Functional coupling of presequence processing and degradation in human mitochondria

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The mitochondrial proteome is built and maintained mainly by import of nuclear-encoded precursor proteins. Most of these precursors use N-terminal presequences as targeting signals that are removed by mitochondrial matrix proteases. The essential mitochondrial processing protease MPP cleaves presequences after import into the organelle thereby enabling protein folding and functionality. The cleaved presequences are subsequently degraded by peptidases. While most of these processes have been discovered in yeast, characterization of the human enzymes is still scarce. As the matrix presequence peptidase PreP has been reported to play a role in Alzheimer’s disease, analysis of impaired peptide turnover in human cells is of huge interest. Here, we report the characterization of HEK293T PreP knockout cells. Loss of PreP causes severe defects in oxidative phosphorylation and changes in nuclear expression of stress response marker genes. The mitochondrial defects upon lack of PreP result from the accumulation of presequence peptides that trigger feedback inhibition of MPP and accumulation of nonprocessed precursor proteins. Also, the mitochondrial intermediate peptidase MIP that cleaves eight residues from a subset of precursors after MPP processing is compromised upon loss of PreP suggesting that PreP also degrades MIP generated octapeptides. Investigation of the PrePR183Q patient mutation associated with neurological disorders revealed that the mutation destabilizes the protein making it susceptible to enhanced degradation and aggregation upon heat shock. Taken together, our data reveal a functional coupling between precursor processing by MPP and MIP and presequence degradation by PreP in human mitochondria that is crucial to maintain a functional organellar proteome.

Abbreviations
PreP, presequence peptidase; MPP, mitochondrial processing protease.
Introduction

Mitochondrial proteostasis is essential for cellular survival and mitochondrial dysfunctions are connected to a multitude of severe human diseases, for example, neurological disorders, metabolic disorders, or cardiomyopathies [1-3]. To maintain a functional mitochondrial proteome, quality control mechanisms exist on several levels including protein biogenesis and protein turnover [4,5]. Mitochondrial proteases play a decisive role in these processes, for example, by cleaving targeting signals upon import for protein maturation and functionality or by the degradation of damaged, superfluous, or misfolded proteins [4,5]. Several mutations in mitochondrial proteases have been identified that result in severe human diseases, often affecting tissues with increased energy demands, like brain or heart, but that have been also linked to global human metabolic disorders [6-8]. However, analyses of the underlying pathomechanisms triggered by these mutations, the determinants of tissue specificity or disease onset and progression are often lacking [9].

The mitochondrial proteome is built mainly by import of precursor proteins from the cytosol [10-12]. For targeting most of these precursors possess cleavable signals that are localized at the proteins’ N termini [12,13]. These presequences are directing import into the mitochondrial matrix, where they are proteolytically removed by the essential mitochondrial processing protease MPP that is composed of the two subunits PMPCA and PMPCB [5,14]. Dysfunctional MPP processing results in the accumulation of unprocessed precursor proteins in the mitochondrial matrix [15]. These unprocessed precursors are prone to rapid aggregation and are therefore not functional [15]. As approximately 70% of all mitochondrial precursors use presequences as targeting signals, proteolytic cleavage of these presequences by MPP is indispensable to build up the mitochondrial proteome [13]. One-step MPP processing of presequence precursors is often sufficient to generate functional proteins. However, several precursors require a second maturation step performed by the mitochondrial intermediate peptidase (MIP, Oct1 in yeast), which removes an octapeptide or the intermediate cleaving peptidase Icp55 (identified in yeast) that cleaves a single amino acid [5,13,15,17]. This two-step processing is required to convert MPP generated unstable processing intermediates into stable and mature proteins. Basis for the instability of the processing intermediates and the stability of their mature counterparts are the identity of the N-terminal amino acid that correlates with the half-life of the protein and follows a mitochondrial N-end rule [13,18,19].

The successive processing by MPP and Oct1 or Icp55 is therefore required to obtain stable and functional mitochondrial proteins.

As a result of presequence processing, not only matured proteins are generated but also cleaved presequences are released into the mitochondrial matrix. Several studies mainly performed in the model organism Saccharomyces cerevisiae have identified peptidases dedicated to degrade these free presequences. In yeast, the concerted action of Cym1, Ste23, and Prd1, all localized in the matrix, is securing efficient peptide degradation [20-22]. Deletion of these peptidases results in growth defects that point into the direction that accumulating presequences are toxic in vivo. In human cells, two mitochondrial peptidases have been identified in the matrix, the Cym1 homologue presequence peptidase (PreP) and the Prd1 homologue, neuremysin (NLN) [23,24]. While deletion strains in yeast enabled investigation of peptidase functions in vivo, analysis of the human enzymes so far mainly relied on in vitro assays using recombinant proteins [23,24]. As decreased proteolytic activity of PreP has been identified in brain mitochondria of Alzheimer’s disease patients, mechanistic analysis of the function of PreP in human cells is of immense interest to understand the disease pathology [25]. Furthermore, recent studies have identified several patients with point mutations in PITRM1, encoding PreP, that present with a neurological disorder characterized by progressive spinocerebellar ataxia, cognitive decline, and psychotic episodes and in one family with severe progressive cerebellar atrophy [26-28]. Conflicting reports on the effect of these mutations on PreP activity exist, suggesting either an enhanced turnover or a decreased enzymatic activity of the mutant protein as underlying cause of mitochondrial dysfunction. In addition, these differing conclusions were based on in vitro degradation assays using recombinant PreP and on assays modeling the disease mutation in the yeast homologous Cym1 protein [26-28]. However, missing knowledge of the functional role of PreP has not permitted analysis of the pathophysiological consequences of the mutations in human cells in vivo so that clarification of these opposing findings has not yet been possible.

