Nonsense mutations in the COX1 subunit impair the stability of respiratory chain complexes rather than their assembly

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Respiratory chain (RC) complexes are organized into supercomplexes forming ‘respirasomes’. The mechanism underlying the interdependence of individual complexes is still unclear. Here, we show in human patient cells that the presence of a truncated COX1 subunit leads to destabilization of complex IV (CIV) and other RC complexes. Surprisingly, the truncated COX1 protein is integrated into subcomplexes, the holocomplex and even into supercomplexes, which however are all unstable. Depletion of the *m*-AAA protease AFG3L2 increases stability of the truncated COX1 and other mitochondrially encoded proteins, whereas overexpression of wild-type AFG3L2 decreases their stability. Both full-length and truncated COX1 proteins physically interact with AFG3L2. Expression of a dominant negative AFG3L2 variant also promotes stabilization of CIV proteins as well as the assembled complex and rescues the severe phenotype in heteroplasmic cells. Our data indicate that the mechanism underlying pathogenesis in these patients is the rapid clearance of unstable respiratory complexes by quality control pathways, rather than their impaired assembly.

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Introduction

The respiratory chain (RC), consisting of four multiprotein complexes, is the main source of the electrochemical transmembrane proton gradient that is utilized by complex V (CV) to generate ATP. Cytochrome *c* oxidase (COX) or complex IV (CIV) catalyses the transfer of electrons from cytochrome *c* (Cyt *c*) to molecular oxygen. Mammalian CIV is composed of 13 subunits encoded by both mitochondrial and nuclear genomes (Tsukihara *et al*, 1996). The catalytic core of the enzyme is formed by mtDNA-encoded subunits COX1 and COX2, which contain the two heme *a* moieties (*a* and *a*₃) and the copper centres (Cu_A and Cu_B) responsible for electron transfer. The third mtDNA-encoded subunit, COX3, also part of the structural core, may play a role in proton pumping (Wilson and Prochaska, 1990), whereas the nuclear encoded remaining 10 subunits surrounding the catalytic core are believed to modulate enzyme activity (Arnold *et al*, 1997) and to confer stability (Galati *et al*, 2009) to the fully assembled complex.

The biogenesis of CIV has been modelled as a sequential assembly process that starts with COX1 and the addition of prosthetic groups heme *a* and Cu_B centre (assembly intermediate S1). Then, COX4 and COX5a are added to form S2 intermediates (Stiburek *et al*, 2005) to which COX2, COX3 and nuclear-encoded subunits are subsequently added, resulting in S3 intermediates. Finally, with the addition of COX6a and COX7b, monomeric holocomplexes (S4) are assembled and can subsequently dimerize to form active complexes (Nijtmans *et al*, 1998).

In the last few years, it was shown that CIV is also incorporated into larger structures containing complexes CI, CII and CIII and the mobile electron carriers Cyt *c* and ubiquinol to form a functional ‘respirasome’ (Schagger and Pfeiffer, 2001; Acin-Perez *et al*, 2008). These supercomplexes have been proposed to stabilize the individual complexes (Acin-Perez *et al*, 2004) and to enhance respiration due to coordinated channelling of electrons (Schafer *et al*, 2006).

Besides numerous specific assembly factors including translation activators, translocases, copper metallochaperones and heme *a* biosynthesis enzymes, a great number of proteases and disassembly chaperones are also necessary for quality control to ensure correct biogenesis and maintenance of complexes and supercomplexes or for the elimination of superfluous or incorrect proteins (Stiburek and Zeman, 2010). Quality control may not only occur at the level of individual subunits, but also at the level of complexes and supercomplexes, since incorrect proteins may become parts of multimeric assemblies. Yeast studies demonstrated that ATP-dependent proteases within the matrix and *m*-AAA and *i*-AAA metalloproteases in the inner membrane recognize and selectively remove non-native proteins (Arlt *et al*, 1996; Guzelin *et al*, 1996; Koppen *et al*, 2007). Such ATP-dependent

proteases, organized in large multimeric protein complexes with a central proteolytic chamber and chaperone like as well as translocase activities, are responsible for the degradation of misfolded proteins to peptides (Tatsuta and Langer, 2008; Moore *et al*, 2008).

Human *m*-AAA protease is composed of paraplegin and AFG3L2 forming either a hetero-oligomeric paraplegin-AFG3L2 or a homo-oligomeric AFG3L2-AFG3L2 complex. Loss of function of paraplegin and missense mutations in AFG3L2 are associated with various neurodegenerative disorders (Casari *et al*, 1998; Salinas *et al*, 2008; Di Bella *et al*, 2010). Besides its proteolytic functions in quality control, AFG3L2 additionally has a regulatory role in mitochondrial protein synthesis and antioxidant defence (Esser *et al*, 2002; Nolden *et al*, 2005) and mammalian AFG3L2 also regulates mitochondrial fusion by controlling the processing of the dynamin-like GTPase OPA1 (Ehses *et al*, 2009).

