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Presequence-dependent folding ensures MrpL32 processing by the *m*-AAA protease in mitochondria

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***m*-AAA proteases exert dual functions in the mitochondrial inner membrane: they mediate the processing of specific regulatory proteins and ensure protein quality control degrading misfolded polypeptides to peptides. Loss of these activities leads to neuronal cell death in several neurodegenerative disorders. However, it is unclear how the *m*-AAA protease chooses between specific processing and complete degradation. A central and conserved function of the *m*-AAA protease is the processing of the ribosomal subunit MrpL32, which regulates ribosome biogenesis and the formation of respiratory complexes. Here, we demonstrate that the formation of a tightly folded domain harbouring a conserved CxxC-X_9-CxxC sequence motif halts degradation initiated from the N-terminus and triggers the release of mature MrpL32. Oxidative stress impairs folding of MrpL32, resulting in its degradation by the *m*-AAA protease and decreased mitochondrial translation. Surprisingly, MrpL32 folding depends on its mitochondrial targeting sequence. Presequence-assisted folding of MrpL32 requires the complete import of the MrpL32 precursor before maturation occurs and therefore explains the need for post-translocational processing by the *m*-AAA protease rather than co-translocational cleavage by the general mitochondrial processing peptidase.**

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Introduction

AAA proteases comprise a conserved family of membrane-bound energy-dependent proteases, which form homo- or hetero-oligomeric, likely hexameric complexes of homologous subunits (Koppen and Langer, 2007; Lee *et al*, 2011). At least two members are present in the mitochondrial inner membrane: *m*-AAA proteases expose catalytic domains, including an AAA domain characteristic of the AAA family of ATPases and a H41 metallopeptidase domain, to the matrix space, while *i*-AAA proteases are active in the intermembrane space (Leonhard *et al*, 1996; Augustin *et al*, 2009). Mutations in subunits of human *m*-AAA proteases are associated with various neurodegenerative disorders. AFG3L2 mutations cause dominant cerebellar ataxia SCA28 (Di Bella *et al*, 2010), characterized by cerebellar degeneration leading to imbalance, progressive gait and limb ataxia, and dysarthria. AFG3L2 subunits can either assemble into homo-oligomeric *m*-AAA proteases or form hetero-oligomeric isoenzymes with paraplegin (Koppen *et al*, 2007), which has been associated with recessive hereditary spastic paraplegia HSP (Casari *et al*, 1998; Salinas *et al*, 2008). HSP patients are characterized clinically by progressive spastic paralysis and suffer of axonal degeneration of motor neurons of the corticospinal tracts and sensory neurons of the fasciculus gracilis (Salinas *et al*, 2008). However, the pathogenic mechanism underlying complex phenotypes associated with a dysfunction of *m*-AAA proteases in human remains poorly understood.

m-AAA proteases are functionally conserved (Atorino *et al*, 2003; Nolden *et al*, 2005; Koppen *et al*, 2007). Expression of human *m*-AAA proteases restores the respiratory deficiency of yeast cells lacking subunits of the hetero-oligomeric yeast *m*-AAA protease, Yta10 and Yta12 (Koppen *et al*, 2007). Originally identified as quality control enzymes degrading non-native inner membrane proteins to peptides in yeast (Pajic *et al*, 1994; Arlt *et al*, 1996; Guzelin *et al*, 1996), *m*-AAA proteases can act additionally as processing enzymes mediating the maturation of proteins with crucial functions during mitochondrial biogenesis (Esser *et al*, 2002; Nolden *et al*, 2005). This was first recognized for the yeast *m*-AAA protease, which promotes processing of cytochrome *c* peroxidase (Esser *et al*, 2002) and the mitochondrial ribosomal subunit MrpL32 (Nolden *et al*, 2005). The mammalian *m*-AAA protease is also involved in maturation of its own subunits. Mouse Afg3l1 and Afg3l2 are essential in processing of paraplegin and mediate autocatalytically their own maturation upon import into mitochondria (Koppen *et al*, 2009). Moreover, the mammalian *m*-AAA protease controls the processing of the dynamin-like GTPase OPA1, a central component of the mitochondrial fusion machinery, in a yet ill-defined manner (Ehses *et al*, 2009).

Despite the increasing number of proteins that are matured by *m*-AAA proteases, it is currently not understood how

specific cleavage by *m*-AAA proteases is ensured and complete degradation prevented. We have therefore analysed maturation of ribosomal MrpL32 that is conserved between yeast and mammals (Nolden *et al*, 2005). It is required for the assembly of mitochondrial ribosomes, for the synthesis of mitochondrial encoded respiratory chain subunits and, concomitantly, for the assembly of respiratory complexes and respiratory growth (Nolden *et al*, 2005). Genetic experiments in yeast revealed that an impaired cleavage of MrpL32 causes growth defects of *m*-AAA protease-deficient yeast cells, identifying MrpL32 processing as the central process controlled by the *m*-AAA protease in yeast. However, it remained unclear why MrpL32 is processed rather than being degraded by the *m*-AAA protease. Here, we describe an unexpected function of the presequence of MrpL32 for the folding of a cysteine-containing domain of MrpL32, which prevents its degradation by the *m*-AAA protease.

Results

MrpL32 accumulation in mitochondria depends on the integrity of a conserved cysteine-containing motif

Eukaryotic homologues of MrpL32 carry an N-terminal mitochondrial targeting sequence which is cleaved off by the *m*-AAA protease upon import into mitochondria (Nolden *et al*, 2005). Alanine residue 72 was identified as the N-terminal amino acid of yeast MrpL32 (Grohmann *et al*, 1991; Graack and Wittmann-Liebold, 1998; Graack *et al*, 1999). The crystal structure of the 50S ribosomal particle of the eubacterium *Deinococcus radiodurans* (Harms *et al*, 2001) revealed that MrpL32 is largely surface exposed with the N-terminal segment protruding deeply into the interior of the ribosome (Figure 1A). The surface exposed C-terminal part of the protein contains four cysteine residues, comprising a CxxC-X_9-CxxC sequence motif that is conserved throughout evolution (Figure 1B). To examine the functional role of this sequence motif, we exchanged each cysteine residue individually or in combination to a serine residue. MrpL32 variants were expressed under the control of the constitutive *ADH1* promoter in haploid yeast wild-type cells, which were crossed with haploid Δ*mrpl32* cells lacking mtDNA. After sporulation and tetrad dissection we isolated haploid cells, which lack the endogenous *MRPL32* gene but express MrpL32 variants, and examined their ability for respiratory growth on non-fermentable carbon sources (Figure 1C). These experiments revealed that replacement of C104, C107 or C120 by serine did not abolish respiratory growth (Figure 1C). However, introducing