Here, we report the generation of a PreP HEK293T knockout cell line (PreP\(^{-/-}\)) that allowed functional characterization of human PreP in vivo. Loss of PreP results in severe mitochondrial dysfunction characterized by defects in the respiratory chain complexes and a decreased mitochondrial membrane potential. Cells lacking PreP also displayed changes in nuclear expression of genes associated with mitochondrial stress responses. Mechanistic analysis of PreP\(^{-/-}\) cells

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demonstrated impaired presequence peptide degradation and as a consequence compromised presequence cleavage by MPP with accumulation of nonprocessed precursor proteins revealing a functional coupling of presequence degradation and precursor processing. Similarly, the activity of MIP was strongly compromised in in organello import assays suggesting that PreP also degrades cleaved octapeptides and that their accumulation in the absence of PreP induces inhibition of MIP. The identification of the close link of PreP and MPP in human cells also enabled analysis of the functional consequences of a PrePR183Q patient mutation. Expression of PrePR183Q in PreP−/− cells revealed an MPP defect that was caused by decreased PrePR183Q protein levels rather than defective proteolytic activity of the mutant protein. Further analyses of the PreP−/− R183Q cells revealed that the severely reduced protein levels of mutant PreP observed in the patients are likely caused by an increased protein turnover and destabilization of the protein upon heat shock.

Results

Loss of human presequence peptidase PreP results in severe mitochondrial dysfunctions

To elucidate the function of PreP in human cells, we generated a knockout of PreP using CRISPR/Cas9-mediated disruption of its alleles in HEK293T cells. For this purpose, we targeted exon 5 of PITRM1 (Fig. 1A). Complete loss of PreP was controlled by sequencing (see Materials and methods) and

immunodecoration of mitochondria isolated from wild-type and PreP knockout (PreP−/−) cells. PreP-specific antisera confirmed the absence of PreP (Fig. 1B). Deletion of PreP resulted in a significant growth defect on glucose medium, which was further enhanced upon growth on medium containing galactose as carbon source (Fig. 1C).

To study changes in mitochondrial physiology upon loss of PreP, we analyzed the integrity of respiratory chain complexes by Blue-native-PAGE (BN-PAGE). Complex III, complex IV, and respiratory chain supercomplexes (SC) were reduced in PreP−/− mitochondria when compared to wild-type, while complex II was not altered (Fig. 2A). To assess whether this decrease in respiratory chain complexes impacts also functionally on oxidative phosphorylation, we measured oxygen consumption by real-time respirometry (Fig. 2B). Already basal respiration was significantly decreased in PreP−/− cells compared to control. Total respiratory capacity can be determined by addition of FCCP uncoupling the respiratory chain complexes from the ATP synthase and revealed a severe reduction of OXPHOS capacity in PreP−/− cells. Measurement of the mitochondrial membrane potential (Δψ) indicated a reduction in PreP−/− cells upon growth on glucose-containing medium, which was further exacerbated when galactose was used as carbon source (Fig. 2C).

The mitochondrial proteome is mainly built by import of precursor proteins that are translated in the cytosol and imported into mitochondria post-translationally [10-12]. Targeting to the organelle is in the majority of proteins encoded in N-terminal signals, termed presequences that are cleaved upon import

Fig. 1. Generation of human PreP−/− cells. (A) Schematic of the strategy to target human PITRM1 encoding PreP by CRISPR-Cas9. The guide RNA was directed against exon 5 of PITRM1. Successful targeting of the gene was confirmed by sequencing. (B) Mitochondria isolated from HEK293T wild-type (WT) and PreP−/− cells were analyzed on SDS/PAGE followed by western blotting. Data are representative of one experiment. The experiment was performed at least in triplicates. (C) Proliferation assay of WT and PreP−/− cells grown on medium supplemented with glucose (left bars) or galactose (right bars) as carbon source. Dashed line reflects starting cell number. Values represent means ± SEM, n = 3. Statistical analysis was performed using Student’s t-test (***P < 0.01, ****P < 0.001).
Import of these presequence precursors into the matrix requires the membrane potential as driving force for translocation across the inner membrane. We wondered if also protein import is affected in the PreP<sup>−/−</sup>/C0 cells due to the reduced membrane potential caused by defective respiratory chain complexes. We assessed protein import by <i>in organello</i> assays using Hsp10 as a model substrate. Hsp10 uses the presequence import pathway and depends therefore on the membrane potential. However, in contrast to most presequence precursors Hsp10 is not processed by MPP upon import [15,29]. Hsp10 is therefore an ideal substrate to test the presequence protein import pathway independent of presequence processing. Import of Hsp10 into mitochondria isolated from PreP<sup>−/−</sup> cells grown on glucose-containing medium revealed a mildly reduced import pointing to a decreased import activity in the absence of PreP (Fig. 2D), which is most likely caused by the decreased membrane potential (Fig. 2C).

