chimera 2 or the plasmid pYX232-SCERV1. Mitochondria were isolated from cells that were grown in selective medium without tryptophan and leucine containing 2% lactate and 0.5% galactose. For the Ni-NTA-agarose pull-down, mitochondria were solubilized in buffer containing 0.5% (w/v) Triton X-100 in buffer A (20 mM Tris, 80 mM KCl, 30 mM imidazole, 1 mM PMSF, pH 7.4) for 30 min at 4 °C. After a clarifying spin, solubilized material was incubated with 25 μL of Ni-NTA-agarose beads for 1 h. Following removal of the supernatant, beads were washed three times with buffer A containing 0.05% Triton X-100 and bound proteins were eluted with Laemmli buffer containing 300 mM imidazole. Total and elution fractions were then analyzed by SDS-PAGE and western blotting.

3. Results

3.1. ERV and small TIM1 cannot be deleted in L. infantum

Kinetoplastid parasites lack Mia40, which raises the question whether they can survive in the absence of Erv or putative substrates. In order to test the relevance of Erv in Leishmania, we tried to disrupt the encoding gene in the human pathogen L. infantum (locus LinJ.15.1020) by homologous recombination (Fig. 1A). Clonal heterozygous erv+/− strains were readily obtained after transfection of L. infantum promastigotes with plasmid pGL345-LIERV and selection with hygromycin as confirmed by PCR analysis (Fig. 1B). Growth curve analysis for liquid cultures of wild-type (wt) and erv+/− strains revealed no morphological abnormalities or drastic growth defects (Fig. 1C), and both strains had similar L erv protein levels (Fig. S1A). The second LI erv allele could not be deleted after subsequent transfection with plasmid pGL726-LIERV and selection with pheleomycin, even after the introduction of an additional gene copy on plasmid pTEX-NEO-LIERV (Fig. 1B). The additional LIERV copy was functional as reflected by a drastic increase in the L erv protein concentration (Fig. S1A), which might explain the short lag phase and decreased maximum cell density for liquid cultures of erv+/− strains that carried the episomal LIERV copy when grown in the presence of the selection antibiotic G418 (Fig. 1C).

To exclude systematic methodological causes for the negative results regarding the generation of homozygous erv+/− knockout parasites, we performed parallel experiments for LIsTIM1 (locus LinJ.25.1610) using the same knockout strategy with plasmids pGL345-LisTIM1 and pGL726-LisTIM1 (Fig. 1A). LIsTim1, the homolog of the IMS marker protein small Tim1 from L. tarentolae, is a candidate substrate for oxidative protein folding in Leishmania mitochondria [42]. Heterozygous hygromycin-resistant stin+/− parasites revealed no abnormal morphology, drastic growth defects or altered protein levels compared to the wt strain (Fig. 1C, Fig. S1B). A homozygous stin+/− knockout strain was only obtained in the presence of an additional gene copy on plasmid pTEX-NEO-LisTIM1 (Fig. 1B). Again, complemented strains had a slight growth defect in the presence of G418 (Fig. 1C) and the protein concentration of LisTim1 was drastically up-regulated (Fig. S1B). The complemented stin+/− knockout strain neither lost its plasmid nor LisTim1 and remained resistant to G418 even after twelve months of continuous culture in the absence of the selection drug, whereas a wt control strain lost the empty plasmid pTEX-NEO over time (Fig. 1D, Fig. S1B). Thus, small Tim1 is probably essential in promastigotes of L. infantum, indicating the importance of the oxidative folding pathway for parasite survival despite the absence of a Mia40 homolog. Repeated negative results for parallel LIERV knockout attempts with LisTIM1 as a control also support the interpretation that Erv is essential in L. infantum.