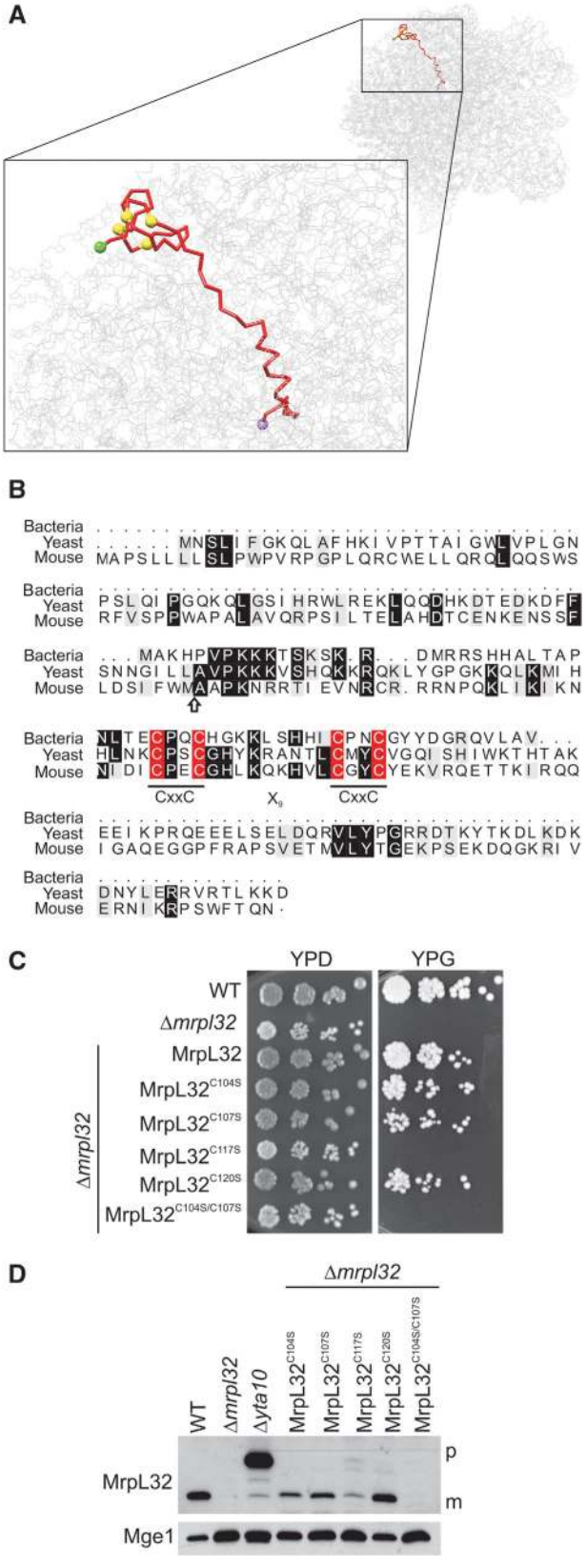

Figure 1 The function of MrpL32 depends on the integrity of a conserved CxxC-X_9-CxxC sequence motif. (**A**) Crystal structure of the 50S large ribosomal subunit from *Deinococcus radiodurans* (Harms *et al*, 2001). The structure of bacterial L32 is highlighted in red, cysteine residues of the CxxC-X_9-CxxC sequence motif are shown in yellow, the N-terminus of MrpL32 is marked in purple and the C-terminus in green. (**B**) Sequence alignment of MrpL32 homologues from yeast (uniprot:rm32_yeast), mouse (uniprot:rm32_mouse) and *E. coli* (uniprot:rl32_ecoli). The alignment was constructed using ClustalW. The conserved CxxC-X_9-CxxC sequence motif is highlighted in red. The arrowhead indicates the processing site in yeast MrpL32. (**C**) Respiratory growth of Δ*mrpl32* cells expressing MrpL32 or mutant variants thereof. Cells were grown on fermentable (YPD) and non-fermentable (YPG) carbon sources at 30°C. (**D**) Steady-state level of MrpL32 in mitochondria isolated from wild-type (WT), Δ*yta10*, Δ*mrpl32* cells and Δ*mrpl32* cells expressing MrpL32 and cysteine variants thereof. To control for gel loading, immunoblotting was performed using antibodies directed against the matrix protein Mge1. To visualize the position of the MrpL32 precursor, Δ*yta10* mitochondria were analysed in parallel; p, precursor and m, mature form.

the mutation of C117S or the combination of C104S and C107S into MrpL32 impaired the respiratory competence of these cells, which did not grow on glycerol-containing media (Figure 1C).

Mitochondria were isolated from wild-type cells, Δ*mrpl32* cells and Δ*mrpl32* cells expressing different MrpL32 variants. Immunoblotting of mitochondrial extracts revealed that—with the exception of MrpL32^{C120S}—the mutant MrpL32 proteins accumulated at reduced levels in mitochondria when compared with wild-type cells (Figure 1D). Notably, the steady-state level of MrpL32^{C117S} was affected more severely and MrpL32$^{C104S/C107S}$ was not detectable in mitochondrial extracts (Figure 1D). As mature MrpL32 is required for respiration, these findings are consistent with the impaired growth of Δ*mrpl32* cells expressing these variants. We, therefore, conclude that the accumulation of MrpL32 within mitochondria and respiratory growth depends on the integrity of the CxxC-X_9-CxxC sequence motif.

Degradation of MrpL32 mutant variants by the m-AAA protease

The decreased steady-state levels of MrpL32 variants harbouring mutations in the conserved cysteine motif may reflect increased turnover of the mutant proteins within mitochondria. We, therefore, determined the stability of MrpL32 and variants thereof after import into isolated mitochondria. MrpL32, MrpL32^{C107S} and MrpL32$^{C104S/C107S}$ were synthesized in a cell-free system in the presence of [^{35}S]-methionine and incubated with mitochondria isolated from wild-type cells (Figure 2). MrpL32 and its variants were imported in a membrane potential-dependent manner and accumulated in a protease-protected form in mitochondria (Figure 2). Mutations in the CxxC-X_9-CxxC sequence motif did not affect the efficiency of import (Figure 2). To assess the stability of the newly imported proteins, mitochondria were further incubated after completion of import at 37°C to allow proteolysis to occur. MrpL32 was matured upon import into mitochondria and accumulated stably upon further incubation at 37°C (Figure 2A). This holds true also in mitochondria lacking mitochondrial DNA (ρ^0), indicating that the assembly of MrpL32 into ribosomes does not affect its processing and turnover (Supplementary Figure S1A). Similarly, MrpL32^{C107S} was imported and processed to the mature form, although processing occurred with reduced efficiency. Newly imported MrpL32^{C107S} remained stable at increased temperatures (Figure 2B). On the other hand, the formation of mature MrpL32$^{C104S/C107S}$ was severely impaired and the precursor form was degraded upon further incubation at 37°C (Figure 2C). Similarly, we observed rapid degradation of the precursor form of MrpL32^{C117S} upon import into mitochondria (data not shown). These results are consistent with our complementation experiments *in vivo* and demonstrate that mutations in the CxxC-X_9-CxxC sequence motif affect the stability of MrpL32 within mitochondria.

To examine if the *m*-AAA protease mediates the proteolytic breakdown of MrpL32 variants, we imported MrpL32$^{C104S/C107S}$ into mitochondria isolated from Δ*yta10*Δ*yta12* cells or from Δ*yta10*Δ*yta12* cells expressing proteolytically inactive Yta10^{E559Q} and Yta12^{E614Q} (Figure 2D). MrpL32$^{C104S/C107S}$ was imported into *m*-AAA protease-deficient mitochondria but, as expected, not converted into its mature form. In contrast to wild-type mitochondria, the precursor form of MrpL32 remained stable upon incubation of the mitochondria at 37°C (Figure 2D). Aggregation of the precursor form of MrpL32 in *m*-AAA protease-deficient mitochondria can be excluded since the imported precursor form remained sensitive to externally added proteinase K in the presence of mild detergent Triton X-100 (Supplementary Figure S1B). We, therefore, conclude that mutations in the conserved cysteine-containing sequence motif of MrpL32 trigger its degradation by the *m*-AAA protease, which mediates maturation of wild-type MrpL32.

Monitoring MrpL32 folding in vitro

The degradation of MrpL32 proteins harbouring mutations in the cysteine-containing sequence motif suggests that an impaired folding of the mature domain of these MrpL32 variants results in their degradation. We, therefore, established a protease-protection assay, which allowed us to directly assess the folding state of MrpL32 and its mutant variants *in vitro*. MrpL32 and variants thereof were synthesized in a cell-free system in the presence of [^{35}S]-methionine and incubated in the presence of proteinase K (Figure 3A–C). MrpL32 displayed a limited resistance against proteinase K, indicating folding (Figure 3A and B). We observed a similar protease resistance for MrpL32^{C107S} (Figure 3A and C), which maintained respiratory growth of Δ*mrpl32* cells (Figure 1C). We next examined the stability against externally added protease of MrpL32^{C117S} and MrpL32$^{C104S/C107S}$, which both accumulated at significantly reduced levels in mitochondria *in vivo* (Figure 1D). Strikingly, both variants exhibited drastically increased protease sensitivity when compared with MrpL32, pointing to an impaired folding (Figure 3A–C).

MrpL32 may bind metal ions, similar to other proteins containing a dual CxxC sequence motif (Won *et al*, 2004). However, although not observed for any other matrix-localized protein, it cannot be excluded that MrpL32 contains disulphide bonds. To distinguish between these possibilities, we assessed the folding of MrpL32 in the presence of the metal chelating agent EDTA (Figure 3A and B). Proteolysis of MrpL32 was significantly enhanced under these conditions when compared with MrpL32 and occurred with similar kinetics as observed for MrpL32^{C117S}, suggesting that the CxxC-X_9-CxxC sequence motif is coordinating a metal ion. Mutational analysis of this sequence motif provided further evidence for the binding of a metal ion. We replaced C117 or, in combination, C104 and C107 in the CxxC-X_9-CxxC motif by histidine, which coordinates metal ions in many proteins but cannot form disulphide bridges. In contrast to MrpL32 variants harbouring serine residues at the corresponding positions, the expression of C117H and C104H/C107H mutants of MrpL32 restored completely the growth phenotype caused by the deletion of *MRPL32* (Figure 3D). The mutant proteins accumulated *in vivo* in their mature form at similar levels as wild-type MrpL32 (Figure 3E). Finally, we directly assessed the redox state of MrpL32 in intact cells and isolated mitochondria, modifying free thiols with 4-acetamido-4′-maleimidylstilbene-2,2′-disulphonic acid (AMS) (Supplementary Figure S2). These experiments revealed that cysteine residues of MrpL32 can be alkylated with AMS, thus are present in a reduced form (Supplementary Figure S2).