Taken together, lack of the presequence peptidase PreP in human cells results in a growth defect and strong reduction in respiratory chain capacity. As a consequence, the mitochondrial membrane potential decreases, compromising the mitochondrial capacity to import newly synthesized precursor proteins from the cytosol.

**PreP activity is required for the maturation of newly imported precursor proteins**

<i>In vitro</i> assays using recombinant PreP have shown that PreP can degrade peptides and cleaved presequences that are generated upon precursor processing by MPP [23]. To assess if lack of PreP results in hampered peptide clearance in mitochondria, we analyzed presequence peptide degradation in soluble mitochondrial extracts [21,29]. For this, isolated mitochondria from control and PreP<sup>−/−</sup> cells were solubilized in digitonin and analyzed on Blue-native (BN-) PAGE using the indicated antibodies. SC, respiratory chain supercomplexes. Data are representative of one experiment. The experiment was performed four times. (B) Oxygen consumption rates (OCR) of WT and PreP<sup>−/−</sup> cells were measured at basal conditions and after addition of indicated compounds. Values represent means ± SEM, n = 6. (C) Membrane potential measurement (Membrane pot.) of WT and PreP<sup>−/−</sup> cells after growth on glucose (Glu, left bars) or galactose (Gal, right bars) as carbon source. Values represent means ± SEM, n = 3. Statistical analysis was performed using Student's t-test (**P < 0.001). (D) Radiolabeled Hsp10 precursor protein was incubated with isolated WT and PreP<sup>−/−</sup> mitochondria for the indicated period of time. Where indicated, the membrane potential (Δψ) was dissipatet prior to the import reaction. Samples were treated with Proteinase K to digest nonimported precursor proteins and analyzed by SDS/PAGE and autoradiography. Quantifications represent means ± SEM, n = 3.

Fig. 2. Analysis of mitochondrial functions in PreP<sup>−/−</sup> cells. (A) Mitochondria isolated from wild-type (WT) and PreP<sup>−/−</sup> cells were solubilized in digitonin and analyzed on Blue-native (BN-) PAGE using the indicated antibodies. SC, respiratory chain supercomplexes. Data are representative of one experiment. The experiment was performed four times. (B) Oxygen consumption rates (OCR) of WT and PreP<sup>−/−</sup> cells were measured at basal conditions and after addition of indicated compounds. Values represent means ± SEM, n = 6. (C) Membrane potential measurement (Membrane pot.) of WT and PreP<sup>−/−</sup> cells after growth on glucose (Glu, left bars) or galactose (Gal, right bars) as carbon source. Values represent means ± SEM, n = 3. Statistical analysis was performed using Student’s t-test (**P < 0.001). (D) Radiolabeled Hsp10 precursor protein was incubated with isolated WT and PreP<sup>−/−</sup> mitochondria for the indicated period of time. Where indicated, the membrane potential (Δψ) was dissipatet prior to the import reaction. Samples were treated with Proteinase K to digest nonimported precursor proteins and analyzed by SDS/PAGE and autoradiography. Quantifications represent means ± SEM, n = 3.
digitonin followed by separation of soluble and membrane fractions via centrifugation [21,29]. Authentic presequence peptides of the dually processed MPP substrate Frataxin (FXN<sup>1–41</sup> and FXN<sup>42–80</sup>) or presequence peptides of mitochondrial malate dehydrogenase (MDH2<sup>2–19</sup>) were added to the soluble extract, incubated for different time points and peptide degradation monitored on Nu-PAGE. While the presequence peptides were efficiently degraded in wild-type extract over the time of incubation, this process was strongly delayed in the absence of PreP (Fig. 3A–C) indicating that human PreP is required for degradation of presequence peptides of different lengths.

We next investigated the functional consequences of impaired presequence peptide degradation on human mitochondria. Deletion of the yeast PreP homologue Cym1 and the matrix peptidase Ste23 was previously shown to result in feedback inhibition of MPP processing revealing a link between presequence degradation and presequence processing in yeast [21,29]. Presequence peptides accumulating upon lack of Cym1 and Ste23 probably compete with incoming precursors for binding to MPP. This results in defective MPP processing, and as a consequence of this MPP inhibition, nonprocessed precursor proteins accumulate [21,29]. Furthermore, PreP can also degrade amyloid-beta peptides and the activity of PreP has been reported to decrease in brain mitochondria of Alzheimer’s disease patients and animal models of Alzheimer’s disease (AD) [23,25]. A potential functional coupling of PreP and MPP could therefore also play a role in disease pathogenesis, since accumulation of nonprocessed precursor proteins was also identified in mitochondria isolated from the temporal cortex of AD patients [29].