3.2. Chimeric L erv can complement the loss of ScErv1

L erv appears to function in Leishmania without a Mia40 homolog and, therefore, may require structural features that are incompatible with the oxidative protein folding system in yeast. To analyze which structure-function relationships might be relevant for the inability of L erv to complement the loss of ScErv1 in yeast [43], we generated a set of chimeras. L erv differs from ScErv1 in two major aspects, (i) the C-terminal addition of a ketoplastida-specific second (KISS) domain and (ii) the position of two redox-active 'distal' or 'shuttle' cysteine residues at the C-terminal instead of the N-terminal arm (Fig. 2A). The shuttle cysteines transfer electrons to the conserved 'proximal' cysteines within the flavodomain [16,43]. To address the relevance of these structural elements, we either replaced the N-terminus before the erv domain of L erv with the N-terminal arm of ScErv1 (chimera 1–3) or the complete N-terminal half of L erv with ScErv1 (chimera 4 and 5). A potential negative effect of the C-terminal arm or the KISS domain was addressed by systematically removing these structural elements in chimera 2, 3 and 5. Furthermore, to check whether the shuttle CQVYC-motif of L erv is incompatible with the oxidative folding machinery in yeast, we replaced the CRSC-motif at the N-terminal arm of ScErv1 in chimera 6. The chimeric constructs were analyzed in complementation assays on agar plates with 5-fluoroorotic acid (FOA) for negative selection against the episomal copy of SCERV1 in a haploid Δ erv knockout strain (Fig. 2B). In contrast to the negative control, which was transformed with wild-type L IERV, viable yeast strains were obtained from FOA agar plates for all chimeras. The absence of ScErv1 and the presence of the chimeric proteins in yeast mitochondria were confirmed for at least three different clones of each construct-harboring strain by subcellular fractionation and western blot analysis (Fig. 2C, Fig. S2A). Furthermore, we confirmed the genotype for chimera 3 and 6 by analytic PCR (Fig. S2B).

Replacing the N-terminus of L erv with the N-terminal arm of ScErv1 allowed L erv to rescue the Δ erv strain in complementation assays (Fig. 2B, Fig. S3A). Yeast cells with chimera 3 grew slowly but were viable, suggesting that the erv domain of L erv in combination with the N-terminal arm of ScErv1 is sufficient to be functional. Addition of the C-terminal arm and/or the KISS domain improved the growth of chimera 1 and 2, which was still not as good as the growth of the positive control with ScErv1. This suggests that the erv domain of L erv might not be able to fully replace the function of the endogenous erv domain (Fig. S3A). Taking into account the improved growth of chimera 1 and 2 compared to chimera 3, it is unlikely that the KISS domain and the C-terminal arm have a deleterious effect on the function of L erv in yeast. Complementation assays for chimera 4 and 5, in which the KISS domain alone or together with the C-terminal arm of L erv were fused to the ScErv1 domain, also support the absence of a dominant-negative effect (Fig. 2B), for example, by inactivating Mia40. Furthermore, the CQVYC-motif of L erv in chimera 6 could functionally replace the CRSC-motif at the N-terminal arm of ScErv1. Thus, the shuttle arm motif of L erv is, at least in part, compatible with yeast Mia40. In summary, attachment of the N-terminal arm of ScErv1 to L erv chimeras suffices to complement and rescue the Δ erv1 strain. Neither the presence of the KISS domain or the C-terminal arm nor the shuttle cysteine motif of L erv render the protein inactive and cause the incompatibility of L erv with the oxidative folding machinery in yeast mitochondria.
3.3. Mutant LtErvc17S can complement the loss of ScErv1

