

# Amyloid- $\beta$ Peptide Induces Mitochondrial Dysfunction by Inhibition of Preprotein Maturation

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## SUMMARY

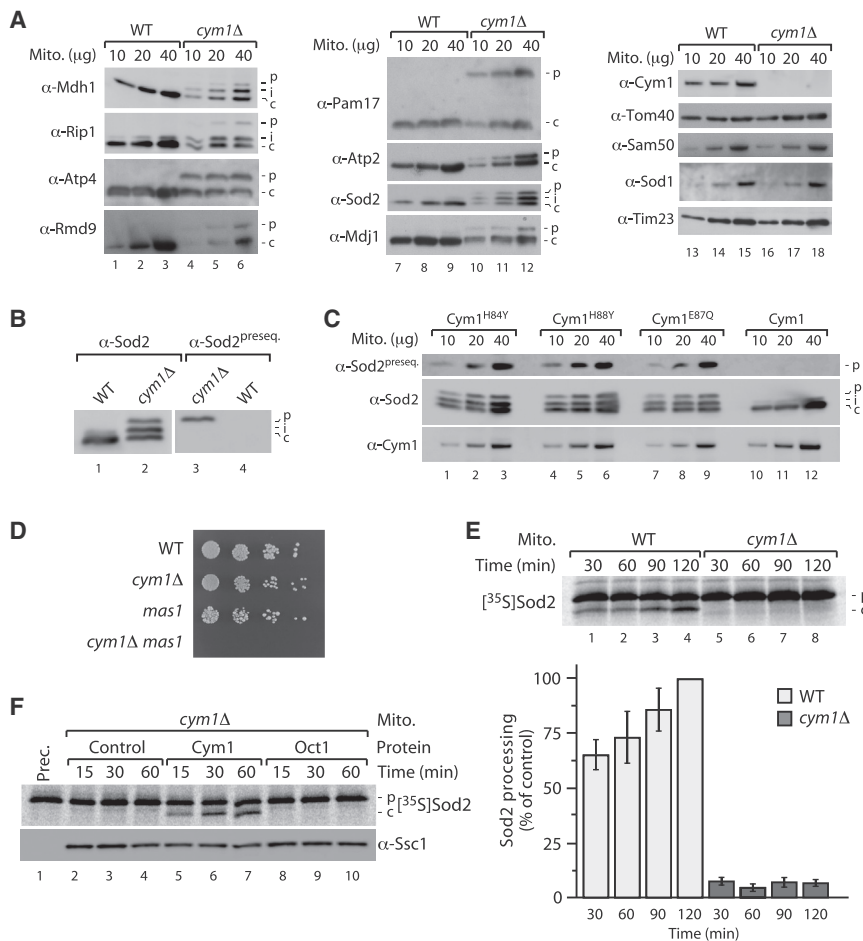
Most mitochondrial proteins possess N-terminal presequences that are required for targeting and import into the organelle. Upon import, presequences are cleaved off by matrix processing peptidases and subsequently degraded by the peptidosome Cym1/PreP, which also degrades Amyloid-beta peptides (A $\beta$ ). Here we find that impaired turnover of presequence peptides results in feedback inhibition of presequence processing enzymes. Moreover, A $\beta$  inhibits degradation of presequence peptides by PreP, resulting in accumulation of mitochondrial preproteins and processing intermediates. Dysfunctional preprotein maturation leads to rapid protein degradation and an imbalanced organellar proteome. Our findings reveal a general mechanism by which A $\beta$  peptide can induce the multiple diverse mitochondrial dysfunctions accompanying Alzheimer's disease.

## INTRODUCTION

The vast majority of mitochondrial proteins is nuclear-encoded and has to be imported into the organelle from the cytosol. Approximately two-thirds of all mitochondrial preproteins possess N-terminal presequences that direct them to the mitochondrial import machineries (Neupert and Herrmann, 2007; Chacinska et al., 2009; Vögtle et al., 2009). Upon import, presequences are typically cleaved by the mitochondrial processing

peptidase MPP in the matrix releasing the mature protein (Hawliitschek et al., 1988; Yang et al., 1991; Vögtle et al., 2009). In several cases, MPP generates import intermediates that are further processed by the octapeptidyl peptidase Oct1/MIP or the intermediate cleaving peptidase Icp55 (Vögtle et al., 2009, 2011; Mossmann et al., 2012; Teixeira and Glaser, 2013). Incomplete processing of mitochondrial preproteins leads to their destabilization and accelerated turnover (Yang et al., 1991; Mukhopadhyay et al., 2007; Vögtle et al., 2009, 2011; Varshavsky, 2011). Presequence peptides that have been cleaved by MPP are subsequently degraded by the matrix peptidosome Cym1/PreP, a metallopeptidase of the pitrilysin family M16 (Alikhani et al., 2011a). PreP catalyzes turnover of peptides larger than 11 amino acids as well as unstructured proteins (Stahl et al., 2002). Recently, it has been shown that PreP also degrades amyloid-beta (A $\beta$ ) peptides that were reported to accumulate in mitochondria of Alzheimer's disease (AD) patients, and a decline in PreP activity has been observed in AD mitochondria (Manczak et al., 2006; Falkevall et al., 2006; Hansson Petersen et al., 2008; Alikhani et al., 2011b). A $\beta$  import depends on the mitochondrial import machinery (Hansson Petersen et al., 2008). Mitochondrial A $\beta$  appears to affect a multitude of different functions in AD, including respiration, detoxification of reactive oxygen species (ROS), and organellar morphology (Lustbader et al., 2004; Manczak et al., 2006; Yao et al., 2009; Rhein et al., 2009; Morais and De Strooper, 2010; Selfridge et al., 2013). How a single peptide like A $\beta$  can impair all of these diverse, important mitochondrial functions remains elusive.