To assess whether presequence processing is compromised in the absence of PreP, we analyzed imports of the presequence containing precursors TFAM (mitochondrial transcription factor A), which is processed by MPP, and OTC (mitochondrial ornithine carbamoyltransferase), which is sequentially processed by MPP and MIP, into isolated mitochondria from wild-type and PreP<sup>–/–</sup> cells. While TFAM was imported into mitochondria in a membrane potential-dependent manner and processed by MPP in the control mitochondria, the mature form was strongly reduced in PreP<sup>–/–</sup> mitochondria (Fig. 4A). Furthermore, nonprocessed TFAM precursor accumulated in PreP<sup>–/–</sup> mitochondria, which was resistant to externally added Proteinase K. Similar to TFAM, import of OTC into isolated mitochondria was also dependent on the membrane potential and a two-step cleavage was observed in wild-type mitochondria. In contrast, processing by MPP from the precursor to intermediate OTC was compromised in PreP<sup>–/–</sup> mitochondria and the second processing by MIP generating the mature protein was strongly impaired (Fig. 4C).

We hypothesized that TFAM and OTC were still imported into PreP<sup>–/–</sup> mitochondria, but MPP and MIP processing in the matrix was impaired. Steady-state protein levels of both subunits of MPP, PMP12 and PMPCB, and also of MIP were not changed in PreP<sup>–/–</sup> compared to wild-type mitochondria (Fig. 4B, D) excluding that the observed processing defects were
caused by a lack of the proteolytic enzymes. As MPP is essential for cell survival and the majority of mitochondrial precursors require MPP activity for their maturation, we further assessed MPP activity in PreP<sup>−/−</sup> mitochondria by using soluble mitochondrial extracts. Soluble extracts have the advantage to uncouple protein maturation by MPP processing from membrane potential dependent import across both mitochondrial membranes and therefore allow direct assessment of MPP activity [21,29]. We generated soluble extract from wild-type and PreP<sup>−/−</sup> mitochondria and added radiolabeled precursors of Frataxin (FXN). Frataxin is processed twice by MPP, making it a sensitive substrate for MPP dysfunction [6]. While 35S-FXN was processed first to the intermediate and then mature form in the wild-type extract, the second MPP processing was not detectable in the PreP<sup>−/−</sup> sample and more unprocessed precursor was observed (Fig. 4E). Intriguingly, we observed appearance of a radiolabeled band below the molecular weight marker of 10 kDa only in the PreP<sup>−/−</sup> sample. We speculate that this band is the Frataxin presequence (FXN<sub>1-41</sub>), which is still partially cleaved off by MPP and then accumulates due to the lack of PreP, which would normally degrade cleaved presequences. Analysis of the kinetics of the two-step Frataxin processing revealed that the first MPP processing (precursor to intermediate) is kinetically faster than the second processing (intermediate to mature) reaction [30]. This difference in kinetics could explain why only the second more sensitive MPP processing is affected in the absence of PreP.

If accumulating presequence peptides in the absence of PreP result in feedback inhibition of MPP, then an overloading of PreP by exogenous addition of presequence peptides to wild-type extract should mimic this feedback inhibition and also result in inhibition of MPP processing. To assess this, we used mitochondrial extract from wild-type cells and added increasing amounts of presequence peptides (FXN<sub>42-80</sub>). MPP activity was monitored by processing of radiolabeled Frataxin (Fig. 4F). While Frataxin was processed to its mature form in the absence of peptides, increasing concentrations of FXN<sub>42-80</sub> resulted in gradual accumulation of the processing intermediate and also presequence form of radiolabeled Frataxin. Concomitantly we observed accumulation of the cleaved presequence in the low molecular weight range that could not be degraded in the wild-type extract due to the saturation of PreP activity by the exogenous peptides.

We wondered if accumulation of peptides upon loss of PreP could result in an MPP processing defect also in vivo and analyzed FXN protein steady-state levels. Indeed, FXN processing was impaired in vivo in PreP<sup>−/−</sup> mitochondria resulting in accumulation of intermediate FXN and reduced levels of the mature form (Fig. 4G).

Defects in mitochondrial protein biogenesis can ultimately result in imbalances of the mitochondrial proteome and compromised mitochondrial proteostasis has been shown to cause mitochondria-to-nucleus signaling that triggers changes in the expression of nuclear genes aiming to ameliorate mitochondrial function [31-33]. We wondered if loss of PreP with its accumulation of presequence peptides and unprocessed precursor proteins could also trigger mitochondrial stress signaling and therefore assessed nuclear expression of stress markers by quantitative reverse transcription-PCR (qRT-PCR). Indeed, transcripts of genes associated with mitochondrial stress and here especially with the integrated stress response (ATF4, CHAC1, ASNS, PCK2) were increased upon loss of PreP (Fig. 4H) [34,35].

In summary, PreP is required for efficient peptide turnover in the mitochondrial matrix of human cells. In the absence of PreP, presequence peptides accumulate and trigger feedback inhibition of MPP and MIP. Dysfunctional MPP processing in turn leads to the accumulation of nonprocessed precursor proteins. Changes in mitochondrial proteostasis induced by loss of PreP further trigger changes in the expression of nuclear genes associated with mitochondrial stress responses likely in order to compensate for mitochondrial dysfunctions. Efficient presequence degradation by PreP therefore appears to be crucially required to build and maintain a functional mitochondrial proteome.