In order to study at which level of the assembly line quality control is exerted, we have used cell lines with missense mutations of COX1 leading to mitochondrial diseases in patients. Only few pathogenic COX deficiencies involve mutations in structural genes of CIV itself, and among these, mutations in all three mtDNA-encoded COX subunits (Hanna *et al*, 1998; Karadimas *et al*, 2000; Kollberg *et al*, 2005; Horvath *et al*, 2005), but only one mutation in the nuclear-encoded COX structural subunit COX6B1 (Massa *et al*, 2008) have been reported.

The current assumption for their pathogenicity is that these mutations disturb the assembly process during CIV biogenesis. However, here we show that a COX1 subunit with a nonsense mutation, which causes its rapid decay, is assembled even into supercomplexes, yet high molecular weight assemblies are unstable and are degraded rapidly. We found that stability of the truncated COX1 and other mitochondrially encoded proteins is dependent on functional AFG3L2 protease, which directly interacts with full-length and truncated COX1 proteins and also other RC subunits, and is thus obviously responsible for their degradation.

Results

A7339G cybrid cells exhibit a high threshold for a RC defect

A heteroplasmic A7339G mutation in the gene encoding the subunit I (*CO1*) of COX has been found in a patient suffering from anaemia, changing the tryptophan into a premature stop codon. Cybrid cell lines containing different mutation load were generated for the functional analysis of this mutation, and the degree of heteroplasmy of the different clones was routinely surveyed during long-term culture by RFLP analysis (Supplementary Figure S1).

Polarographic studies showed comparable respiration rates in wild-type (WT) cells and in cells with up to 80% mutation load. In contrast, in cells containing >90% mutant mtDNA, respiration was severely impaired, whereas 100% mutant cells did not show any respiration (Figure 1A). A similar threshold value was observed when we measured the rate of respiration in digitonin permeabilized cells in the presence of substrates for different complexes (Figure 1A). The approximate threshold value calculated according to a Bayesian change point model varies from 84 to 89% of mutation.

In contrast, the activity of CIV was significantly reduced already with 40% and further dropped with increasing mutation loads (Figure 1B, upper left). A similar threshold was observed for CI activity (Figure 1B, upper right), and CIII activity was lower in cells containing >68% mutated mtDNA (Figure 1B, lower right). On the other hand, activities of CII were only lowered significantly in clones containing 100% mutated mtDNA (Figure 1B, lower left). In conclusion, the high threshold for a RC defect suggests the existence of an excess pool of intact COX1 subunits derived from WT mtDNA that could be used as a 'reserve' to compensate for a deficit, as long as the mutation load did not exceed 85% heteroplasmy.

Steady-state levels of RC subunits in A7339G cybrids

Although various antibodies directed against the holo-CIV and the N-terminus of COX1 were used, the mutant COX1 protein with a predicted loss of -35 AA at the C-terminus could not be detected in cells carrying 100% of the A7339G mutation (Figure 2A; Supplementary Figure S2), suggesting that it does not accumulate to detectable steady-state levels. In the absence of COX1, levels of other CIV subunits were also reduced: COX4 was nearly undetectable, while COX3 and COX2 were markedly decreased. Interestingly, levels of NDUFA9 (CI), SDHA (CII) and CORE2 protein (CIII) were also reduced in 100% mutant cells (Figure 2A). In contrast, Cyt c was rather increased, while no pronounced differences were seen in the mtDNA-binding protein TFAM, the ATPase subunit ATP5B, the heat shock protein HSP60 and the voltage-dependent anion channel VDAC (Figure 2A). In summary, steady-state levels of subunits of all RC complexes are affected by the A7339G mutation in the *CO1* gene, while other mitochondrial proteins not assembled into RC complexes were unaffected.

The A7339G mutation leads to reduced steady-state levels of assembled RC complexes

To investigate how the A7339G mutation affects steady-state levels of assembled RC complexes, we solubilized mitochondria with dodecylmaltoside (DDM) and performed BN-PAGE followed by western blot analysis, using representative antibodies against each of the five OXPHOS complexes. Steady-state levels of CIV were reduced to one-fifth in the 90% clone, while no CIV at all was seen in the 100% mutant cells (Figure 2B). The levels of assembled CI were also lower in the absence of CIV (Figure 2B). In accordance with reduced steady-state levels of the SDHA and CORE2 subunits (Figure 2A), the amounts of assembled CII and CIII were also decreased in these cells (Figure 2B). The content of CV was not significantly decreased, and smaller subassemblies were clearly seen (Figure 2B). In-gel-activity assays showed that the 90% mutant clone exhibited normal activities of CI and CII, while activities of both complexes were significantly decreased in 100% mutant cybrids (Figure 2C). Thus, the decrease in enzyme activities (Figures 1B and 2C) was caused by the reduced amounts of assembled CI and CII.