Taken together, we conclude from these experiments that the conserved CxxC-X_9-CxxC sequence motif binds a metal ion, stabilizing the folded state of MrpL32. Mutations in this

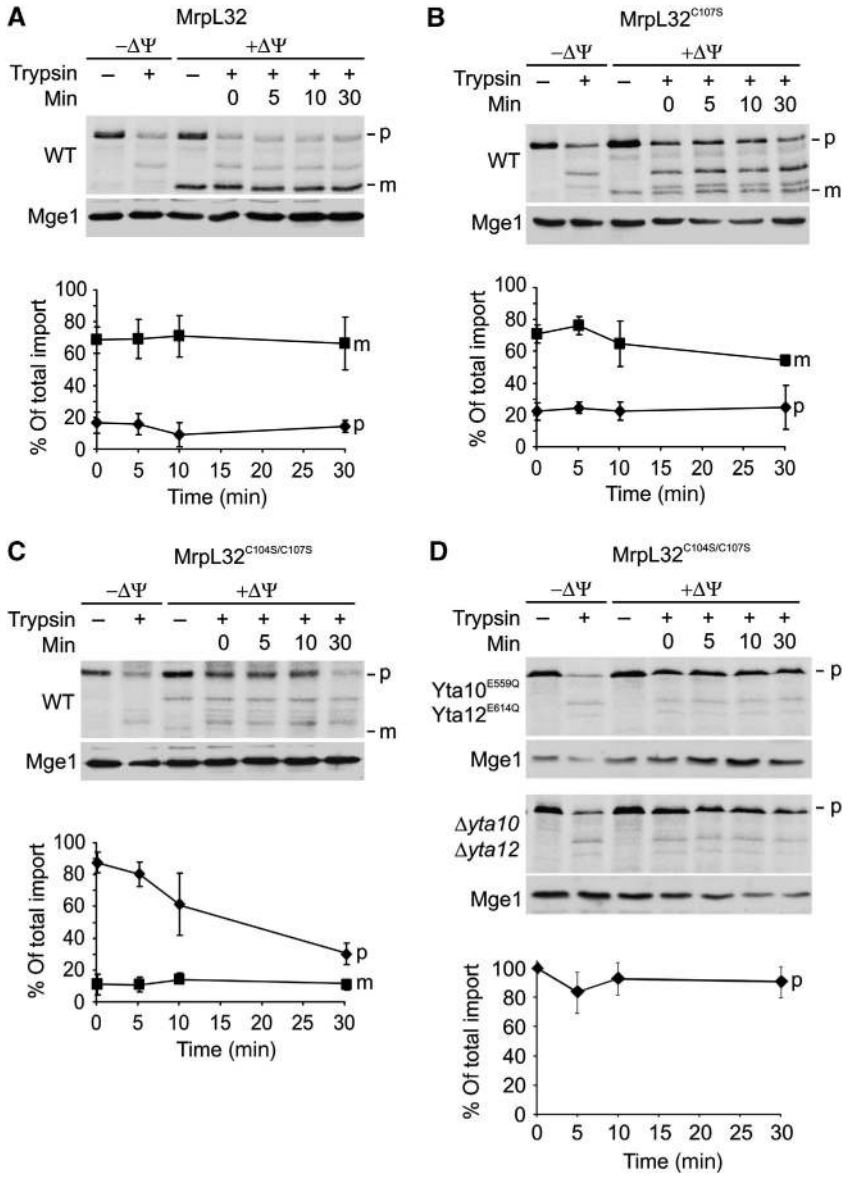

Figure 2 Processing and turnover of MrpL32 variants by the *m*-AAA protease. [^{35}S]-labelled MrpL32 (**A**), MrpL32^{C107S} (**B**) or MrpL32^{C104S7C107S} (**C**, **D**) were imported for 30 min at 25°C into wild-type mitochondria (**A**–**C**) or (**D**) mitochondria isolated from Δ*yta10*Δ*yta12* cells or from Δ*yta10*Δ*yta12* cells expressing Yta10^{E559Q} and Yta12^{E614Q} in the absence (−ΔΨ) or presence (+ΔΨ) of a mitochondrial membrane potential. After stopping the import reaction by dissipation of the membrane potential, the stability of the newly imported proteins was assessed by incubating the samples at 37°C for indicated time points. Non-imported precursor proteins were digested with trypsin and samples were subjected to SDS–PAGE. Immunoblotting with antibodies directed against Mge1 was used to control for gel loading. Lower panels: quantification of [^{35}S]-labelled MrpL32 variants present in mitochondria after completion of import. Precursor (p) and mature (m) forms were quantified by phosphorimaging in four independent experiments (± s.e.m.). The sum of precursor and mature levels of MrpL32 present within mitochondria at time point 0 was set to 100%. Quantification of MrpL32$^{C104S/C107S}$ in Δ*yta10*Δ*yta12* cells expressing Yta10^{E559Q} and Yta12^{E614Q} is shown in (**D**).

sequence motif or the depletion of metal ions impair folding of MrpL32. Destabilized MrpL32 is not matured within mitochondria but subjected to degradation by the *m*-AAA protease.

Domain folding defines the processing site in MrpL32

It is conceivable that folding of mature MrpL32 serves as a downstream signal, which halts proteolysis by the *m*-AAA protease and therefore determines the site of maturation. To examine this possibility, we introduced linker regions of either 20 or 40 amino acids after the MrpL32 processing site into the protein sequence of mature MrpL32 (Figure 4A). Notably, these linker regions are predicted to be unstructured and lack apparent sequence specificity. The amino-acid sequence of the 20 amino-acid spacer was selected randomly, whereas the 40 amino-acid spacer consists of the unstructured region of the *lacI* gene from *E. coli* (Figure 4A; Prakash *et al*, 2004). If the *m*-AAA protease recognizes a specific sequence element at the processing site, the MrpL32^{20aa} and MrpL32^{40aa} variants will be converted into significantly larger, mature forms. However, if MrpL32 cleavage was solely determined by a folded domain located C-terminally, the processing site should be shifted towards the C-terminal domain of MrpL32, resulting in the formation of similar-sized mature MrpL32 variants. MrpL32^{20aa} and MrpL32^{40aa}