**PreP patient mutation is proteolytically active**

The identification of the functional consequences of PreP loss and its impact on mitochondrial physiology by inhibition of MPP processing enabled us for the first time to investigate the pathological mechanism triggered by mutations in PITRM1 that have been identified in several patients in vivo [26,28]. Functional analyses of these PreP mutations have so far only been performed in vitro or in yeast models and yielded contradictory results [26-28]. While one study claimed a destabilization of PreP by the PreP<sup>R183Q</sup> mutation [26], another study proposed that the same mutation affects the catalytic activity of PreP [27]. However, PreP stability was not directly tested, but deduced from analysis of protein steady-state levels of the yeast homologue Cym1, that did not include assays to determine the half-life of the protein [26]. Furthermore, PreP activity of wild-type and mutant was only measured in vitro using recombinant PreP and fluorogenic
substrates but not authentic presequence peptides [27]. The identification of a functional coupling of PreP and MPP in human cells allowed now investigation of the downstream consequences of the patient mutation in vivo using MPP activity as read-out for changes in PreP activity.
We re-expressed wild-type (PreP) and the mutant version (PreP<sub>R183Q</sub>) in PreP<sup>-/-</sup> cells to assess impact of the mutation on MPP function. Re-expression of PreP in the PreP<sup>-/-</sup> cells (PreP<sup>-/-</sup> Resc) partially rescued the MPP defect restoring FXN processing to its mature form (Fig. 5A, lane 3). Interestingly, re-expression of PreP did not yield similar protein steady-state levels as detected in the wild-type cells and also MPP processing of Frataxin was only partially restored (compare lanes 1 and 3 in Fig. 5A). MPP activity, assessed by the ratio of intermediate to mature Frataxin, therefore seems to directly correlate with PreP protein levels. We speculated that the amount of PreP<sub>R183Q</sub> protein levels might be the underlying cause for the pathophysiological consequences in the patients and not a compromised proteolytic activity [26,27]. Therefore, we re-expressed wild-type (PreP<sup>-/-</sup> Resc) and mutant PreP (PreP<sub>R183Q</sub>) in PreP<sup>-/-</sup> cells and compared cells that had equal protein steady-state levels of mitochondrial PreP (Fig. 5A, lanes 5 and 6), to cells with strongly reduced PreP<sub>R183Q</sub> protein levels (Fig. 5A, lane 4), which reflects the PreP<sub>R183Q</sub> levels in patient fibroblasts [26]. When the expression levels of PreP<sub>R183Q</sub> reached almost wild-type levels, no significant difference in FXN processing was detectable (Fig. 5A, lanes 5 and 6). In contrast, if PreP<sub>R183Q</sub> was expressed to a much lower extent, accumulation of intermediate and reduced mature FXN protein levels was observed (Fig. 5A, lanes 3 and 4). This demonstrates that the patient mutation is fully functional in vivo, implicating that the strong reduction of mutant PreP protein levels in the patients is the underlying cause for mitochondrial dysfunction, and not loss of enzymatic activity.

Taken together, the patient mutation PreP<sub>R183Q</sub> does not impact on peptidase activity in vivo. Instead, rather the low protein levels detected in the patients and eventually degradation capacity of PreP seem to be triggering mitochondrial dysfunction by feedback inhibition of MPP.

PreP patient mutation results in a destabilized and heat-shock-sensitive protein

Based on our results that the severe reduction of mutant PreP protein levels and not a compromised catalytic activity is likely responsible for the pathophysiological consequences in the patients, we aimed to identify the underlying cause of this low protein abundance as it might be opening up a new avenue for therapeutic intervention. We first assessed protein turnover in organello and compared degradation of PreP in mitochondria isolated from wild-type and mutant PreP cells. Indeed, PreP<sub>R183Q</sub> was degraded faster than its wild-type form (Fig. 5B) indicating that the mutation destabilizes PreP and triggers degradation of the otherwise functional protein. However, reduced protease levels can often be tolerated due to a fast turnover of substrates. We wondered why the residual amounts of mutant PreP still result in severe MPP inhibition and speculated that the mutation elicits additional restraints on the enzyme that render the PreP<sub>R183Q</sub> mutant nonfunctional under certain conditions in vivo. We therefore assessed the sensitivity of PreP<sub>R183Q</sub> upon stress conditions, for example, heat shock. For this, isolated mitochondria were incubated for 30 min at 39 °C followed by solubilization with TX-100 and nonsoluble and soluble fractions were...
separated via centrifugation. Wild-type and mutant PreP were found in the soluble fraction (supernatant) if mitochondria had not been exposed to the heat shock (Fig. 5C). However, when isolated mitochondria were subjected to the mild *in organello* heat shock, a major part of PreP R183Q was found in the pellet fraction containing nonsoluble, for example, aggregating proteins, while the wild-type protein was still predominantly recovered in the soluble fraction (Fig. 5D). These results indicate that PreP R183Q is less stable than MIP.
the wild-type protein and additionally prone to misfolding and aggregation under heat-shock conditions. The low protein amounts still detectable in the patient would therefore be labile under, for example, fever, which might trigger the detrimental circuit of disease onset and progression by further compromising peptide clearance in the mitochondrial matrix.