Here, we investigated the effects of impaired peptide turnover and of A $\beta$  peptide accumulation on mitochondrial functions. We report that mitochondrial preprotein maturation depends on efficient peptide degradation. Moreover, we find that mitochondrial A $\beta$  leads to inhibition of peptide turnover, thereby causing



**Figure 1. Mitochondrial Presequence Processing Depends on Peptide Turnover**

(A) Immunoblot analysis of wild-type (WT) and *cym1Δ* mitochondria isolated from yeast strains grown on YPD at 30°C. Right panel shows Cym1 and control proteins that are not imported via presequences. p, precursor; i, intermediate; c, cleaved protein.

(B) Sod2 presequence specific antibody recognizes the larger precursor form accumulating in *cym1Δ* mitochondria.

(C) Immunoblot showing Sod2 precursor accumulation in yeast with mutations in the catalytic center of Cym1 (HXXEH).

(D) Synthetic lethality of *cym1Δ mas1* double mutant. The indicated strains were grown under respiratory growth condition (YPG plates, 30°C).

(E) In vitro processing of [<sup>35</sup>S]Sod2 precursor in soluble extracts of wild-type (WT) and *cym1Δ* mitochondria in the presence of 10 μM Cox4 presequence peptide. Quantifications represent mean ± SEM (n = 4).

(F) In vitro synthesized Cym1 protein restores Sod2 precursor processing in *cym1Δ* mitochondrial extract. Reaction was performed as described in (E). Oct1, wheat germ lysate with synthesized Oct1 protein; Control, without wheat germ lysate.

accumulation of nonprocessed preproteins and processing intermediates within mitochondria. Impaired preprotein maturation modulates protein turnover and changes the global organellar protein composition, which might finally explain the pleiotropic mitochondrial defects observed in AD mitochondria.

## RESULTS

### Mitochondrial Presequence Processing Depends on Peptide Turnover

In order to analyze the physiological impact of peptide turnover on mitochondrial functions, we characterized a yeast mutant that lacks the PreP homolog Cym1 (Kambacheld et al., 2005). We isolated mitochondria from *cym1Δ* and wild-type strains and analyzed various mitochondrial proteins by western blotting. Several proteins showed a strong accumulation of their precursor forms and processing intermediates as well as decreased levels of cleaved, mature proteins in *cym1Δ* mitochondria in comparison to wild-type (Figure 1A). An antibody raised against the presequence peptide of Sod2 revealed a specific signal of the precursor form in *cym1Δ* mitochondria, but not in wild-type (Figure 1B). Analysis of *cym1* mutants that lack critical residues of the metal binding motif (HXXEH) (Table S1, available online; Kambacheld et al., 2005) indicated that accumulation of precursor proteins depends on Cym1 protease activity (Figure 1C). This

implied that impaired peptide degradation in *cym1Δ* might affect the presequence processing activity of MPP. To further support this assumption, we performed a global mass spectrometric analysis of mitochondrial N termini in *cym1Δ* mitochondria using COFRADIC (combined fractional diagonal chromatography) (Vögtle et al., 2009) (Table S2). Indeed, a large number of N termini in *cym1Δ* mitochondria corresponded to nonprocessed preproteins or processing intermediates of dually processed proteins when compared to the N-proteome of wild-type mitochondria (Tables S2 and S3) (Vögtle et al., 2009). We conclude that the lack of the peptidase Cym1 leads to impaired preprotein maturation in vivo.

As an additional assay to link preprotein maturation and peptide turnover, we generated a yeast mutant that lacks Cym1 and harbors a temperature-sensitive allele of the essential MPP subunit Mas1 (Hawlicschek et al., 1988; Yang et al., 1991; Vögtle et al., 2009). The mutant was not able to grow under respiratory conditions (i.e., when mitochondrial energy metabolism is essential for cell viability), indicating a genetic interaction of the presequence peptidase MPP and the peptidosome Cym1 (Figure 1D).

The proteins that were accumulating as precursors or processing intermediates in *cym1Δ* samples encompass a variety of mitochondrial functions, including respiration, ATP synthesis, mtDNA maintenance, and gene expression or oxidative stress response (Table S3; Figures 1A and 1B). Assessment of various mitochondrial functions in *cym1Δ* mitochondria revealed increased levels of ROS, decreased membrane potential, and impaired O<sub>2</sub> consumption compared to wild-type (Figure S1A).