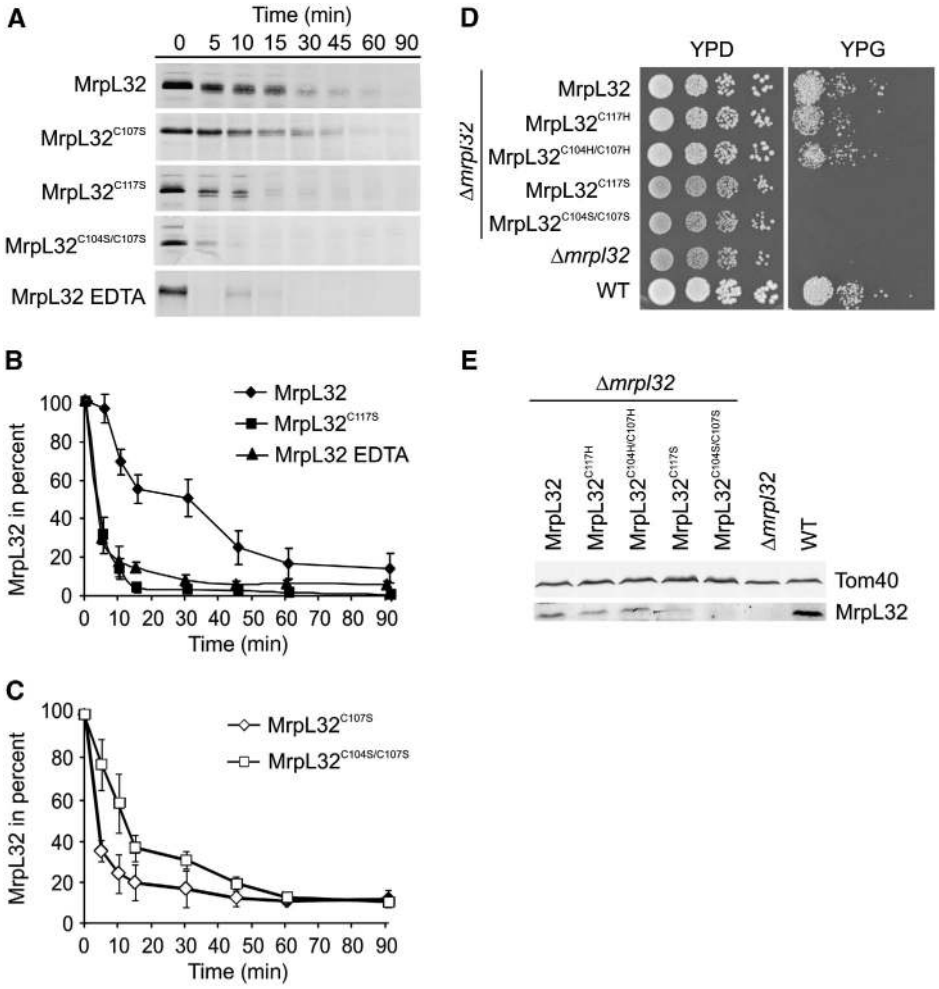

Figure 3 The role of the cysteine cluster for folding of MrpL32. (**A**–**C**) Monitoring MrpL32 folding *in vitro*. MrpL32 and variants thereof were synthesized in a cell-free system in the presence of [^{35}S]-methionine and subjected to limited proteolysis using proteinase K (0.4 μg/ml). EDTA (5 mM) was added when indicated. (**A**) Samples were analysed by SDS–PAGE and autoradiography. (**B**, **C**) Three independent experiments were quantified by phosphorimaging (± s.e.m.). (**D**) Respiratory growth of Δ*mrpl32* cells expressing MrpL32^{C117H}, MrpL32$^{C104H/C107H}$, MrpL32^{C117S} and MrpL32$^{C104S/C107S}$. Cells were grown on fermentable (YPD) and non-fermentable (YPG) carbon sources at 30°C. (**E**) Steady-state level of MrpL32 and MrpL32 mutant variants expressed in Δ*mrpl32* cells. To control for gel loading, immunoblotting was performed using antibodies directed against Tom40.

variants harbouring spacer regions and, for control, MrpL32 were synthesized in a cell-free system in the presence of [^{35}S]-methionine. The radiolabelled proteins were imported in a membrane potential-dependent manner into mitochondria, which was accompanied by proteolytic maturation (Figure 4B). Maturation of MrpL32^{20aa} and MrpL32^{40aa} variants was mediated by the *m*-AAA protease as revealed by import experiments using Δ*yta10*Δ*yta12* mitochondria (Figure 4B). Interestingly, the electrophoretic mobility of mature MrpL32^{20aa} and MrpL32^{40aa} was indistinguishable from the mature form of wild-type MrpL32, despite the presence of up to 40 additional amino acids after the cleavage site in wild-type MrpL32 (Figure 4B).

To assess the *in vivo* relevance of these findings, we complemented Δ*mrpl32* cells with MrpL32^{20aa} and examined their respiratory competence (Figure 4C). Expression of MrpL32^{20aa} allowed at least to some extent the growth of Δ*mrpl32* cells on non-fermentable carbon sources, indicating that the variant is functional (Figure 4C). This is remarkable as the mature forms of MrpL32 and MrpL32^{20aa} likely differ in their N-terminal amino acids. Immunoblotting of mitochondrial extracts isolated from these cells revealed that MrpL32^{20aa} accumulated in its precursor form, but was cleaved to some extent to a mature form, whose apparent molecular mass was similar to endogenous MrpL32 (Figure 4D). Cleavage of MrpL32^{20aa} was mediated by the *m*-AAA protease and inhibited when MrpL32^{20aa} was expressed in *m*-AAA protease-deficient cells (Figure 4D).

These findings are in agreement with the mitochondrial import experiments *in vitro* and demonstrate that processing of MrpL32 by the *m*-AAA protease is determined by the folding of the cysteine-containing domain of MrpL32 rather than by a specific sequence motif at the cleavage site. Consistently, mutations in amino-acid residues at the cleavage site or a deletion of amino acids flanking the cleavage site did not interfere with the maturation of MrpL32 (data not shown). As the folding of mature MrpL32 located C-terminal to the cleavage site is crucial for maturation, it appears likely that the *m*-AAA protease initiates proteolysis from N-terminal amino acids of MrpL32 before a folded domain of MrpL32 halts further degradation resulting in the release of mature MrpL32.

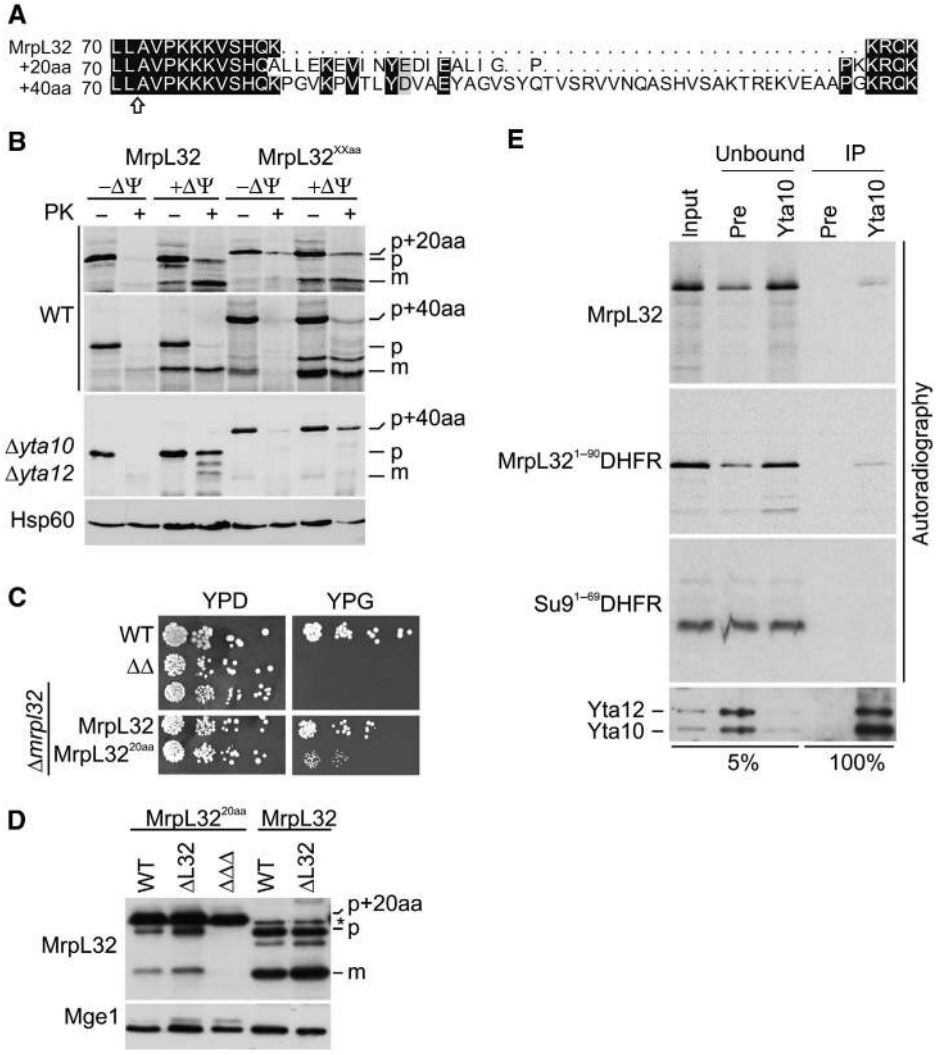

Figure 4 Domain folding defines the processing site of MrpL32. (**A**) Sequence alignment of MrpL32 with variants containing either +20 or +40 additional amino acids, inserted C-terminal to the cleavage site in MrpL32 (arrowhead). The alignment was constructed using ClustalW. (**B**) MrpL32, MrpL32^{20aa} and MrpL32^{40aa} are processed by the *m*-AAA protease to similar-sized, mature forms upon import into mitochondria. [^{35}S]-labelled MrpL32, MrpL32^{20aa} or MrpL32^{40aa} were imported into wild-type or Δ*yta10*Δ*yta12* mitochondria as described in Figure 2. p, precursor form of MrpL32; p+20aa, precursor form of MrpL32^{20aa}; p+40aa, precursor form of MrpL32^{40aa} and m, mature MrpL32. Immunoblotting using antibodies against mitochondrial Hsp60 was used to control for gel loading. (**C**) Growth of Δ*mrpl32* cells expressing MrpL32 or MrpL32^{20aa} when indicated on fermentable (YPD) and non-fermentable (YPG) carbon sources at 30°C. ΔΔ, Δ*yta10*Δ*yta12*. (**D**) Accumulation of MrpL32 and MrpL32^{20aa} in mitochondria *in vivo*. Mitochondria isolated from Δ*mrpl32* cells expressing MrpL32 or MrpL32^{20aa}, or from Δ*yta10*Δ*yta12*Δ*mrpl32* mitochondria harbouring MrpL32^{20aa} were analysed by SDS–PAGE and immunoblotting using antibodies directed against MrpL32 or for control, Mge1. The precursor forms of MrpL32 and MrpL32^{20aa} likely accumulate due to the overexpression of both proteins. The asterisk marks an unspecific band detected by MrpL32 antibodies in Δ*mrpl32* mitochondria. p+20aa, precursor form of MrpL32^{20aa}; p, precursor form of MrpL32; m, mature form of MrpL32; WT, wild-type mitochondria; ΔL32, Δ*mrpl32* mitochondria and ΔΔΔ, Δ*yta10*Δ*yta12*Δ*mrpl32* mitochondria. (**E**) Binding of the *m*-AAA protease to N-terminal regions of MrpL32. [^{35}S]-labelled MrpL32, MrpL32$^{1–90}$DHFR, and, for control, Su9$^{1–69}$DHFR were imported into wild-type mitochondria as described in Figure 2. Mitochondrial membranes were solubilized and extracts were subjected to coimmunoprecipitation using affinity purified Yta10-specific antibodies (Yta10) or preimmune serum (pre). The total sample (input, 5%), of unbound material (5%), and the immunoprecipitate were analysed by SDS–PAGE and autoradiography. The efficiency of the immunoprecipitation was monitored by immunoblotting using Yta10- and Yta12-specific antibodies.