The chimera complementation assays suggested that LtErvc, at least in principle, functionally replace ScErvc1. To further address the structural requirements for a successful complementation, we analyzed additional LtErvc constructs in which we removed the N-terminal or C-terminal half of the protein (LtErvc-term and LtErvcN-term) or replaced the different cysteine residues (Fig. 2D). Neither LtErvc-term nor LtErvcN-term was able to complement the loss of ScErvc1 (Fig. 2E, Fig. S3A). The latter result indicates that the flavodoxin plus the N-terminal sequence of LtErvc is not sufficient to complement for ScErvc1 in yeast. It also excludes that the inability of full-length LtErvc to complement is due to a negative effect of the C-terminal half on its otherwise functional N-terminal half (in accordance with the growth of the truncated chimera in Fig. 2B). Systematic mutation of the distal, proximal, structural, and clamp cysteine residues of LtErvc revealed that replacement of clamp residue Cys17 results in a functional mutant that was able to complement the loss of ScErvc1 on FOA agar plates (Fig. 2E, Fig. S3A). The absence of ScErvc1 and the presence of LtErvcC17S in yeast mitochondria were confirmed for different LtErvcC17S clones by subcellular fractionation and western blot analysis. Clones of all other LtErvc mutants were either not viable or still contained ScErvc1 (Fig. 2F, Fig. S4). Noteworthy, mutations of the distal cysteine residues in LtErvcC300S/C304S or of one of the proximal cysteines in LtErvcC63S appeared to have no effect.

**Fig. 1.** Knockout attempts for ERV and sTIM1 in *Leishmania infantum*. (A) Schematic summary of the LIERV and LisTIM1 knockout strategy by homologous recombination using excised replacement cassettes from plasmids pGL345 and pGL726. Different selectable marker genes (HygR and PheoR) were used in two rounds of transfection to target both alleles in diploid promastigotes. Experiments were performed with and without episomal copies of LIERV or LisTIM1 on rescue plasmid pTEX-Neo. Dashed lines indicate the sites of homologous recombination at the 5′ and 3′ untranslated regions (UTRs). Expected product sizes from analytic PCR reactions 1–12 are highlighted. (B) PCR analysis of the indicated parasite lines for (i) the absence or presence of chromosomal LIERV (PCR 1) and LisTIM1 (PCR 5 and 6), (ii) the 5′ and 3′ integration of the selectable marker genes (PCR 2, 3 and 7–10), and (iii) the absence or presence of episomal LIERV (PCR 4) or LisTIM1 (PCR 11). (C) Growth curve analysis for synchronized liquid cultures of the indicated parasite lines. Parasites that were complemented with an episomal copy of LIERV or LisTIM1 were grown with or without 50 µg/mL G418 (+/- G418). Values represent the mean and standard deviation of three independent biological replicates. (D) PCR analysis of the indicated parasite lines for the absence or presence of episomal LisTIM1 (PCR 11) or the pTEX-Neo selection marker NeoR (PCR 12) after twelve months of continuous culture in the absence or presence of 15 µg/mL G418.
on the mitochondrial localization of the proteins, whereas LtErvC17S was also found in the cytosolic fraction.

In order to test whether the presence of Cys17 might have masked a positive complementation for the other LtErv mutants, we repeated the SCERV1 complementation experiments with the C17S constructs LtErvN-term/C17S, LtErvC17S/C63S, LtErvC17S/C300S, LtErvC17S/C300S/C304S, and LtErvC17S/C92S/C109S. However, none of these constructs could complement the loss of ScErv1 (Fig. S5).

In summary, Cys17 renders LtErv non-functional in the yeast system, and the presence of this cysteine residue is, at least in part, the cause for the incompatibility between LtErv and the oxidative folding machinery in yeast mitochondria. Replacing either residue Cys17 with serine or residues 1–16 of LtErv with the N-terminus of ScErv1 in chimera 1–3 partially rescues the functionality of LtErv and its compatibility with the yeast system.