Remarkably, similar diverse mitochondrial dysfunctions have been observed in mitochondria from AD patients and AD mouse models (Morais and De Strooper, 2010; Manczak et al., 2006; Rhein et al., 2009; Alikhani et al., 2011b). These results indicate that physiological consequences of impaired mitochondrial peptide turnover activity correlate with pathological phenotypes observed in AD mitochondria.

To test if presequence processing is affected in the absence of Cym1, we performed in organello imports of radiolabelled preproteins into isolated mitochondria of wild-type and *cym1 $\Delta$*  yeast cells. Presequence processing of Sod2 preprotein was impaired in *cym1 $\Delta$*  mitochondria, and nonprocessed preprotein accumulated as a Proteinase K-resistant form within the organelle (Figure S1B). Similar defects were observed when import was performed in *mas1* mitochondria (Figure S1C). The general import efficiency of the presequence import pathway was not compromised in *cym1 $\Delta$*  or *mas1* mitochondria (tested by the nonprocessed Hsp10 preprotein) (Figures S1B and S1C). The processing defect in *cym1 $\Delta$*  appeared to be specific for MPP because presequence cleavage of Mrp132 that does not depend on MPP (Nolden et al., 2005) was fully functional in *cym1 $\Delta$*  mitochondria (Figure S1D). To directly analyze the dependency of preprotein maturation on peptide turnover, we employed an in vitro processing assay in mitochondrial extracts (Figure S1E) (Falkevall et al., 2006) from wild-type and *cym1 $\Delta$*  mitochondria. This allowed the analysis of presequence processing independent of protein import. Presequence peptides were rapidly degraded in wild-type, but not *cym1 $\Delta$* , extracts (Figure S1F). In the presence of a typical presequence peptide (Cox4<sup>preseq</sup>) (Yang et al., 1991), the in vitro processing of radiolabelled Sod2 precursor by MPP was efficiently blocked in the absence of Cym1 (Figure 1E). Cox4 presequence peptides were able to inhibit purified MPP in similar concentrations (Figures S1G and S1H) (Yang et al., 1991). To exclude that the impaired presequence processing activity in *cym1 $\Delta$*  is caused by an indirect effect, we added cell-free translated Cym1 protein to the in vitro processing assay. We found that MPP processing activity was restored in *cym1 $\Delta$*  extracts in the presence of Cym1 protein, indicating a direct functional link between Cym1 activity and presequence processing (Figure 1F). In addition, overexpression of MPP in the *cym1 $\Delta$*  strain, as well as presence of purified MPP in mitochondrial extracts of *cym1 $\Delta$* , was able to suppress the impaired preprotein processing activity (Figures S1I and S1J). We conclude that impaired turnover of presequence peptides leads to inhibition of MPP processing activity. This functional coupling explains the preprotein accumulation observed in *cym1 $\Delta$*  mitochondria in vivo (Figures 1A and 1B; Table S3).

### Maturation of Precursor Processing Intermediates Depends on Cym1

We wondered why preprotein processing intermediates also accumulated in the *cym1 $\Delta$*  mutant (Figure 1A). It has been proposed that PreP/Cym1 requires a minimal substrate length of 11 amino acids (Stahl et al., 2002). However, the intermediate peptidase Oct1 cleaves off octapeptides (Vögtle et al., 2011). We constructed an *oct1 $\Delta$ cym1 $\Delta$*  double mutant, and the observed synthetic growth defect pointed to a functional link between both enzymes (Figure 2A). Furthermore, we found that processing activity of purified MPP was not affected in the pres-

ence of octapeptides (derived from the Oct1 substrate Sdh1; Figure 2B). In contrast, the presence of a presequence peptide efficiently inhibited MPP activity (Figure S1H). However, in vitro processing of the Cox4 precursor that is cleaved sequentially by MPP and Oct1 revealed a specific impairment of the Oct1-dependent processing step in *cym1 $\Delta$*  in the presence of octapeptides (Figure 2C). This indicated that Cym1 activity is also affected by accumulation of octapeptides and that an impaired turnover of MPP generated presequences, and Oct1-derived octapeptides leads to inhibition of presequence processing, causing accumulation of preproteins as well as processing intermediates.