N-terminal binding of the m-AAA protease to MrpL32

To corroborate this hypothesis, we analysed binding of newly imported MrpL32 to the *m*-AAA protease in coimmunoprecipitation experiments (Figure 4E). Mitochondria were isolated from Δ*yta10*Δ*yta12* cells harbouring proteolytically inactive *m*-AAA protease, which is composed of Yta10^{E559Q} and Yta12^{E614Q} subunits and acts as a substrate trap (Nolden *et al*, 2005). Newly imported, radiolabelled MrpL32 accumulated in its precursor form in mitochondria and was precipitated from mitochondrial extracts with antibodies directed against Yta10, but not with preimmune serum (Figure 4E). To examine whether the *m*-AAA protease can bind to the N-terminal region of MrpL32, we generated a chimeric protein that is composed of amino-acid residues 1–90 of MrpL32 fused to mouse DHFR. For control, a similar fusion protein of mouse DHFR and the unrelated mitochondrial presequence of the ATP synthase subunit 9 of *Neurospora crassa* was used in parallel experiments. Both hybrid proteins were synthesized in a cell-free system in the presence of [^{35}S]-methionine and imported into *yta10*^{E559Q}*yta12*^{E614Q}

mitochondria (Figure 4E). Newly imported MrpL32$^{1-90}$DHFR was not processed upon import and specifically co-precipitated with Yta10-specific antibodies with a similar efficiency as MrpL32 (Figure 4E). In contrast, the *m*-AAA protease did not interact with newly imported DHFR (Figure 4E) generated upon proteolytic processing of Su9$^{1-69}$DHFR. We conclude from these experiments that the *m*-AAA protease can bind to N-terminal amino-acid residues including the mitochondrial presequence of MrpL32 and, thus, can initiate proteolysis from the N-terminus of MrpL32.

Decreased accumulation of MrpL32 under oxidative stress

Our findings identify a critical role of a conserved cysteine-containing sequence for folding and maturation of MrpL32. We reasoned that the presence of this conserved sequence motif renders MrpL32 susceptible to modification by reactive oxygen species (ROS), which may interfere with MrpL32 folding and processing, and concomitantly mitochondrial translation. A decreased synthesis of mitochondrial encoded respiratory chain subunits during oxidative stress could attenuate ROS production by respiratory chain complexes under these conditions. We, therefore, examined the accumulation of MrpL32 under conditions of oxidative stress. Yeast cells were incubated for 2 h in the presence of increasing concentrations of H_2O_2, which resulted in the accumulation of carbonylated proteins within mitochondria (Figure 5A). Notably, mitochondria that were isolated from H_2O_2-treated cells contained decreased levels of MrpL32 (Figure 5A and B). Steady-state levels of another ribosomal protein, MrpL13, or of Hsp60 and Tom20, localized to the mitochondrial matrix and outer membrane, respectively, were unaffected (Figure 5A). These results indicate that oxidative stress impacts on the stability of MrpL32 *in vivo*. It is conceivable that oxidation of cysteine residues in the CxxC-X$_9$-CxxC sequence motif impairs MrpL32 folding. AMS-modification experiments provided indeed direct evidence for oxidation of MrpL32 in H_2O_2-treated cells (Supplementary Figure S2C). Interestingly, treatment of *m*-AAA protease-deficient yeast cells with H_2O_2 did not affect the accumulation of MrpL32 within mitochondria, suggesting degradation of MrpL32 by the *m*-AAA protease under oxidative stress (Figure 5A).

To corroborate these findings, we analysed the accumulation of MrpL32 in cells lacking *SOD2*. *SOD2* encodes for a manganese-superoxide dismutase that is localized to the mitochondrial matrix and involved in oxygen radical detoxification (Luk *et al*, 2005). Δ*sod2* cells show increased sensitivity to oxidative stress and a decreased lifespan (Longo *et al*, 1996, 1999). If MrpL32 folding is sensitive to oxidative stress, deletion of *SOD2* should impair its accumulation within mitochondria. Mitochondria were isolated from wild-type and Δ*sod2* cells grown on fermentable or non-fermentable carbon sources and analysed by SDS–PAGE and immunoblotting (Figure 5C). The steady-state level of MrpL32 was decreased by ∼50% in Δ*sod2* mitochondria when compared with wild-type mitochondria, irrespective of the cellular growth conditions (Figure 5C). Other mitochondrial proteins, including MrpL13, Tom20 or Hsp60, remain unaffected (Figure 5C). MrpL32 accumulated at normal levels in Δ*sod2* mitochondria lacking Yta12, providing further evidence that MrpL32 is degraded by the *m*-AAA protease under conditions of oxidative stress (Figure 5C).

As MrpL32 is an essential subunit of mitochondrial ribosomes, we examined in further experiments whether the decreased steady-state level of MrpL32 was associated with an impaired synthesis of mitochondrial encoded proteins under oxidative stress. Mitochondrial-translation products were radiolabelled incubating yeast cells in the presence of [^{35}S]-methionine and cycloheximide to inhibit cytosolic translation. We observed a significant impairment of the synthesis of mitochondrial encoded proteins upon incubation of the cells with increasing concentration of H_2O_2 (Figure 5D). As treatment with H_2O_2 likely affects various proteins involved in mitochondrial translation, we wanted to unravel the relevance of MrpL32 for the maintenance of protein synthesis under these conditions. Therefore, we overexpressed MrpL32 in yeast cells and monitored mitochondrial translation in the presence of different concentrations of H_2O_2 *in vivo* (Figure 5D). Overexpression of MrpL32 did not affect mitochondrial protein synthesis in untreated cells, but MrpL32 appears to become rate limiting under conditions of oxidative stress. Quantification of newly synthesized mitochondrial encoded polypeptides revealed a modest but statistically significant protective effect of MrpL32 overexpression against oxidative stress at an intermediate concentration of H_2O_2 (Figure 5D).

We conclude from these experiments that mitochondrial protein synthesis is susceptible to oxidative stress, which is at least partially caused by oxidation of MrpL32 and its degradation by the *m*-AAA protease. This mechanism offers an opportunity for regulation of mitochondrial translation under conditions of oxidative stress.

Presequence-dependent folding of MrpL32

While these experiments unravel the mechanism of MrpL32 processing by the *m*-AAA protease and its relevance for mitochondrial translation under oxidative stress, it remains still enigmatic why MrpL32 is processed by the *m*-AAA protease and not by the general mitochondrial processing peptidase (MPP), as the vast majority of presequence-containing mitochondrial matrix proteins (Hawlitschek *et al*, 1988; Yang *et al*, 1988; Ou *et al*, 1989). We reasoned that additional, specific roles of the MrpL32 presequence may exist and examined a putative function of the presequence for folding of mature MrpL32. MrpL32 and the truncated variant MrpL32$^{72-183}$ corresponding to mature MrpL32 were synthesized in a cell-free system in the presence of [^{35}S]-methionine and subjected to limited proteolysis by externally added proteinase K to monitor the folding state (Figure 6A). Strikingly, MrpL32$^{72-183}$ exhibited significantly increased protease sensitivity, which was similar to unfolded MrpL32 variants harbouring mutations in the conserved CxxC-X$_9$-CxxC sequence motif (Figures 3 and 6A). A heterologous presequence could not substitute for the presequence of MrpL32 and restore protease resistance: a hybrid protein composed of the presequence of murine ornithine carbamoylase (OCT) was highly susceptible to externally added proteinase K (Figure 6A). We conclude that efficient folding of MrpL32 *in vitro* depends on its presequence.