Steady-state levels of CI, CII and CIII seem to depend on CIV. To test whether this observation is also true for other COX mutations, we analysed the levels of complexes in cells containing a different stop codon mutation in the *CO1* gene. The G6930A mutation, previously found in a patient suffering from multisystem disorder (Bruno *et al*, 1999), results in the

Figure 1 Respiration and RC enzyme activities in A7339G cybrid cells with different percentages of mutated mtDNA. **(A)** Endogenous respiration of intact cells and complex I, II and III dependent respiration after permeabilization with substrates as indicated ($n \geq 3$). **(B)** Activities of complex IV, I, III and the citrate synthase (CS) measured by spectrophotometry ($n \geq 3$). Threshold values were calculated according to a Bayesian change point model. * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$.

loss of the last 170 AA (33%) of the polypeptide and was supposed to cause a complete disruption in COX assembly (D'Aurelio *et al*, 2001). Similar to the A7339G mutation, G6930A cells also exhibited reduced steady-state levels of CI, CII and CIII₂ in the absence of CIV (Supplementary Figure S3). In contrast, mitochondria isolated from a muscle biopsy of a patient with the G6708A mutation in the *COI* gene (Kollberg *et al*, 2005) still contained ~40% of assembled

CIV (Supplementary Figure S3) and did not show any abnormality of other complexes. Thus, other complexes are only affected when COX levels are dramatically reduced.

Supercomplexes are almost depleted in A7339G cells

To analyse how the mutation influences levels of supercomplexes, digitonin-solubilized mitochondrial samples were subjected to BN-PAGE, followed by western blot and in-gel

Figure 2 Steady-state levels of RC subunits and assembled complexes in A7339G cybrids. **(A)** Western blot analysis of whole cell lysates from WT cells and 100% mutant clones. **(B)** BN-PAGE of DDM-solubilized mitochondria followed by western blot analysis using representative antibodies against OXPHOS complexes. In parallel, the same amount of solubilized mitochondria was run in a SDS-PAGE followed by western blot analysis with an antibody against VDAC. Band intensities of RC complexes were normalized to VDAC and were expressed relative to intensities in WT mitochondria. **(C)** In-gel enzyme activity assay of RC complexes I and II. ns ($P > 0.05$), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, $n = 3$.

activity analysis. In WT mitochondria, CIV was found to be present in its various supercomplex forms SC1 (CIII₂ + CIV) and SC3 (CI + CIII₂ + CIV₍₁₋₄₎), but predominantly as a monomer (Figure 3A, upper left panel). CIII was detected in its dimeric form CIII₂ (Figure 3A, upper right panel), as well as in its various supercomplexes SC1 (CIII₂ + CIV), SC2 (CI + CIII₂) and SC3 (CI + CIII₂ + CIV₍₁₋₄₎). Only few CI was seen in its monomeric form (Figure 3A, lower left panel), the majority was associated with other complexes building the SC2 (CI + CIII₂) and SC3 (CI + CIII₂ + CIV₍₁₋₄₎) supercomplexes. The majority of CII was found to migrate as a free monomer.

No monomeric CIV and no other CIV containing supercomplexes were detectable in 100% mutant cells. Parallel to the loss of the SC3 (CI + CIII₂ + CIV₍₁₋₄₎) and SC1 (CIII₂ + CIV) supercomplexes, a significant increase in the SC2 (CI + CIII₂) supercomplex was evident in the 90 and 100% mutant clone (Figure 3A). In the 100% mutant clone, the reduction of CIV occurred at the levels of the supercomplexes as well as of the individual complex, whereas in the 90% mutant clone, amounts of CIV in the SC3 (CI + CIII₂ + CIV₍₁₋₄₎) supercomplexes seemed to be less decreased compared with SC1 (CIII₂ + CIV) and monomeric CIV.

Figure 3 Depletion of RC supercomplexes in cybrids containing the A7339G and G6930A mutation (**A**, **C**). BN-PAGE and western blot analysis of digitonin-treated mitochondria. Blots were probed with anti COX4 (CIV), CORE2 (complex III), NDUFA9 (complex I) and 70 kDa (complex II). (**B**, **D**). In-gel-activity (IGA) assay of Complex IV and I. CI, complex I; CII, complex II; CIII₂, complex III dimer; CIV, complex IV; #, new CI + CIII₂ * supercomplex.