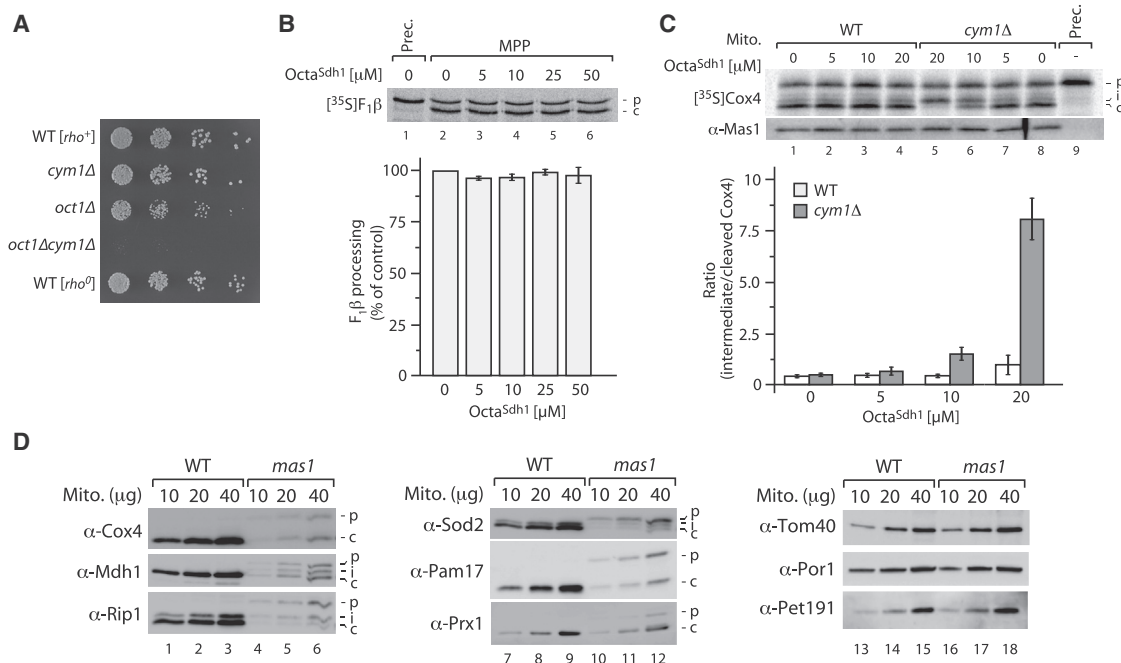
To investigate the functional consequences of impaired presequence processing activity, we employed mitochondria from the temperature-sensitive *mas1* strain that had been shifted to nonpermissive growth temperature for 24 hr to inactivate MPP activity in vivo. Western blot analysis revealed an accumulation of nonprocessed preproteins and reduced levels of mature proteins similar to the phenotype observed in *cym1 $\Delta$*  mitochondria (Figure 2D). Thus, inactivation of either peptide turnover (*cym1 $\Delta$* ) or presequence processing (*mas1*) leads to the phenotype of impaired preprotein maturation and reduced amounts of mature proteins (Figures 1A, 1B, and 2D).

### A $\beta$ Impairs Mitochondrial Peptide Turnover, Leading to Feedback Inhibition of Presequence Processing

Next, we asked if A $\beta$  peptide that accumulates in mitochondria of AD patients and represents a substrate of human PreP might cause a delay in matrix peptide degradation and thereby induce feedback inhibition of presequence processing enzymes. We found that A $\beta$  peptide was degraded by Cym1 in mitochondrial extracts and by the recombinant enzyme (Figures 3A and 3B). However, degradation of A $\beta$  was slower compared to turnover of presequence peptides (Figure S2A). We then analyzed degradation of Cox4 presequence peptides in wild-type mitochondrial extracts and found that the presence of A $\beta$ , but not of a scrambled form, impaired the peptide turnover capacity of Cym1 (Figure 3C, lanes 5–13 versus 18–26). Scrambled A $\beta$  was not degraded by Cym1 (Figure S2B). We further asked if A $\beta$  can also affect presequence processing activity of MPP. We tested the processing of radiolabelled Sod2 preprotein and observed a striking delay in presequence processing in the presence of A $\beta$  (Figure 3D). We noticed that A $\beta$ <sup>1–40</sup> (unlike the shorter version A $\beta$ <sup>1–28</sup>) slightly inhibited activity of purified MPP at higher concentrations (Figures S2C and S2D) and therefore included the shorter version in our functional assays. Taken together, our findings show that A $\beta$  peptide inhibits peptide turnover in mitochondrial extracts, which causes impaired maturation of preproteins.

### Mitochondrial A $\beta$ Inhibits Precursor Maturation In Vivo

To analyze the effect of A $\beta$  on precursor maturation in vivo, we attempted to reconstitute the entire mechanism described above in a yeast model that allowed galactose-induced expression of an eGFP-A $\beta$ <sup>1–42</sup> fusion protein harboring a cleavage site for TEV protease (Figure 4A). Coexpression of TEV protease led to release of A $\beta$  peptides from the fusion protein (Figure S3A). To analyze A $\beta$ -induced mitochondrial dysfunctions, we employed the aging-prone strain *coa6 $\Delta$* , which showed a moderate instability of respiratory chain complexes in order to mimic



**Figure 2. Maturation of Precursor Processing Intermediates Depends on Cym1**

(A) Synthetic growth defect of *cym1Δ oct1Δ* double-mutant yeast strain under fermentable conditions (23°C, YPD). [*rho*<sup>0</sup>], wild-type strain lacking mitochondrial DNA.

(B) Processing of [<sup>35</sup>S]F<sub>1</sub>β precursor by purified MPP is not inhibited by octapeptides. Quantifications represent mean ± SEM (n = 3).