We have previously observed that overexpression of a hybrid protein composed of the mitochondrial presequence of ATP synthase subunit 9 of *N. crassa* and MrpL32$^{72-183}$ can

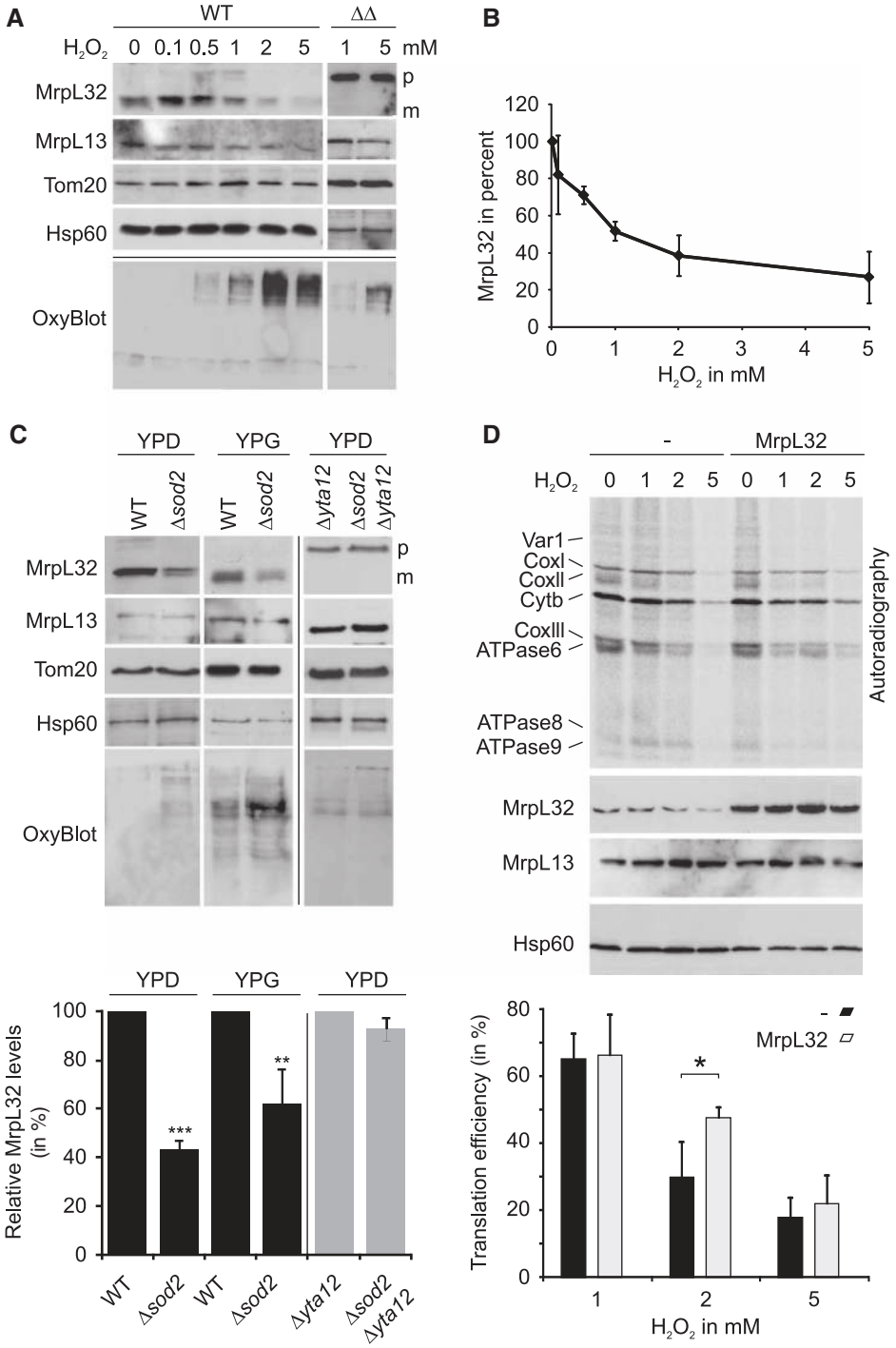

Figure 5 Oxidative stress triggers degradation of MrpL32 by the *m*-AAA protease. (**A**) Accumulation of MrpL32 under oxidative stress. Wild-type (WT) and Δ*yta10*Δ*yta12* cells were incubated for 2 h in the presence of different concentrations of H_2O_2. Cell extracts were analysed by immunoblotting using antibodies directed against MrpL32, the mitoribosomal protein MrpL13, Tom20 and Hsp60. The accumulation of carbonylated proteins was monitored using the Protein Oxidation Detection Kit (OxyBlot^{TM}, Millipore); p, precursor form of MrpL32 and m, mature form of MrpL32. (**B**) The steady-state level of MrpL32 was quantified in three independent experiments (± s.e.m.). The MrpL32 level in untreated cells was set to 100%. (**C**) Degradation of MrpL32 by the *m*-AAA protease in Δ*sod2* mitochondria. Extracts of wild-type (WT), Δ*sod2* or Δ*sod2*Δ*yta12* cells grown on YPD or YPG medium were analysed by SDS–PAGE and immunoblotting as in (**A**). The accumulation of mature MrpL32 (in WT and Δ*sod2* cells) or of the precursor form of MrpL32 (in Δ*sod2*Δ*yta12* cells) were quantified in three independent experiments (± s.e.m.), and the statistical significance was assessed by a paired Student's *t*-test *P*-value: **, 0.01; ***, 0.005. (**D**) Labelling of mitochondrial-translation products *in vivo*. Wild-type cells and cells overexpressing MrpL32 (MrpL32) cells were grown at 30°C and incubated for 2 h in the presence of different concentrations of H_2O_2. Mitochondrial encoded proteins were labelled for 10 min with [^{35}S]-methionine in the presence of cycloheximide. Protein synthesis was monitored by SDS–PAGE and autoradiography. The total amount of newly translated proteins in the absence of H_2O_2 was quantified by phosphorimaging and is given as percentage of mitochondrial-translation products in the absence of H_2O_2 in the respective mitochondria. Steady-state levels of MrpL32, MrpL13, and, for control, Hsp60 were analysed on SDS–PAGE and immunoblotting. The efficiency of translation was quantified in five independent experiments (± s.e.m.) and the statistical significance was assessed by a paired Student's *t*-test (*$P<0.05$).