The same observations could be made in G6930A cybrid cells. Moreover, in both mutant cell lines, we detected a faster-migrating band (labelled with #), which showed reac-

tivity with antibodies directed against the NDUFA9 subunit of CI and the CORE2 subunit of CIII (Figure 3A and C). No signals were found in G6930A cybrid cells when probed with

an antibody against the COX4 subunit (Figure 3C) and in in-gel-activity assays for CIV (Figure 3B and D, upper panels), excluding the presence of CIV in this new association. However, this faster-migrating CI + CIII₂ association exhibited no CI activity (Figure 3B and D, lower panels), suggesting that it could be either a breakdown product or an assembly intermediate of the normal CI + CIII₂ supercomplex (termed SC2* hereafter). The absence of the CI activity in SC2* might be caused by the lack of the NADH dehydrogenase module. Indeed, western blot analysis with antibodies against the NDUFS4 and NDUFB2 subunits of the NADH dehydrogenase module, which were able to detect the fully assembled CI and the SC2 (CI + CIII₂) supercomplex, failed to detect SC2* (Supplementary Figure S4), suggesting the absence of the NADH dehydrogenase module in this supercomplex.

The truncated COX1 protein is synthesized and associated with higher molecular assemblies

To analyse whether a ~35 AA truncated protein is synthesized at all, mitochondrially encoded proteins were specifically labelled with ³⁵S-methionine in cells with various mutation loads. After a 2-h pulse, an ~38 kDa band was clearly observed in addition to the WT COX1 protein (~43 kDa) in mutant cells (Figure 4A, COX1*). No differences in the rate of mitochondrial protein synthesis were observed between WT and mutant cells (Supplementary Figure S5).

To investigate whether the truncated COX1 protein can be incorporated into higher molecular assemblies, we performed immunoprecipitation of native CIV after pulse labelling of mitochondrially encoded proteins using antibodies directed against COX4 and the holo complex. Besides WT COX1 and COX2/3, the ~35 AA truncated COX1 was also found in the elution fractions (Figure 4B), suggesting that the truncated COX1 was also associated with the native complex. Using an antibody directed against the C-terminus of the COX1 protein, native CIV could be purified from WT cells, but not from 100% mutant cells (Figure 4C). Labelled signals corresponding to ND2, ATP6 and ATP8 seemed to be nonspecific contaminants as they were also visible in the preclearing fractions.

Furthermore, labelled signals corresponding to CI (ND4L, ND3 and ND6) and CIII (Cyt b) subunits (Figure 4B) were also seen in the elution fractions, supporting the idea that CI and CIII could be associated with native CIV. The presence of CI and CIII in the eluate was confirmed by western blot analysis showing that not only CIV subunits like COX2 and COX4, but also nuclear-encoded subunits of CIII and CI, namely Core2 and NDUFB8, were copurified with the native CIV (Figure 4D).

Not the assembly, but the stability of CIV is impaired in A7339G cells

To understand the mechanism how the A7339G mutation disturbs the assembly of CIV, we labelled the 13 mtDNA-encoded proteins and analysed the time course of their incorporation into the individual RC complexes as well as into the smaller supercomplexes by BN-PAGE/2D-SDS-PAGE and autoradiography (Supplementary Figure S6). The relative positions of labelled mtDNA-encoded subunits are indicated according to their molecular weight, additionally the identi-

ties of the spots for COX1 and COX2/3 were verified by western blot analysis (Supplementary Figure S6C).

Overall signal intensities dropped in the course of the experiment, starting from 24 h of chase when the cells had divided once, and after 72 h of chase, signal intensities were even lower due to several cell divisions (Figure 5; Supplementary Figure S7). In WT cells (Figure 5A), after a 2-h pulse (0 h) and a short period of chase (1.5 h), most of the newly synthesized proteins of CI, CIII and CIV were still in the process of being assembled into RC complexes and were progressing to higher molecular weight associations with time. For CIV (blue, uncoloured original figures see Supplementary Figure S7), most of the newly synthesized COX1 subunit was rapidly integrated into the S2 subcomplex, but only after a 24-h chase, most of it was incorporated into the mature CIV (S4, ~200 kDa). Even at short chase periods, signals corresponding to COX2/3 were almost exclusively found in the S3 or S4 stage, suggesting that these subunits are assembled in later stages. The smaller supercomplex SC1 (CIII₂ + CIV, ~700 kDa) was labelled with newly made subunits COX1, COX2/3 and the CIII subunit cytochrome b (Cyt b) already after 1.5 h chase. Similar observations were made for the assembly kinetics of CIII (green). At 0 h chase, the majority of Cyt b migrated in an ~120 kDa species. Following 72 h chase, Cyt b was found at the positions corresponding to the homodimer CIII₂, the SC1 (CIII₂ + CIV) and the SC2 (CI + CIII₂, ~1500 kDa) supercomplexes.

Assembly of CI seems to require a longer maturation time (red). Radiolabelled CI subunits were detectable in several intermediate complexes migrating between 300–550 kDa or in an 800-kDa complex before being chased into a fully assembled CI (~980 kDa) after 6 h chase. After 24 h chase, the SC2 supercomplex (CI + CIII₂, ~1500 kDa) could be clearly seen. Only the CV subunits ATP6 and ATP8 had been incorporated in the mature complex already at very short chase periods, suggesting either very rapid assembly kinetics of CV or their incorporation only at higher molecular weight levels (yellow). The dimerization of this complex (V₂, ~1300 kDa) from newly made subunits was also completed after 6 h.