(C) In vitro processing assay of [<sup>35</sup>S]Cox4 precursor in WT and *cym1Δ* mitochondrial extracts in the presence of octapeptides. Quantifications represent mean ± SEM (n = 4).

(D) Impaired preprotein maturation leads to imbalanced mitochondrial proteome. Wild-type and temperature-sensitive *mas1* strains were grown on YPD medium at 23°C and shifted to nonpermissive temperature (37°C) for 24 hr. Inactivation of the essential MPP subunit Mas1 causes accumulation of precursor proteins that are rapidly degraded. This leads to decreased amounts of mature, fully cleaved proteins (lanes 4–6 and 10–12). In contrast, proteins that do not contain presequences were not affected (lanes 13–18). p, precursor; i, intermediate; c, cleaved protein.

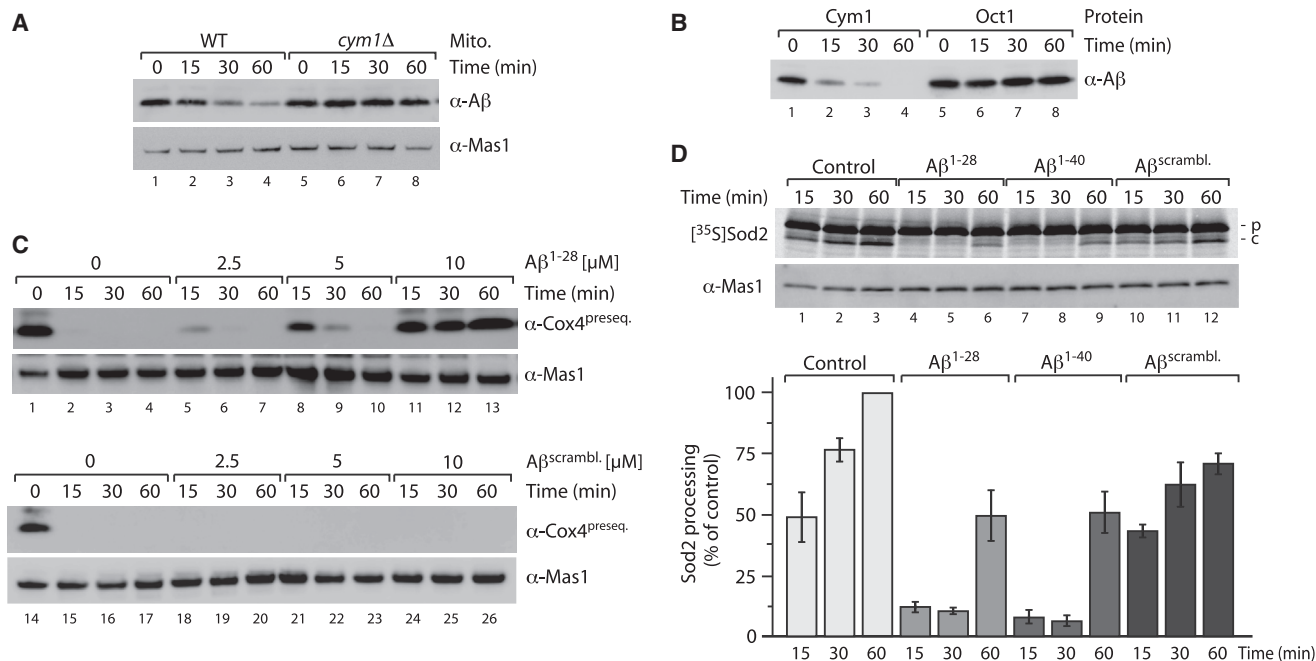
conditions in aged humans (Larsson, 2010; Vögtle et al., 2012), and induced eGFP-A $\beta$ <sup>1–42</sup> expression by growth on galactose. A $\beta$  localized to mitochondria (Figures S3B and S3C) and impaired maturation of Sod2 precursor in soluble mitochondrial extracts (Figures 4B and 4C). The presequence import pathway was not compromised by A $\beta$  (Figure S3D). After induction for 3 days, we observed accumulation of several precursor proteins in mitochondria of the A $\beta$ -expressing strain (Figure 4C), indicating that A $\beta$  inhibits preprotein maturation in vivo. After induction for 5 days, several mitochondrial dysfunctions were observed in mitochondria isolated from the A $\beta$ -expressing strain compared to the control strain. This included increased levels of ROS, a decrease in membrane potential, and impaired oxygen consumption (Figure S3E). Overexpression of Cym1 in the A $\beta$ -expressing strain led to an increased turnover of A $\beta$ , substantial reduction of the accumulating preproteins in vivo (Figure S3F), and increased MPP processing activity in vitro (Figure S3G). We conclude that A $\beta$ -mediated inhibition of the peptidase Cym1 leads to an impaired presequence processing activity of MPP, which results in accumulation of preproteins in vivo.