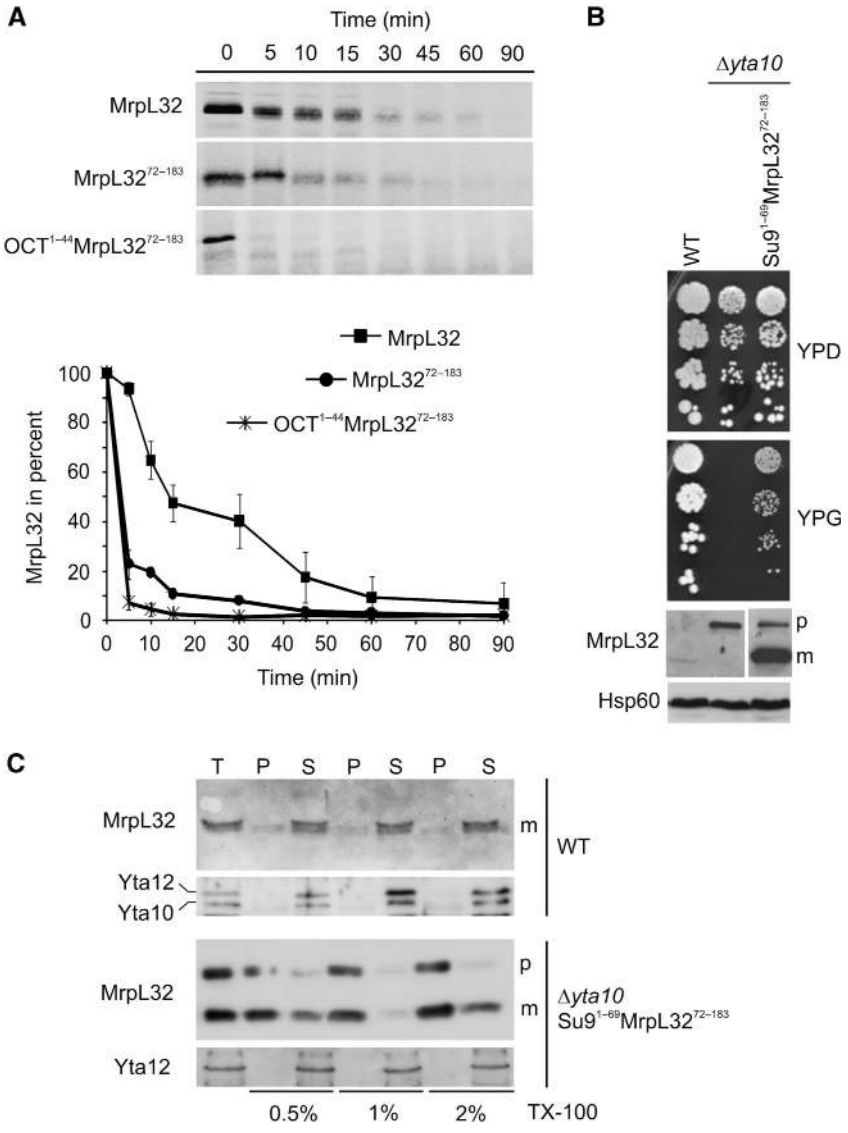

Figure 6 Presequence-assisted folding of MrpL32. (**A**) MrpL32, MrpL32$^{72–183}$ and OCT$^{1–44}$MrpL32$^{72–183}$ were synthesized in a cell-free system in the presence of [^{35}S]-methionine and subjected to limited proteolysis as in Figure 3. Samples were analysed by SDS–PAGE and autoradiography and three independent experiments were quantified by phosphorimaging (±s.e.m.). (**B**) Respiratory growth of Δ*yta10* cells expressing Su9$^{1–69}$MrpL32$^{72–183}$. Wild-type (WT) cells, Δ*yta10* and Δ*yta10* cells expressing Su9$^{1–69}$MrpL32$^{72–183}$ were grown on fermentable (YPD) and non-fermentable (YPG) carbon sources at 30°C. Cell extracts were analysed by SDS–PAGE and immunoblotting using antibodies directed against MrpL32 and Hsp60; p, precursor form of MrpL32 and m, mature form of MrpL32. (**C**) Aggregation of mature MrpL32 derived from Su9$^{1–69}$MrpL32$^{72–183}$ *in vivo*. Mitochondria isolated from wild-type cells (WT) or Δ*yta10* cells expressing Su9$^{1–69}$MrpL32$^{72–183}$ were lysed in the presence of different concentrations of Triton X-100 and split into a pellet (P) and supernatant (S) fraction by centrifugation. Fractions were analysed by SDS–PAGE and immunoblotting using antibodies directed against MrpL32 and, to control for solubilization of mitochondrial membranes, against Yta10 and Yta12.

restore respiratory growth of Δ*yta10* cells suggesting functional complementation (Figure 6B; Nolden *et al*, 2005). Immunoblotting of mitochondria isolated from these cells revealed that mature MrpL32 accumulates in *m*-AAA protease-deficient Δ*yta10* mitochondria at significantly increased levels when compared with wild-type mitochondria (Figure 6B). However, respiratory growth of these cells on non-fermentable carbon sources was only partially restored (Figure 6B). These findings are consistent with an inefficient folding of MrpL32$^{72–183}$ lacking the endogenous presequence *in vivo*, which accumulates rather than being degraded in *m*-AAA protease-deficient mitochondria.

As inefficient folding of MrpL32$^{72–183}$ may result in its aggregation, we determined the detergent solubility of MrpL32$^{72–183}$ *in vivo*. Mitochondria were isolated from wild-type cells and Δ*yta10* cells expressing Su9$^{1–69}$MrpL32$^{72–183}$. After solubilization of mitochondrial membranes using different concentrations of Triton X-100, extracts were split by centrifugation into pellet and supernatant fractions. Immunoblotting using Yta10- and Yta12-specific antibodies revealed that these integral membrane proteins were present in the supernatant fraction indicating complete solubilization of mitochondrial membranes (Figure 6C). As expected, mature MrpL32 was recovered from wild-type mitochondria as a soluble protein (Figure 6C). In contrast, only a minor part of MrpL32$^{72–183}$ generated from Su9$^{1–69}$MrpL32$^{72–183}$ was soluble in mitochondria. The majority of MrpL32$^{72–183}$ was aggregated and was detected in the pellet

fraction after solubilization of mitochondrial membranes (Figure 6C). These experiments provide compelling evidence that the formation of a soluble form, that is, folding, of MrpL32 *in vivo* depends on its mitochondrial presequence, which thus exerts functions in both targeting of MrpL32 to and folding within mitochondria.

Discussion

While previous experiments revealed dual activities of the *m*-AAA protease as quality control and processing enzyme, it remained enigmatic how partial degradation of specific substrates is ensured. We demonstrate here that processing of the ribosomal subunit MrpL32 by the *m*-AAA protease depends on the folding of MrpL32 rather than on the specific recognition of the cleavage site. The *m*-AAA protease binds to N-terminal regions of newly imported MrpL32 and initiates proteolysis, which is halted by a tightly folded, cysteine-containing domain of MrpL32. Mutations in conserved cysteine residues of MrpL32 or the depletion of metal ions impair MrpL32 folding, resulting in the complete degradation by the *m*-AAA protease. On the other hand, the insertion of spacer regions N-terminal of the folded domain shifts the maturation site further downstream. Such a processing mechanism explains how the *m*-AAA protease, which has likely degenerate cleavage specificity and processively degrades misfolded proteins to peptides (Young *et al*, 2001), can mediate the maturation of specific proteins. Our findings are reminiscent of 26S proteasomes that regulate various signalling cascades by processing a number of transcription factors, including mammalian NF-κB, Cubitus interruptus of *D. melanogaster* or yeast Spt23 and Mga1 (Tian *et al*, 2005; Piwko and Jentsch, 2006). Similarly, bacterial Clp proteases release their substrate when they reach protein domains that are hard to unfold (Kenniston *et al*, 2003). In these cases, tightly folded domains in substrate proteins in combination with an amino-acid sequence with low compositional complexity ensure partial degradation (Tian *et al*, 2005). The simple sequence is thought to limit the unfolding force exerted on substrate proteins, and thus, act as a processing signal. Such a sequence is absent from the N-terminal region of MrpL32. However, membrane-bound AAA proteases have a low capacity to unfold their substrate proteins (Herman *et al*, 2003; Koodathingal *et al*, 2009), which may lead to substrate release from the *m*-AAA protease when the protease encounters a tightly folded domain.

Folding of MrpL32 depends on the integrity of the conserved CxxC-X_9-CxxC sequence motif. Several lines of evidence suggest that this sequence binds metal ions and does not form disulphide bridges: first, chelating agents like EDTA destabilize MrpL32 *in vitro*; second, MrpL32 remains functional active *in vivo*, if cysteine residues are replaced by histidine which coordinates metal ions in many proteins but cannot form disulphide bonds; third, AMS-modification experiments demonstrate that MrpL32 is present in a reduced state in the mitochondrial matrix. It appears likely that MrpL32 coordinates zinc ions, as has been found for other proteins containing related cysteine-containing domains (Won *et al*, 2004; Maret, 2006). Structurally related zinc binding sites in bacterial Hsp33 serve as redox switches and sense high levels of ROS *in vivo* (Maret, 2006). Zinc binding renders cysteine ligands more susceptible to electrophilic ROS, which can explain the decreased stability of MrpL32 under oxidative stress. Strikingly, we observed that, at least under certain circumstances, folding and accumulation of MrpL32 can be rate limiting for mitochondrial protein synthesis. It is, therefore, conceivable that increased ROS production by respiratory chain complexes impacts on mitochondrial translation, resulting in attenuation of the synthesis of mitochondrial encoded subunits of respiratory complexes under oxidative stress.

Our experiments revealed an unexpected role of the mitochondrial presequence in MrpL32 folding. MrpL32 was unable to attain a protease-protected, folded conformation in the absence of the presequence *in vitro*. Consistently, mature MrpL32 that was targeted to mitochondria *in vivo* by a heterologous mitochondrial sorting signal exhibited to a strong tendency to aggregate. The expression of this hybrid protein restored respiratory growth of *m*-AAA protease-deficient cells only partially, although the chimeric protein was overexpressed and mature MrpL32 accumulated at significantly higher levels in mitochondria. The presequence of MrpL32 thus exerts dual activities: it ensures the targeting of newly synthesized MrpL32 to the mitochondrial matrix space and its folding after translocation across mitochondrial membranes. How the presequence assists the folding of newly imported MrpL32 remains to be determined. It may act in a similar manner as pro-domains that promote folding of a wide variety of proteases and other proteins (Baker *et al*, 1993; Eder and Fersht, 1995). In contrast to molecular chaperone proteins which facilitate folding by decreasing the rate of aggregation (Hartl and Hayer-Hartl, 2002), pro-regions were found to interact with the mature part of a protein and directly increase the forward folding reaction (Baker *et al*, 1993).