3.4. LtErvC17S and chimera 1, 2 and 6 have specific carbon source-dependent growth defects

To further characterize the complemented yeast strains, we compared their growth in liquid or on agar minimal medium with glucose (SD), galactose (SGal) or glycerol (SG) as a carbon source (Fig. 3 and Fig. S6). Chimera 1 had a pronounced growth defect on all three carbon sources. This phenotype was observed in liquid medium and on agar plates. In contrast, chimera 2 showed a growth defect in SG liquid medium but not in SGal or SD medium. Chimera 2 also grew normal on SG agar plates. Chimera 3 revealed no striking phenotype, whereas chimera 4 and 5 grew slightly faster on SGal and SG agar plates than the control. This phenotype was absent in liquid medium. Chimera 6 revealed a growth defect on SG agar plates and in SD liquid medium. Cells with LtErvC17S grew normally in SD medium but reached a much lower
final cell density in SGal or SG liquid medium. Noteworthy, the strain grew faster during the initial 12 h in SG medium than the control. The phenotype of LrErv<sup>CT75</sup> was specific for liquid medium and was absent on agar plates. The different phenotypes for LrErv<sup>CT75</sup> on SD and SGal medium are quite surprising taking into account that galactose is converted to glucose-6-phosphate by the Leloir pathway [58], which should not be directly affected by the mitochondria. In summary, pronounced growth defects on SG medium for chimera 1 and 2 in liquid medium as well as for chimera 6 on agar plates point towards an impaired mitochondrial respiration in these strains.

3.5. Chimera 1, 2 and 6 have an impaired oxidative folding pathway

Analysis of the substrates of the Mia40/Erv1 system by western blotting revealed reduced steady-state protein levels in mitochondria with chimera 1, 2 and 6 compared to control mitochondria with ScErv1 (Fig. 4A). In contrast, mitochondria with chimera 4 and 5 had normal steady-state substrate concentrations. The results suggest that the CRSC-motif of ScErv1 has been optimized for the interaction with ScMia40 and/or the ScErv1 flavodomain during the course of evolution, as reflected by chimera 6, which functions with a decreased efficiency compared to the control. Furthermore, the N-terminal arm of ScErv1 and the proximal cysteines of the flavodomain of LtErv in chimera 1 and 2 are not perfectly compatible.

The altered steady-state concentrations were specific for class II IMS proteins, which are oxidatively trapped [2]. Other mitochondrial proteins were not affected by the chimeras (Fig. 4A). This also includes ScMia40 itself, which has an N-terminal mitochondrial targeting signal and a transmembrane segment that is inserted into the inner mitochondrial membrane by the TIM23 complex before ScMia40 is oxidatively folded by the Mia40/Erv1 system [11,12,18,25,59]. An impaired protein import of the class II reference protein ScTim13 was confirmed using in vitro import assays (Fig. 4B). Again, mitochondria with chimera 1, 2 and 6, but not with chimera 4 and 5, imported less ScTim13 compared to mitochondria with ScErv1.

The underlying cause for the impaired protein import of class II proteins was presumably the altered ratio between reduced and oxidized ScMia40, as revealed by redox mobility shift assays and western blot analysis (Fig. 4C). The redox state of ScMia40 from isolated mitochondria with chimera 1, 2 and 6 was shifted to the reduced state compared to the redox state of ScMia40 from mitochondria with ScErv1 or chimera 4 and 5, which were predominantly oxidized. Thus, oxidation of ScMia40 by ScErv1 or chimera 4 and 5 is probably more efficient than the oxidation of ScMia40 by chimera 1, 2 and 6. This interpretation is also supported when isolated mitochondria were challenged with GSH. GSH treatment yielded almost completely reduced ScMia40 for chimera 1 and 2 in contrast to mitochondria with chimera 4 or ScErv1 (Fig. 4C).

Oxidation of ScMia40 by ScErv1 requires the physical interaction of both proteins. We previously showed that LrErv cannot replace ScErv1 and that wild type LrErv does not interact with ScMia40 [43]. Taking into account that an attachment of the N-terminal arm of ScErv1 to the flavodomain of LrErv rescued the deletion of SCERV1 (Fig. 2), we tested an interaction with ScMia40 for one of these chimera in a Ni-NTA pull-down experiment. Indeed, His-tagged ScMia40 co-precipitated with chimera 2, albeit with reduced efficiency compared to ScErv1 (Fig. 4D). As negative control, a hydrophobic inner membrane protein, the ATP/ADP-carrier (ScAAC), was not co-precipitated, indicating the specificity of the pull-down experiment. The experiment supports the hypothesis that the N-terminal arm of ScErv1 of the chimera oxidizes ScMia40 and subsequently transfers the electrons to the flavodomain of LrErv. However, the functionality of the N-terminal arm of ScErv1 appears to be impaired by the presence of the flavodomain of LrErv, which shifts the steady-state equilibrium between oxidized and reduced ScMia40 towards the reduced form.