As a further in vivo system, we tested freshly isolated matrix extracts from brain mitochondria of PS2APP mice. This AD model harbors mutations in the PS2 (N141I) and APP (Swedish

FAD) genes and shows A $\beta$  accumulation in mitochondria (Figure S3H) (Rhein et al., 2009; Ozmen et al., 2009). Indeed, processing of the Cox4 preprotein was significantly impaired in PS2APP samples compared to that in age-matched wild-type mice (Figure 4D).

Finally, we asked if mitochondrial precursor accumulation caused by A $\beta$  can be observed in AD patients. Therefore, we isolated mitochondria from post mortem brain samples of four AD patients and four age-matched non-AD controls (Table S4). Western blot analysis showed the presence of higher molecular species of the matrix protein MDH2 in all patient samples, but not in controls (Figure 4E). An antibody raised against the presequence peptide of MDH2 that recognizes only the precursor, but not the mature, cleaved protein, confirmed the specific accumulation of the MDH2 preproteins in AD mitochondria (Figure 4F). In order to identify further mitochondrial proteins that might accumulate as preproteins or processing intermediates in AD patients, we screened various antibodies by western blotting and performed a ChaFRADIC analysis (Venne et al., 2013). Several proteins could be identified, including the mitochondrial ribosomal subunit MRPL23 and NDUFA9, a subunit of the respiratory complex I (Figure S3I and Table S5). Taken together, our results show that mitochondrial A $\beta$  causes impaired maturation of preproteins in vivo.





**Figure 3. A $\beta$  Impairs Mitochondrial Peptide Turnover, Leading to Feedback Inhibition of Presequence Processing**

(A) A $\beta$  degradation in soluble extracts of wild-type (WT) and *cym1* $\Delta$  mitochondria.

(B) A $\beta$  degradation by cell-free translated Cym1 (wheat germ lysate). Oct1, Oct1 translated in wheat germ lysate.

(C) A $\beta$ <sup>1-28</sup>, but not A $\beta$ <sup>scrambled</sup>, peptide impairs Cox4 presequence peptide degradation in WT soluble mitochondrial extract. Cox4 presequence peptide (10  $\mu$ M) was added to each reaction. Mas1, loading control.

(D) In vitro processing of [<sup>35</sup>S]Sod2 precursor in WT mitochondrial extract in the presence of the indicated A $\beta$  peptides (10  $\mu$ M) and 10  $\mu$ M Cox4 presequence peptide. The control 60 min value was set to 100%; mean  $\pm$  SEM (n = 3).

## DISCUSSION

So far, mitochondrial presequence processing and peptide turnover have been considered independent reactions. Our findings indicate a functional coupling of both processes. Presequence processing capacity seems to depend directly on efficient peptide clearance in the matrix, as addition of the peptide-degrading enzyme Cym1 to mitochondrial *cym1* $\Delta$  extracts immediately restored MPP presequence processing activity in vitro. Moreover, lack of Cym1 leads to accumulation of immature preproteins in vivo, reflecting the *mas1* mutant phenotype with impaired presequence processing activity. Accumulating presequence peptides likely bind to the active site of MPP, thereby competing directly with incoming preproteins (Yang et al., 1991; Taylor et al., 2001). In both cases—mutations in Cym1 and in Mas1—impaired preprotein maturation causes accelerated protein degradation and results in an imbalanced organellar proteome. As a consequence, various mitochondrial functions are affected, e.g., decreased respiration and membrane potential and increased levels of ROS. Interestingly, similar phenotypes have been observed in AD mitochondria (Morais and De Strooper, 2010; Manczak et al., 2006; Rhein et al., 2009; Alikhani et al., 2011b). It is still unclear if mitochondrial dysfunction is the cause or consequence of AD and how mitochondrial damage is connected to other cellular dysfunctions observed in AD (Lin and Beal, 2006; Morais and De Strooper, 2010; Treusch et al., 2011). However, it has been shown that A $\beta$  accumulates in mitochondria of AD patients, where it seems to affect similar diverse