In striking contrast, A7339G 100% mutant cells showed completely different assembly kinetics (Figure 5B). Compared with WT cells, all spots representing mitochondrially encoded subunits were already visible in high molecular weight associations after a short chase time. In A7339G 100% mutant cells, the newly synthesized, truncated COX1 was rapidly (Figure 5B, 0 h) incorporated into all subcomplexes as well as into the holoenzyme and, surprisingly, even into supercomplex SC1 (CIII₂ + CIV). However, the signal corresponding to truncated COX1 vanished rapidly during the chase, starting from supercomplexes at 1.5 h to lower molecular subcomplexes (S2), and was hardly visible after a 3.5-h chase. Signals for COX2/3 were also disappearing, together with truncated COX1. In contrast to WT cells, no fully assembled CIV and no SC1 (CIII₂ + CIV) supercomplex were detectable after 24 h chase. On the other hand, the synthesis of CI, CIII and CV and the assembly into the supercomplexes CI + CIII₂ and CV₂ were not disturbed in 100% mutant cells. These data strongly suggest that the truncated COX1 protein was integrated into pre-existing subcomplexes as well as into the COX holoenzyme and even into supercomplex SC1 (CIII₂ + CIV), but failed to remain stably incorporated.

Figure 4 Synthesis and association of truncated COX1 with high molecular weight assemblies. (A) Synthesis of full-length and truncated COX1 protein in A7339G cells containing different levels of mutated mtDNA. Mitochondrially encoded proteins were pulse labelled with ^{35}S -methionine and separated in a SDS gradient gel (4–16%). COX1*, truncated COX1 protein. (B, C). Mitochondrially encoded proteins were labelled in A7339G WT and A7339G 100% mutant cells. Mitochondria were solubilized in DDM and subjected to immunoprecipitation with antiserum against holo-CIV or with an antibody against COX4 (B) or against the C-terminus of COX1 (C) followed by SDS-PAGE and autoradiography. PC, preclearing of lysates with beads coupled to an irrelevant antibody; IP, elution of immunoprecipitate. (D) Western blot analysis of the same membrane (B) probed with antibodies directed against Core 2, COX2, NDUFB8, COX4 and VDAC. #, unspecific signals.

Stability of mitochondrially encoded proteins depends on the m-AAA protease AFG3L2

The decreased stability of complexes and supercomplexes should also be reflected at the individual subunit level. Thus, pulse-chase experiments were performed to analyse the stability of mitochondrial-encoded subunits in cells containing the A7339G mutation, but also the G6930A mutation. As shown in Figure 6A, in WT cells most mitochondrial proteins like the full-length COX1 (full arrowhead), Cyt b (full circle), ND2 and ATP6 exhibited a high stability. The band intensity of these proteins normalized to the intensity obtained at 0 h chase dropped to 65–70% after 3.5 h and to 50–60% after 24 h chase, when the cells had divided once (Figure 6B). The COX2/3 proteins exhibited a slightly higher turnover, with band intensity reduced to ~36% after 3.5 h and to ~20% after 24 h chase. In contrast, the 35 AA truncated COX1 polypeptide (A7339G mutation, open arrowhead) was almost completely absent after 3.5 h chase (Figure 6A). In parallel, the newly synthesized COX2/3 subunits were

also rapidly degraded in A7339G 100% mutant cells, emphasizing the enhanced instability of other CIV subunits in the absence of full-length COX1. We also observed an accelerated degradation rate for ND4 in A7339G 100% mutant cybrids, and a similar tendency could be seen with ND1 (diamond) and Cyt b.

In G6930A 100% mutant cells, no faster-migrating band corresponding to a truncated protein with the predicted loss of 170 AA at the C-terminus could be detected in pulse-chase experiments (putative position given by double arrow in Figure 6A), confirming that it is highly unstable (Bruno *et al*, 1999). Indeed, in G6930A 100% mutant cybrids, the degradation rate of other labelled mitochondrial proteins was even faster compared with A7339G 100% mutant clones. COX2/3 were almost completely degraded after 1.5 h chase (Figure 6A and B), and ND4 and ND1 subunits of CI and Cyt b showed a significantly increased decay after 3.5 and 24 h chase. Only ATP6 subunit of CV was not significantly altered, in both WT and mutant cell lines.