Discussion

Most mitochondrial precursor proteins have N-terminal presequences for targeting and translocation into mitochondria that are proteolytically removed upon import. This presequence processing by MPP is required to generate functional proteins as nonprocessed precursors are rapidly aggregating [15]. Presequence processing is therefore essential to build and maintain a functional mitochondrial proteome. The importance of precursor maturation is also underlined by identification of several patients with mutations in one of the two MPP subunits that suffer from neurodegeneration [6,36-38]. As a side product of MPP processing free presequence peptides are generated that are degraded by matrix-localized peptidases. Studies in yeast revealed that this presequence degradation is required to maintain MPP activity [21,29]. Here, we identify a functional coupling between the presequence peptidase PreP and the presequence proteases MPP and MIP in human mitochondria. Loss of PreP results in accumulation of presequence peptides that in turn induce feedback inhibition of MPP and MIP (Fig. 5E).

Analysis of yeast mitochondria revealed that three peptidases, Cym1, Ste23, and Prd1, degrade cleaved presequences. Intriguingly, the studies that analyzed the function of the yeast PreP homologue Cym1 used in vitro assays with addition of exogenous presequence peptides to elicit feedback inhibition of MPP [21,29]. This suggests that the three yeast presequence peptidases have overlapping substrate spectra and can partially compensate each other. In contrast, lack of PreP in human cells resulted in a severe impairment of MPP processing already in the absence of additional peptides. Therefore, PreP seems to have a predominant role in peptide degradation in human cells as the deletion of PreP alone already results in the accumulation of a significant amount of peptides that induce MPP dysfunction in organello and in vivo.

Also in human mitochondria, two further peptidases have been reported to be capable of presequence degradation besides PreP. An isoform of human IDE, homologous to yeast Ste23, has been suggested to localize to mitochondria [39]. In vitro analysis using recombinant IDE and a synthetic presequence peptide implicated degradation of the cleaved targeting signal by IDE [39]. Furthermore, the Prd1 homologue mitochondrial NLN has also been shown to cooperate with PreP in degradation of long presequence and also amyloid-beta peptides in vitro [24]. While the role of these two peptidases in presequence turnover and the consequences of their lack in vivo await analysis, the characterization of PreP<sup>−/−</sup> cells revealed that loss of PreP is sufficient to trigger MPP dysfunction.

We observed the accumulation of presequence peptides, nonprocessed precursors, and processing intermediates that all can cause disturbances in mitochondrial proteostasis in PreP<sup>−/−</sup> mitochondria. It has been suggested that mammalian cells sense and respond to mitochondrial dysfunctions by activation of protective transcriptional responses that promote, for example, synthesis of nuclear-encoded mitochondrial chaperones and proteases [31-35]. We found a strong transcriptional increase of components of the integrated stress response involving the transcription factor ATF4 and its targets CHAC1, ASNS, and PCK2, while other previously reported stress marker genes (ATF5, HSPA9, CLPP, LONP, HSPD1) showed no or only very mild changes. Therefore, loss of PreP elicits a mitochondria-to-nuclear signaling response adapting nuclear gene expression to support mitochondrial function. The discovery of the changed nuclear expression upon loss of PreP further suggests that PreP is playing a dominant role for peptide degradation and mitochondrial proteostasis in human cells.

The identification of the functional coupling of presequence degradation and presequence processing in human cells also enabled characterization of a PreP mutation (PreP<sup>R183Q</sup>), which was identified in patients suffering from a neurological disorder characterized by slowly progressive spinocerebellar ataxia, mental retardation, and psychosis [26]. While analysis in yeast and in vitro assays using the recombinant enzyme resulted in contradictory results regarding the effect of the patient mutation, we were now able to analyze the consequences of impaired PreP activity in vivo by assessing feedback inhibition of MPP activity. Expression of PreP<sup>R183Q</sup> at the low protein levels detected in patient cells was resulting in a strong impairment of MPP activity in vivo. In contrast, expression of the PreP<sup>R183Q</sup> mutation in PreP<sup>−/−</sup> cells at wild-type levels restored MPP processing. Therefore, low PreP protein levels are likely responsible for the mitochondrial defects observed in the patient and not a reduced proteolytic activity. Using HEK293T cells, we could also investigate the underlying nature of the decreased protein levels in the PreP patients and found that
PreP<sup>R183Q</sup> was faster degraded compared to the wild-type protein. Furthermore, PreP<sup>R183Q</sup> was also prone to aggregation upon heat shock, which could render the residual amounts identified in the patients non-functional upon stresses like fever that might be relevant in triggering disease onset or further exacerbate disease progression.

Taken together, we identified a functional coupling of presequence processing by MPP and MIP and presequence degradation by PreP in human mitochondria that is likely playing a role in the pathogenesis of several diseases. The identification of the crucial role of PreP for the activity of the essential mitochondrial processing protease MPP opens up new avenues for treatment of dysfunctions not only caused by mutations in PreP but also for MPP dysfunctions and Alzheimer’s disease, in which a decreased PreP activity has been reported [6,25,26].