In summary, oxidation of ScMia40 by chimera 1, 2 and 6 appears to be less efficient than oxidation by chimera 4 and 5. The subsequent increase of reduced ScMia40 in mitochondria with chimera 1, 2 and 6 most likely impairs the import of substrates of the Mia40/Erv1 system in vitro and in vivo and results in decreased levels of these proteins in mitochondria.
3.6. \textit{LtErv} cannot functionally replace yeast Mia40

Our results show that the KISS domain does not interfere with the function of \textit{LtErv} in yeast. A potential function of the KISS domain, which lacks a cysteine residue \cite{43}, might be the recruitment of protein thiol substrates before they interact with the shuttle disulfide. Thus, the KISS domain and the shuttle arm might functionally replace Mia40, which does not only introduce disulfide bonds but also serves as a receptor and binds to a hydrophobic patch of the imported substrate \cite{26,28,29,31,49,60–63}. To test the hypothesis whether protist Erv homologs are able to replace Mia40, we tried to complement a \textit{Δmia40} strain with genes encoding the Erv homolog from \textit{P. falciparum} (which lacks a KISS domain), full length \textit{LtErv}, \textit{LtErvC-term}, \textit{LtErvN-term}, and \textit{LtErvC17S} (Fig. 5). Negative selection on FOA agar plates against the episomal copy of a short functional version of \textit{SCMIA40} in the \textit{Δmia40} knockout strain revealed that none of the constructs could complement the loss of ScMia40 (Fig. 5C). This was also the case when alternative temperatures were tested (Fig. 5D, Fig. S3B). In a subsequent set of experiments, we fused the N-terminal mitochondrial targeting signal and transmembrane segment of ScMia40 to our protist Erv constructs in order to localize the proteins to the same location in the inner mitochondrial membrane as endogenous ScMia40 strain with genes encoding the Erv homolog from \textit{P. falciparum} (which lacks a KISS domain), full length \textit{LtErv}, \textit{LtErvC-term}, \textit{LtErvN-term}, and \textit{LtErvC17S} (Fig. 5).
(Fig. 5E). However, none of these Mia40N-fusion constructs was able to complement the loss of ScMia40 (Fig. 5F,G). In a last set of experiments, we addressed the hypothesis whether some of the chimeric LtErv constructs from Fig. 2 have a Mia40-like activity. We therefore tested chimera 1–6 as well as their fusion constructs with the N-terminal mitochondrial targeting signal and transmembrane segment of ScMia40 in SCMIA40 complementation assays. Again, none of the twelve constructs could complement the loss of ScMia40 (Fig. S7). In summary, neither PfErv nor LtErv or structural elements of LtErv, such as the KISS domain and the C-terminal arm, can functionally replace ScMia40.

4. Discussion

Our knockout studies for LiErv and LtTIM1 suggest that both genes are essential for parasite survival and underline the potential of mitochondrial protein import pathways for future intervention strategies against important pathogens. An analogous knockout strategy has been previously used to show that a cytosolic tryparedoxin is essential for L. infantum survival [64]. Furthermore, RNAi studies in the related kinetoplastid parasite T. brucei suggested that TbErv is essential [44,45,65]. Nevertheless, we are aware that viable knockout strains with a drastic growth defect can be missed without negative selection and that the recent establishment of CRISPR/Cas9 systems in Leishmania (66–68) could be extremely helpful to get a definite answer regarding the essentiality of Erv and StTim1 in Leishmania pathogens.