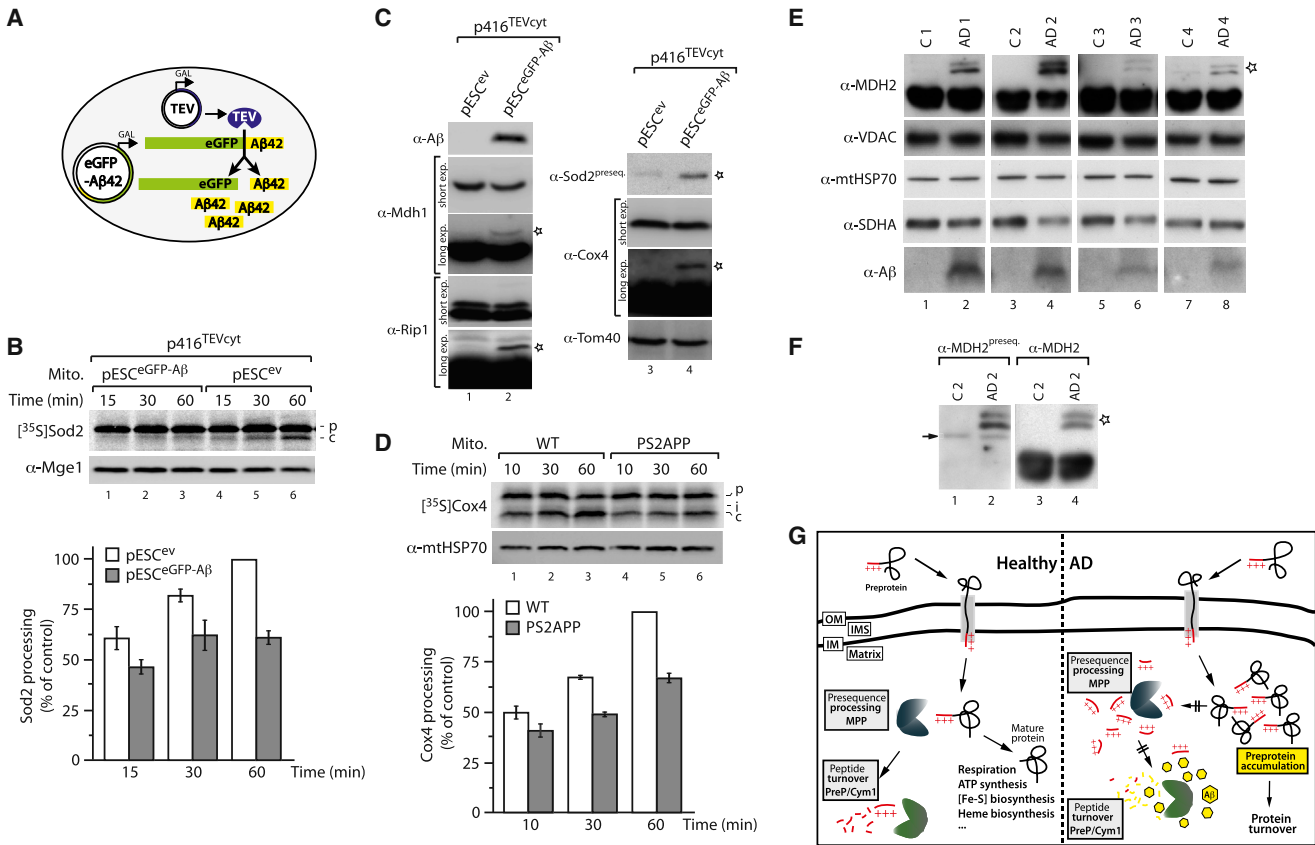
functions that we found here in *cym1* $\Delta$  mitochondria. Our results reveal that A $\beta$  causes inhibition of the peptidosome PreP/Cym1. In turn, accumulation of presequence peptides leads to impaired maturation and therefore cumulation of mitochondrial preproteins by inhibition of the presequence processing machinery. Indeed, global proteomic studies of AD samples indicated an imbalance of the mitochondrial proteome; presequence-containing mitochondrial proteins in particular were found affected (Rhein et al., 2009; Begcevic et al., 2013).

The results presented here provide a mechanistic model that can explain the diversity of mitochondrial dysfunctions observed in AD (Figure 4G). Herein, the presequence processing machinery plays a central role in the age-dependent vicious cycle of mitochondrial dysfunction in AD. Our findings offer exciting perspectives on the development of diagnostic markers (e.g., to detect preproteins in blood cells of patients that were found to possess mitochondrial dysfunctions; Leuner et al., 2012) and therapeutic strategies (e.g., stimulation of the presequence processing machinery) in AD.

## EXPERIMENTAL PROCEDURES

### Isolation of Mitochondria from Yeast Cells and Mouse and Human Brain Tissue

Mitochondria from *S. cerevisiae* strains (Table S1), mouse, and human brain tissues (Table S4) were isolated by differential centrifugation. Yeast cells were grown in YPD or YPG medium. Cells were harvested by centrifugation. Wild-type C57Bl/6 mice and transgenic mice (line PS2APP, homozygous for human PS2 N141I and APP Swedish FAD transgenes; Rhein et al., 2009;



**Figure 4. Mitochondrial Aβ Inhibits Preprotein Maturation In Vivo**

(A) Inducible expression system for generation of free Aβ<sup>1-42</sup> peptide in the yeast cytosol.  
 (B) In vitro processing assay of [<sup>35</sup>S]Sod2 precursor in yeast mitochondrial extracts isolated from *coa6Δ* strains harboring empty vector pESC<sup>ev</sup> or pESC<sup>eGFP-Aβ</sup> (1 day induction on galactose medium). Both strains coexpress TEV protease (p416<sup>TEVcyt</sup>). Quantifications represent mean ± SEM (n = 3).  
 (C) Immunoblot analysis of purified mitochondria from strains described in (B) after induction for 3 days on galactose medium. exp., exposure time. Stars indicate accumulating precursor proteins.  
 (D) In vitro processing assay of Cox4 precursor in WT and PS2APP mouse brain mitochondrial extract. mtHSP70, loading control. Quantifications represent mean ± SEM (n = 3).  
 (E) Immunoblot analysis of various mitochondrial proteins in purified brain (temporal cortex) mitochondria from AD and age-matched non-AD control brains (isolated pairwise). Star indicates precursor protein.  
 (F) Validation of MDH2 precursor accumulation (star) in AD brain mitochondria using presequence-specific antibody. Arrow, nonspecific signal.  
 (G) Model of Aβ-induced inhibition of mitochondrial preprotein maturation. In healthy cells (left), mitochondrial preproteins are imported from the cytosol and presequences are efficiently cleaved off by presequence processing enzymes. Presequence peptides (shown in red) are then degraded by the peptidosome PreP, which constitutes the mitochondrial peptide turnover machinery. Peptide turnover is impaired in the presence of Aβ (AD, right), leading to the inhibition of presequence processing and accumulation of preproteins. This results in their destabilization and turnover.