Figure 5 Kinetics of incorporation of mtDNA-encoded proteins into RC complexes and supercomplexes. Two-dimensional display of OXPHOS complexes and supercomplexes after BN-PAGE/SDS-PAGE 2D electrophoresis. Mitochondrial-encoded proteins were pulse labelled in A7339G WT (A) and A7339G 100% mutant cells (B) with ^{35}S -methionine and chased for the indicated time period followed by solubilization of isolated mitochondria in DDM and BN-PAGE/SDS-PAGE 2D electrophoresis. S1–S4, assembly intermediates of CIV; CI, complex I; CIII2, complex III dimer; CV2, CV dimer; SC1, CIII2 + CIV; SC2, CI + CIII2. OXPHOS subunits as indicated on the right side. Shown are representative pictures from three independent experiments.

The accelerated degradation of proteins in both mutant cybrid cell lines was accompanied by increased steady-state levels of the AAA proteases AFG3L2 (fold-change relative to

WT: 1.71 ± 0.18 for A7339G, $P < 0.01$, $n = 4$ and 3.52 ± 0.60 for G6930A, $P < 0.01$, $n = 4$) and YME1L1 (fold-change relative to WT: 1.78 ± 0.28 for A7339G, $P < 0.05$, $n = 4$, and

Figure 6 Degradation of RC subunits and role of the *m*-AAA protease AFG3L2. (A) Mitochondrial translation products of WT and 100% mutant cells were labelled with 35 S-methionine, chased for the indicated time period, separated in a SDS gradient gel (4–16%) and transferred to a PVDF membrane (autoradiogram). Full arrowhead, full-length COX1; open arrowhead, –35 AA truncated COX1; double arrow, putative position of –120 AA truncated COX1; open circle, COX2/3; diamond, ND4; full circle, Cyt b. (Lower panel) Western blot of the same membrane probed against VDAC as loading control. (B) Band intensities of RC subunits in cells containing WT mtDNA or 100% mutated mtDNA from different time points normalized to 0 h chase and to VDAC ($n=3$). Statistically significant comparisons for the A7339G and the G6930A mutation against WT are indicated with * and #, respectively. #/* $P<0.05$, ##/* $P<0.01$. (C) Western blot analysis of whole cell lysates from WT cells and 100% mutant clones probed with antibodies against mitochondrial proteases. (D) G6930A WT cells were transfected with siRNA directed against AFG3L2 transcripts, pulse labelled with 35 S-methionine and chased for 3 h. Autoradiogram and western blot analysis of the same membrane probed with antibodies directed against AFG3L2 and against HSP60 as loading control. Lower panel indicates quantification of three exemplary RC subunits after 3 h of chase normalized to 0 h for control and transfected cells as a degree for protein stability. ns = not significant, * $P<0.05$, $n=3$. (E) AFG3L2 was transiently overexpressed in G6930A 100% mutant cells, pulse labelled with 35 S-methionine and chased for 3 h. Autoradiogram and western blot analysis of the same membrane probed with antibodies directed against AFG3L2, and against HSP60 as loading control. (Lower panel) Quantification of three exemplary RC proteins after 3 h of chase normalized to 0 h for control and transfected cells as a degree for protein stability. * $P<0.05$, ** $P<0.01$, $n=4$. (F) After overexpression of AFG3L2^{E408Q}, WT or 100% mutant cells were labelled with 35 S-methionine. Mitochondrial extracts were fractionated by chromatography. Autoradiogram of mitochondrial lysates before purification, wash fraction and eluted fractions. *, –35 AA truncated COX1; #, –170 AA truncated COX1. (Lower panel) Western blot analysis of the same membrane probed with antibodies directed against AFG3L2 and against HSP60. Shown are representative pictures of three independent experiments.

2.92 ± 0.65 for G6930A, $P<0.05$, $n=4$), while the protease LONP1 and the chaperone HSP60 were not significantly altered (Figure 6C). Treatment with RC inhibitors rotenone (CI), antimycin (CIII), KCN (CIV), oligomycin (CV) and chloramphenicol (mt translation inhibitor) did not induce higher expression levels of AFG3L2 (Supplementary Figure S8A), suggesting that the upregulation of the protease might be a rather specific mitochondrial unfolded protein response.

To characterize the role of both proteases in quality control in human cells, we used specific siRNAs to deplete YME1L1 and AFG3L2 in WT cells. Knockdown of the *i*-AAA protease YME1L1 did not alter significantly the turnover of mitochondrially encoded proteins (data not shown). In contrast, depletion of AFG3L2 increased the stability of mitochondrial proteins in WT cells, with band intensities of ND4L and COX2 being significantly higher after 3 h chase (Figure 6D). To further confirm its role in the degradation of mitochondrial proteins, we overexpressed AFG3L2 in WT cells followed by pulse-chase labelling. Indeed, this decreased the stability of mitochondrial proteins; after 3 h chase, signals corresponding to ND1, ND4L and COX1 were significantly reduced in AFG3L2 overexpressing cells (Figure 6E).