### Materials and methods

#### Cell culture

Human embryonic kidney cell lines HEK293T were cultured in DMEM (Gibco, Carlsbad, CA, USA) containing 4.5 g L<sup>−1</sup> glucose or galactose and supplemented with 10% (v/v) fetal bovine serum (FBS) (Sigma, F7524, St. Louis, MO, USA) and 2 mM L-glutamine (Sigma, G7513) at 37 °C in a humidified incubator with 5% CO<sub>2</sub>. All functional analyses were performed with cells grown on glucose as carbon source.

#### Generation of knockout and stable cell lines

CRISPR/Cas9 genome editing was used to generate PreP knockout HEK293T cell line as described previously and the pSpCas9(BB)-2A-GFP (PX458) was a gift from Feng Zhang (Addgene plasmid, #48138) [40]. Guide RNA sequence (GCGTAAACATGGGAAAAAGGTGG) targeting human PITRM1 exon 5 was cloned into pSpCas9(BB)-2A-GFP vector. HEK293T cells were transfected by Lipofectamine 2000 (Invitrogen, #11668-027, Waltham, MA, USA). Five hours after transfection, GFP-positive cells were obtained and screened for PreP expression by western blotting and indel mutations were verified by sequencing. The obtained PreP<sup>−/−</sup> cells were complemented by retroviral transduction. PITRM1 and PITRM1-R183Q cDNA were cloned into pBABE-puromycin vector. NIH293T cells were transfected by retroviral constructs using Lipofectamine LTX (Invitrogen, #15338-100). After 48 h, viral supernatant was collected and used to infect PreP<sup>−/−</sup> cells in the presence of 8 µg mL<sup>−1</sup> polybrene. Positive clones were selected using 0.5 µg mL<sup>−1</sup> puromycin.

#### Cell proliferation assay

Growth analysis was performed by seeding cells at a density of 200 000 cells per well into a 6-well plate. Cells were counted after 3 days by Countess II FL-automated cell counter (Thermo Fisher, Waltham, MA, USA).

#### Isolation of mitochondria

Mitochondria were isolated as previously described [41]. Cells were harvested and resuspended in solution A (220 mM mannitol, 70 mM sucrose, 20 mM HEPES-KOH (pH 7.6), 1 mM EDTA, 0.5 mM PMSF, and 2 mg mL<sup>−1</sup> BSA). Subsequently, cells were homogenized using a glass potter. Samples were centrifuged at 800 g for 5 min at 4 °C to remove cellular debris, and the supernatant was subjected to centrifugation at 10 000 g for 15 min at 4 °C. Mitochondrial pellet was resuspended in solution B (solution A without BSA).

#### MPP activity assay

Isolated mitochondria were solubilized in reaction buffer (10 mM HEPES-KOH (pH 8), 1 mM MnCl<sub>2</sub> and 1 mM DTT) containing 1% (w/v) digitonin, incubated on ice for 15 min, and centrifuged at 10 000 g for 10 min at 4 °C. Supernatant was incubated with radiolabeled Frataxin precursor at 37 °C for different time points and analyzed by SDS/PAGE followed by autoradiography. The PageRuler prestained marker (Thermo Fisher Scientific, #26616) was used as molecular weight marker.

#### Peptide degradation assay

Isolated mitochondria were solubilized in reaction buffer (20 mM HEPES-KOH (pH 8), 10 mM MgCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>) containing 1% (w/v) digitonin, incubated on ice for 15 min, and centrifuged at 10 000 g for 10 min at 4 °C. Obtained supernatant was incubated with 30 μM Frataxin presequence peptides 1-41 (MWTLGRRAVAGLLASPSPAQATLTRVPRAELAPLCGRRG), 20 μM 42-80 (LRTD IDATCTPRRASSNQRGLNQIWNVKKQSVYLMNLRK), or 80 μM MDH2 presequence peptide 1-19 (MLSALARPASAALRRSFST) at 37 °C for different time points separately. Samples were analyzed by Nu-PAGE (Invitrogen) followed by immunoblotting.

#### Blue-native-PAGE

Respiratory chain complexes were analyzed by Blue-native polyacrylamide gel electrophoresis. Mitochondria were...
solubilized in solubilization buffer containing 1% (w/v) digitonin, 20 mM Tris/ HCl (pH 7.4), 0.5 mM EDTA, 10% glycerol, 50 mM NaCl and incubated on ice for 15 min. Samples were centrifuged at 20 000 g for 5 min at 4 °C and supernatant was loaded on 4–10% gradient Blue-native-PAGE followed by immunoblotting.

**Mitochondrial respiration**

Mitochondrial respiration was measured by XF96 Extracellular Flux analyzer (Seahorse Bioscience, Billerica, MA, USA). Cells were seeded on a poly-D-lysine-coated plate at a density of 20 000 cells per well the day before the measurement. Basal levels of oxygen consumption rate (OCR) and OCR in the presence of electron transport chain inhibitors and uncouplers (2 µM oligomycin, 0.3 µM carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone, 1 µM antimycin, and 1 µM rotenone) were measured. Bradford assay was performed after the assay and OCR results were normalized to protein content.