The presequence-dependent folding of MrpL32 provides also an attractive answer to the puzzling question as to why MrpL32 is processed by the *m*-AAA protease and not by the general MPP, as the vast majority of presequence-containing mitochondrial proteins (Figure 7). It is well established that MPP cleaves mitochondrial precursor proteins during their import into the mitochondrial matrix space (Ungermann *et al*, 1994). Mature forms of newly imported mitochondrial proteins fold into their native state upon completion of import. In case of MrpL32, however, processing during membrane translocation would impair presequence-assisted folding after import and therefore would be detrimental (Figure 7). The need to ensure maturation after completion of the import reaction can therefore explain the unusual processing of MrpL32 by the *m*-AAA protease. It will be of interest to examine whether presequences of other mitochondrial matrix proteins lacking an apparent cleavage site for MPP (Vogtle *et al*, 2009) assist protein folding as well.

Materials and methods

Cloning procedures

The plasmid pCRII-MrpL32 for SP6-RNA polymerase-driven expression *in vitro* was previously described (Nolden *et al*, 2005). Mutations in conserved cysteine residues were introduced by site-directed mutagenesis. A random sequence of 20 amino acids was introduced into pCRII-MrpL32 by ex-site-PCR. To introduce a 40 amino-acid spacer into the sequence of MrpL32, the

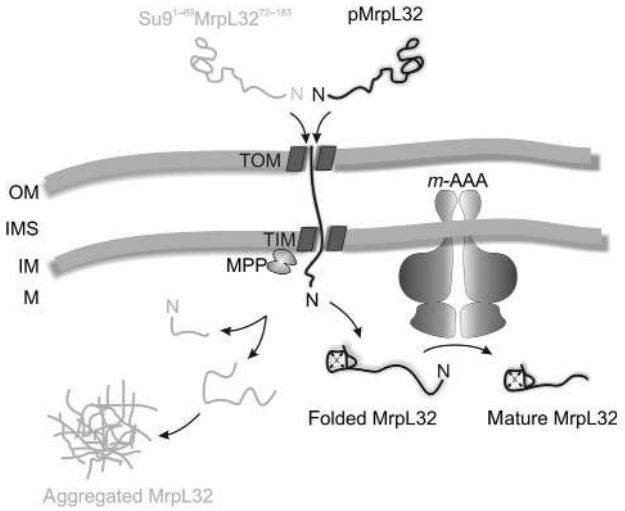

Figure 7 MrpL32 processing by the *m*-AAA protease after completion of import allows folding of MrpL32 within mitochondria. Mature MrpL32 accumulates in a soluble form, if processing occurs by the *m*-AAA protease after completion of import, allowing presequence-assisted folding of MrpL32 within mitochondria. In contrast, co-translocational processing of Su9$^{1-69}$MrpL32 by MPP results in aggregation of mature MrpL32. See text for details. IM, inner membrane; IMS, intermembrane space; M, matrix; MPP, mitochondrial processing peptidase; OM, outer membrane; TIM, translocase of the inner membrane; TOM, translocase of the outer membrane.

unstructured region of the *E. coli lacI* gene (Prakash *et al*, 2004) was amplified by PCR and cloned into a *Xma*I site previously integrated into pCRII-MrpL32. For expression of MrpL32 or its variants in yeast, genes were cloned into the yeast expression vector YEplac181 and expressed under the control of an *ADH1* promotor (Nolden *et al*, 2005). The presequence from murine OCT, residues 1–44, was cloned into pGEM-T Su9$^{1-69}$MrpL32$^{72-183}$ (Nolden *et al*, 2005), replacing the Su9$^{1-69}$ presequence and obtaining pGEM-T_OCT$^{1-44}$MrpL32$^{72-183}$.

Yeast strains and growth conditions

All *S. cerevisiae* strains used in this study are derivatives of W303, except for Δ*sod2,* Δ*sod2*Δ*yta12* that are derived from an S288c wild-type strain. Yeast cells were grown according to standard procedures at 30°C either in YP medium (1% (w/v) yeast extract, 2% (w/v) peptone) or in minimal medium (0.67% yeast nitrogen base, 0.15% amino-acid mix) supplemented with 40 μg/ml of adenine and tryptophan, 20 μg/ml of histidine and uracil, 60 μg/ml of leucine and 30 μg/ml lysine in different combinations, which contained 2% (w/w) glucose. For isolation of mitochondria, glucose was replaced by 2% (w/w) galactose and 0.5% (w/v) lactate. For testing the respiratory activity, yeast cells were grown on YP medium containing 3% (w/v) glycerol as the sole carbon source.

To examine for growth complementation of Δ*mrpl32* cells, YEplac181 plasmids encoding MrpL32 or its variants were transformed into diploid *MRPL32*/Δ*mrpl32* cells. Haploid spores harbouring the deletion of *MRPL32* and the plasmid YEplac181 were isolated by sporulation and tetrad dissection and tested for their ability to grow on non-fermentable carbon sources.

Assessment of MrpL32 folding in vitro

The cell-free synthesis of MrpL32 and variants thereof was performed in the presence of [^{35}S]-methionine using the TNT® Sp6 or T7 coupled reticulocyte lysate system (Promega) according to the manufacturer's instructions. For limited proteolysis, [^{35}S]-labelled proteins (1 μl) were incubated for the time indicated at 4°C in 5 mM Tris/HCl pH 7.4 (10 μl) in the presence of proteinase K (0.4 μg/ml). Addition of PMSF (1 mM) inhibited proteolysis at indicated time points. EDTA (5 mM) was present during the reaction when indicated. Samples were analysed by SDS–PAGE and quantified by phosphorimaging analysis.

Protein import into isolated mitochondria

For *in vitro* protein import, mitochondria were isolated as previously described (Tatsuta and Langer, 2007) and resuspended to a concentration of 1 mg/ml in import buffer (Tatsuta and Langer, 2007) that was supplemented with NADH (5 mM), creatine phosphate (10 mM), creatine kinase (100 μg/ml) and ATP (2.5 mM). For control, the membrane potential was dissipated by addition of valinomycin (0.5 μM) when indicated. Samples were incubated at 30°C for 3 min before [^{35}S]-labelled precursor proteins were added. Import was performed for 30 min at 25°C and halted by the addition of valinomycin (0.5 μM). To assess the stability of newly imported proteins, samples were further incubated at 37°C for the indicated time. Non-imported precursor proteins were degraded by trypsin (50 μg/ml) for 20 min at 4°C. Proteolysis was inhibited by adding soybean trypsin inhibitor (1 mg/ml). Mitochondria were washed with SHKCl buffer (0.6 M sorbitol, 50 mM HEPES/KOH pH 7.4, 80 mM KCl) and analysed by SDS–PAGE, autoradiography and immunoblotting.

Assessment of MrpL32 aggregation

In order to assess aggregation of MrpL32 *in vivo*, membrane fractions (from 10OD cells) containing mitochondria were isolated from wild-type cells or Δ*yta10* cells expressing Su9$^{1-69}$MrpL32$^{72-183}$ and lysed for 20 min at 4°C (T) in the presence of different concentrations of Triton X-100 and PMSF (1 mM). The sample was split into a pellet (P) and supernatant (S) fraction by centrifugation for 20 min at 18 000 *g*. Fractions were analysed by SDS–PAGE and immunoblotting using antibodies directed against MrpL32 and, to control for solubilization of mitochondrial membranes, against Yta10 and Yta12.

Coimmunoprecipitation

To examine binding of newly imported precursor proteins to the *m*-AAA protease, mitochondria (500 μg) were lysed after completion of import at a concentration of 1 mg/ml in 1% (w/v) digitonin, 50 mM K-phosphate buffer pH 7.0, 50 mM NaCl, 4 mM Mg-acetate, 10% (w/v) glycerol, 1 mM PMSF supplemented with protease inhibitor cocktail (Roche). After a clarifying spin for 15 min at 125 000 *g*, extracts were loaded onto 20 μl protein-A sepharose beads (100 mg/ml) coupled with affinity purified antibodies against Yta10 or preimmune serum and incubated for 12 h at 4°C under gentle shaking. Beads were washed subsequently with 0.5% (w/v) digitonin, 50 mM K-phosphate buffer pH 7.0, 50 mM NaCl, 4 mM Mg-acetate, 1 mM PMSF and with 10 mM Tris–HCl, pH 7.4. Antibody–antigen complexes were eluted from the beads and analysed by SDS–PAGE, autoradiography and immunoblotting.

Miscellaneous

Polyclonal antibodies directed against MrpL13 were raised in rabbits (Biogenes, Germany) using a purified fusion protein of MrpL13 and GST that had been expressed in *E. coli*. The specificity of the obtained antisera was verified by immunoblotting using Δ*mrpl13* cells. The following procedures were performed essentially as described in Tatsuta and Langer (2007): labelling of mitochondrial-translation products in yeast cells; alkaline extraction of proteins from yeast cells.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: FB performed most of the experiments. TT performed the experiments on the histidine variants of MrpL32. CR and JR performed the analysis of the redox status of MrpL32. FB and TL designed the research and wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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