These observations led us to postulate that mitochondrially encoded proteins, and particularly the truncated COX1

proteins, could be substrates of the *m*-AAA protease. We used the dominant negative variant AFG3L2^{E408Q} (Ehse *et al*, 2009) as a trap to identify short-lived substrate proteins. The *m*-AAA protease complexes formed upon assembly are still capable of substrate binding, but lack proteolytic activity, and a hexahistidine peptide fused to the C-terminus of the AFG3L2^{E408Q} variant allows their purification. Mitochondrial extracts of WT, A7339G and G6930A 100% mutant cells expressing the tagged AFG3L2^{E408Q} were fractionated by metal chelating chromatography. As shown in Figure 6F (left panel), the labelled -35 AA truncated COX1 (A7339G mutation), but also the full-length COX1 proteins were detected in the eluate. The labelled -170 AA truncated COX1 protein (G6930A mutation), undetectable under normal conditions, was clearly visible upon inhibition of AFG3L2 protease activity and was copurified with the protease (Figure 6F, right panel). In addition, other mitochondrially encoded subunits for CI (ND5, ND4, ND2 and ND4L) and CV (ATP6 and ATP8) were also coeluted in small amounts (Figure 6F), while Cyt b seemed not to be associated with the protease. Pull down assays using labelled cells without transfection with AFG3L2^{E408Q} encoding vectors showed no labelled signals in the eluted fractions (Supplementary Figure S8B), excluding the possibility that copurification was nonspecific.

Inhibition of AFG3L2 activity promotes stabilization of COX proteins and rescues the phenotype in A7339G 90%, but not in A7339G 100% mutant cells

To test whether inhibition of AFG3L2 activity would increase the stability of its substrates, we performed pulse-chase experiments after overexpression of AFG3L2^{E408Q} and found a significantly slower degradation rate of truncated COX1 and COX2/3 proteins in 100% mutant cells (Figure 7A, right panel). Similar to its depletion (Figure 6A), stability of mitochondrially encoded proteins for CIV (COX2/3) and CI (ND1, ND4L) was markedly increased in WT cells upon expression of the dominant negative AFG3L2^{E408Q} (Figure 7A, left panel).

Based on the observation that inhibition of the *m*-AAA protease activity may indeed promote stabilization of CIV and CI proteins, we tested whether inhibition of AFG3L2 can rescue the phenotype of the COX1 mutation. As shown in Figure 7B, overexpression of AFG3L2^{E408Q} indeed produced a substantial increase in assembled CIV levels and resulted in higher COX activity in A7339G 90% mutant cells (Figure 7C, upper panel). Moreover, basal respiration was restored to control levels (Figure 7C, lower panel).

However, inhibition of AFG3L2 activity did neither restore activity nor steady-state levels of CIV in A7339G 100% cells (Figure 7B and C). Expression of the dominant negative AFG3L2^{E408Q} variant led to a substantial reduction in cell number and cell vitality in mutant cells, while WT cells were much less affected (Figure 7D). Indeed, overexpression of AFG3L2^{E408Q} induced increased cell death in 100% mutant cells containing truncated COX1 proteins (Figure 7E), however, without the activation of caspase 3 (Supplementary Figure S8C).

Discussion

The stability of multimeric protein complexes is defined by a dynamic equilibrium of assembly and disassembly.

Mutations in CIV structural genes and accessory factors are generally believed to disturb the assembly process during its biogenesis (Fernandez-Vizarra *et al*, 2008). However, we could observe the incorporation of a truncated COX1 protein into various subcomplexes, into the holocomplex and even into supercomplexes after pulse labelling (Figure 5), demonstrating that assembly is not impaired. The comigration of proteins in discrete regions of BN-PAGE indicate *in vivo* associations of the truncated COX1 proteins with higher molecular assemblies, which was further confirmed by immunoprecipitation experiments (Figure 4B).

Additionally, the newly made subunits seem to be assembled faster into higher molecular assemblies in mutant mitochondria (Figure 5). As previously reported (D'Aurelio *et al*, 2001), we also did not observe differences in mitochondrial transcript levels (Supplementary Figure S5A) and mitochondrial protein synthesis rate (Supplementary Figure S5B) between WT and mutant cells, excluding a compensatory upregulation of mitochondrial transcription and translation as possible reasons for this phenomenon. However, it is conceivable that an upregulation of important assembly factors like OXA1L in mutant mitochondria may lead to the accelerated assembly rate. Alternatively, the pool of remaining subunits may have undergone more rapid depletion in mutant mitochondria (Figure 2A), thus allowing newly synthesized subunits to be channelled faster into higher associations, since they do not compete with a large pool of pre-existing subunits. Indeed, a smaller pool of free CIV subunits was suggested previously in the mitochondria of patients with a mutation in the *SURF1* gene (Williams *et al*, 2004).