**Membrane potential measurement**

Membrane potential was measured by TMRE mitochondrial potential assay kit (Abcam, ab113852, Cambridge, UK). Tetramethylrhodamine, ethyl ester (TMRE) was added to cells at a final concentration of 50 nM and incubated 15 min at 37 °C. Cells were analyzed by FLOWJO software (BD, Becton Dickinson Company, Ashland, OR, USA).

**In organello import of radiolabeled precursor proteins**

Radiolabeled precursor proteins (human TFAM, human Frataxin, rat OTC, yeast Hsp10) were synthesized in vitro with rabbit reticulocyte lysate system (Promega, Madison, WI, USA) in the presence of [35S]-methionine. Radiolabeled precursor proteins and isolated mitochondria were incubated in import buffer (250 mM sucrose, 5 mM magnesium acetate, 80 mM potassium acetate, 10 mM sodium succinate, 20 mM HEPES-KOH (pH 7.4)) supplemented with 1 mM DTT and 5 mM ATP for the indicated time points at 37 °C. Membrane potential was disrupted prior to the import reaction by addition of AVO (8 µM antimycin, 1 µM valinomycin, 20 µM oligomycin). Samples were treated with 20 µg·mL⁻¹ Proteinase K to digest nonimported precursor proteins. Mitochondria were re-isolated by centrifugation at 10 000 g for 10 min at 4 °C. Samples were analyzed by SDS/PAGE and autoradiography.

**RNA isolation and qRT-PCR**

Total RNA was isolated using the RNeasy Mini Kit (QIA-GEN, Venlo, Netherlands) and treated with DNase to prevent DNA contamination. cDNA was synthesized with High-Capacity cDNA kit (Applied Biosystems, Foster City, CA, USA) from 1 µg RNA. PCR amplification and detection were done by CFX384 Real-time PCR detection system (Bio-Rad, Hercules, CA, USA) using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene for normalization and relative mRNA levels were calculated using the delta-delta Ct method. Each experiment was performed in triplicates. The following primer pairs were used: ATF4-F, CAGCAAGGAGGA TGCCCTTCT; ATF4-R, CCAACAGGGCATCACAAGTC; CHAC1-F, GTGTTGACGCTCCTTGAAGA; CHAC1-R, TTCAGGGGCTCTGTTACCTG; ASNS-F, GATGAACT TACGCAGGTACCTACA; ASNS-R, CACTCCTCTCG GTTT; PCK2-F, AACACCTGAAACTGTGTTG; PCK2-R, CAATGGGACACCTCCTGCT; ATFS-F, CAGGCCCTTG CAGGTGAT; ATFS-R, CAGAGGGAGGAGAGCTGT GA; CLPP-F, AAGGCACCAAACAGCAGCT; CLPP-R, AAGATGCCAACACTCCTTGG; HSPA9-F, TGTTGACGAC TTGTGGGAAT; HSPA9-R, ATTTGGAGCCACGGACA ATTTT; HSPDI-F, ACTCGGAGGCGGAAAG; HSPDI-R, TGTGGGTAACCAAGGACATTT; LONP-R, AGAAAGACGCCGA CTTT; LONP-F, CCCG CCGTCTTATCAAGATT; LONP-R, AGAAAGACGCCGA CATAGG GAPDH-F, AGGGTCATCATCTCTGCCCCT CTC; and GAPDH-R, TGTGTCATGAGCTTCCAC GAT; TOMM70-F, TTTTGATTTGACCGCCAGG and TOMM70-R, ATAGCCTTCCGCCACCTTG.

**Protein aggregation assay**

Isolated mitochondria (40 µg) were lysed in PBS containing 0.5% Triton X-100 and incubated 5 min on ice. Samples were centrifuged at 16 000 g for 10 min at 4 °C. The pellet was resuspended in PBS containing 0.5% Triton. All fractions were subjected to TCA precipitation. Alternatively, mitochondria were subjected to an in organello heat shock (39 °C for 30 min) prior to the aggregation assay. Samples were analyzed by SDS/PAGE and immunoblotting.

**In organello degradation assay**

Isolated mitochondria were incubated in sucrose buffer (10 mM HEPES-KOH (pH 7.6), 0.5 mM sucrose) at 37 °C. Samples were collected at indicated time points, and mitochondria were re-isolated by centrifugation at 10 000 g for 10 min at 4 °C. Samples were analyzed by SDS/PAGE and immunoblotting.
Statistical analysis

Data shown represent means ± standard error of the mean (SEM). Statistical details of each experiment can be found in the figure legends. Student’s t-test was applied to compare between two groups. Significance are indicated with asterisks: ***P < 0.001, **P < 0.01, *P < 0.05, not significant (n.s.) P > 0.05.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

CK, AAT, and AM performed the experiments. CK, TB, SD, and FNV designed experiments and analyzed and interpreted the data. CK and FNV developed the project. FNV wrote the manuscript and coordinated and directed the project. All authors approved the final version of the manuscript.

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