Ozmen et al., 2009) of 12 months were sacrificed, and brains were removed. Tissue samples of the temporal cortex of human brains were collected post mortem for diagnostic purposes. Cells or tissues were homogenized by 15–30 strokes in a glass potter. Cell debris and nonbroken cells were removed in two consecutive centrifugation steps (484–1,500 × g, 4°C). Mitochondria were isolated by centrifugation at 7,000–16,000 × g at 4°C. The mitochondrial fractions were aliquoted, snap frozen in liquid nitrogen, and stored at –80°C.

**Preprotein Processing and Peptide Degradation Assays in Soluble Mitochondrial Extracts**

Isolated mitochondria were washed, reisolated, and suspended in reaction buffer (250 mM sucrose, 10 mM MOPS/KOH [pH 7.2], 80 mM KCl, 1–5 mM MgCl<sub>2</sub>, 5 mM KH<sub>2</sub>PO<sub>4</sub>). Mitochondrial extracts were obtained by sonication on ice (five times for 30 s with 30 s breaks; Sonifier250, Branson) or by solubilization in 1% digitonin. Samples were centrifuged at 100,000 × g for 45 min or 20,000 × g for 10 min at 4°C, respectively. The supernatant was incubated with radiolabelled preproteins, various peptides, and/or chemical amounts

of peptidases. Reactions were stopped by the addition of 4× Laemmli buffer. Samples were analyzed via SDS-PAGE followed by autoradiography and immunodecoration.

**In Vivo Reconstitution of Aβ-Induced Mitochondrial Dysfunction in Yeast**

Yeast strains were transformed with p416<sup>TEVcyt</sup> and pESC<sup>eGFP-Aβ</sup> or pESC<sup>ev</sup> (empty vector). For selection, cells were grown in selective medium lacking histidine and uracil. Expression of plasmids was induced by shift to selective medium containing 2% galactose. Cells were grown at 30°C for 1, 3, or 5 days. Cell extracts were obtained by post-alkaline extraction, and mitochondria were isolated as described above. Samples were analyzed on SDS-PAGE followed by immunodecoration.

**In Vitro MPP Activity Assay**

β-MPP (with N-terminal poly-His tag) and α-MPP (Mas1 and Mas2) subunits from *S. cerevisiae* were expressed in *E. coli* BL21 cells. Cells were grown in

LB medium (1% [w/v] tryptone, 0.5% [w/v] yeast extract, 1% [w/v] NaCl) at 37°C, and expression was induced by 1 mM IPTG. Cells were lysed and incubated with Ni-NTA resin (QIAGEN). MPP was eluted and further purified by size-exclusion chromatography. For activity assays, purified MPP was incubated in processing buffer with various peptides prior to the addition of <sup>35</sup>S-labeled F<sub>1</sub> $\beta$  preprotein (*Nicotiana plumbaginifolia*). Reactions were stopped with Laemmli buffer, and samples were analyzed by SDS-PAGE and autoradiography.

#### Generation of Radiolabelled Precursors and Chemical Amounts of Peptidases

Radiolabelled preproteins were synthesized by in vitro transcription/translation using the rabbit reticulate lysate system (Promega) in the presence of <sup>35</sup>S-methionine. Chemical amounts of Cym1 (aa 8–989) and control protein (Oct1; aa 29–772) were synthesized using the RTS wheat germ system (5 PRIME).

#### Statistical Analysis

Quantified data are shown as mean  $\pm$  SEM and were obtained from at least three independent experiments.

#### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and five tables and can be found with this article online at <http://dx.doi.org/10.1016/j.cmet.2014.07.024>.

#### AUTHOR CONTRIBUTIONS

D.M., F.-N.V., A.A.T., P.F.T., J.R., J.M.B., N.B., C.M.P., J.T., and R.P.Z. performed the experiments. D.L., C.G., F.-N.V., and O.K. isolated human and mouse samples. D.M., F.-N.V., A.A.T., P.F.T., J.R., J.M.B., N.W., R.P.Z., F.M., E.G., and C.M. designed experiments and interpreted the data. P.F.T., F.M., A.S., N.W., R.P.Z., F.M., and E.G. reviewed and edited the manuscript. C.M., D.M., and F.-N.V. developed the project and wrote the manuscript. C.M. coordinated and directed the project. All authors approved the final version of the manuscript.

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