Previous studies on Erv homologs from human, yeast, L. tarentolae, T. brucei, and Arabidopsis thaliana revealed that the redox-active shuttle cytoxines are present in highly variable motifs, which are sometimes close to the N- or the C-terminus [30,37,43,44,69–73]. Wild-type Erv homologs from A. thaliana and L. tarentolae both carry a C-terminal shuttle arm and were unable to complement the loss of TbErv1 [43,72]. Even though complementation with LiErv1 resulted in a growth defect on FOA agar plates, the viability of the strain clearly shows that the position of the shuttle arm is not a criterion for exclusion for the functionality in yeast. This is also the case regarding the CBSR shuttle arm motif, which was successfully replaced in chimera 6, although at the cost of a drastically impaired mitochondrial protein import. Residue Cys17 of LtErv is partially conserved in Erv homologs [16,43] and was shown to form a clamp disulfide bond in mammalian ALR [33,37,74]. Notably, heterologous human ALR was found in the yeast cytosol and was unable to functionally replace ScErv1 unless a bipartite pre-sequence was fused to its N-terminus or residues 1–80 of ALR were replaced with residues 1–93 of ScErv1 [75,76]. How residue Cys17 of LtErv exactly impairs its function in yeast and whether this neglected residue exerts a physiological function in a subset of Erv homologs remains to be studied. Chimera 1 and 4 both contain an N- and C-terminal shuttle arm, but only yeast cells with chimera 1 had a growth defect. Maybe both shuttle arms compete for the proximal cytoxines of the flavodomain of chimera 1 or form long-lived disulfide-bonded intermediates in contrast to chimera 4. In summary, growth and import defects for chimera 1, 2 and 6 suggest that the shuttle arm motif and the interactions between the shuttle arm and the flavodomain and/or Mia40 have been optimized for different Erv homologs in the course of evolution. Nevertheless, the mitochondrial protein import machinery in yeast is robust enough to allow certain degrees of incompatibility.

Can protist Erv homologs act alone and do protists have simplified oxidative folding machineries mediating the IMS protein import as recently suggested [65,77]? This possibility would require an efficient interaction of Leishmania class II IMS proteins with LtErv, including their direct oxidation by LtErv. In this case one might expect that LtErv is able to complement the function of ScMia40, because the import signals of IMS proteins are functionally conserved in the course of evolution [42]. Our complementation studies do not provide evidence for a simplified oxidation and folding machinery, because neither the KISS domain nor full length LtErv, LtErvC-term, LtErvC-term or chimera 1–6 could rescue the Δmia40 strain. This rather points to the presence of a Mia40 replacement in protists (compound Z in one of our previously discussed evolutionary scenarios [42]). Such a model suggests that protists without Mia40 have a replacement for this substrate receptor. So far we were unable to identify a stable disulfide-bonded interaction partner between LtErv and another protein in Leishmania mitochondria using a variety of experimental approaches (Liedgens et al., unpublished). However, just because redox mobility shift assays and pull-down experiments do not reveal a stable interaction, we cannot exclude a transient or non-covalent interaction with a Mia40 replacement. The “sluggish” behavior of ScMia40 with its metastable intermolecular disulfide species is actually rather an exception compared to oxidative protein folding in the periplasm or the endoplasmic reticulum [7,31]. What could be a reasonable replacement of Mia40? A recent study showed that a subpopulation of the yeast peroxidase Gpx3 interacts with ScMia40 and the oxidative folding pathway in the IMS [78]. This situation somehow resembles the transient interaction between peroxiredoxin IV and protein disulfide isomerase (PDI) for oxidative protein folding in the endoplasmic reticulum of mammals [79,80]. A tryparedoxin peroxidase (Tb927.9.5750) was also identified in a pull-down assay with TbErv [65], and this protein was 1.34-fold up-regulated in the TbErv knockout strain [45]. Whether this is just a false positive hit due to the high abundance of the cytosolic peroxidase or points to a dual localization and alternative function in oxidative protein folding remains to be studied. However, Gpx3 and peroxiredoxin IV are both downstream electron acceptors of Mia40 and PDI so that an analogous involvement of the tryparedoxin peroxidase in the IMS of kinetoplastid parasites would still necessitate the existence of an alternative receptor for the incoming protein substrates. Further studies are obviously necessary to identify the receptor for incoming IMS proteins and to decipher the oxidative folding machineries in protist mitochondria.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2017.12.010.

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