Most of newly assembled CIV might be formed via the assembly line proposed by Nijtmans *et al* (1998); however, it is conceivable that COX1 can be also incorporated into already partially assembled complexes (Figure 5). For instance, a subcomplex containing COX4 and COX5a was found to be stable before being associated to COXI resulting in the S2 intermediate (Stiburek *et al*, 2005). Our observation that most of the newly synthesized COX1 was rapidly integrated into the S2 subcomplex supports the idea that a large pool of subcomplex containing COX4 and COX5a may already exist before the addition of COX1 (Figure 5A). As proposed first for the biogenesis of yeast CIV (Mick *et al*, 2007), assembly intermediates may also already exist in supercomplex forms (Figure 5). Indeed, more recently newly imported nuclear DNA-encoded subunits COX6a and COX7a were shown to be integrated into the CIV holoenzyme and supercomplex forms by associating with pre-existing subunits and intermediate assemblies (Lazarou *et al*, 2009).

Truncated COX1 proteins lacking only 11 and 15 residues at the C-terminus have been reported to be assembled into active CIV in yeast (Shingu-Vazquez *et al*, 2010). Although we could see the assembly of the truncated protein into subcomplexes and complexes, it remains unclear whether the fully assembled CIV and supercomplexes containing the truncated COX1 are functional as these multimeric protein assemblies were very unstable (Figures 5B, 6A and B). In contrast to the transmembrane domains, the hydrophilic C-terminus of COX1 is less conserved overall and seemingly is crucial for COX activity in mammals, whereas it does not affect OXPHOS in yeast at all and is rather required for the assembly mediated regulation of COX1 translation (Perez-Martinez *et al*, 2009; Shingu-Vazquez *et al*, 2010).

Figure 7 Stabilization of COX proteins and rescue of phenotype in A7339G 90% mutant cells after inhibition of AFG3L2 activity. (A) A7339G WT and 100% mutant cells were transfected with AFG3L2^{E408Q}, followed by pulse-chase experiment. Autoradiogram and western blot analysis of the same membrane probed with antibodies directed against AFG3L2 as control of transfection efficiency, and against HSP60 as loading control. (Lower panel) Quantification of three exemplary RC proteins after 2 h of chase normalized to 0 h. **P* < 0.05, *n* = 3. (B) BN-PAGE of DDM-solubilized mitochondria of A7339G WT, 100% and 90% mutant cells after transfection with AFG3L2^{E408Q} followed by western blot analysis using antibodies against RC complexes, against AFG3L2 and against HSP60. (Lower panel) Quantification of CIV intensity normalized to HSP60 after AFG3L2^{E408Q} transfection compared with untransfected mutant and to WT cells. **P* < 0.05, *n* = 3. (C) COX activity and endogenous respiration of intact cells were measured after transfection with AFG3L2^{E408Q} in A7339G 100% and 90% mutant cells in comparison to untransfected mutant and to WT cells. **P* < 0.05, *n* ≥ 3. (D) Number of A7339G WT and 100% mutant cells after transfection with AFG3L2^{E408Q} (upper panel). Cell vitality measured with the neutral red assay in G6930A WT and 100% mutant cells (lower panel). **P* < 0.05, ****P* < 0.001, *n* ≥ 3. (E) Flow cytometry analysis of Annexin V-FITC and propidium iodide staining in G6930A WT, G6930A 100% and A7339G 100% mutant cells 24 h after transfection with AFG3L2^{E408Q} in comparison to untransfected cells. Shown are representative dot blots of three independent experiments.

Our data clearly suggest a shift of the equilibrium towards disassembly and degradation. It still remains to be elucidated how RC (sub)complexes or supercomplexes containing incorrect proteins are recognized and then destabilized. In contrast to disassembly of protein complexes by remodelling reactions (Hartman and Vale, 1999), which only unfold but do not destroy the individual components, no decomposition of large associations into smaller associations during the chase period was observed (easier seen in Supplementary Figure S6B). Thus, it appears likely that subunits are rapidly degraded into small peptides without a significant accumulation of breakdown products after disassembly of large RC structures.

The destabilization of high molecular weight assemblies was also mirrored at RC subunit levels (Figure 6A). The observed accelerated degradation rate was accompanied by the upregulation of *m*-AAA and *i*-AAA metalloproteases AFG3L2 and YME1L1 (Figure 6C), which were shown to perform protein quality control in the mitochondrial inner membrane in yeast (Koppen *et al*, 2007). Here, we demonstrated that particularly AFG3L2 is crucial for the degradation of mitochondrially encoded subunits as their stability was highly dependent on the expression levels of this protease (Figure 6D and E). Furthermore, we found that impairment of AFG3L2 activity resulted in decreased cell number and increased cell death in 100% mutant (Figure 7D and E), pointing out that this *m*-AAA protease becomes especially important if mutated proteins are present.

Unassembled RC complex components and F1Fo ATPase subunits have been reported to be substrates of the yeast *m*-AAA protease (Arlt *et al*, 1996), while the physiological substrates of the mammalian enzyme are still largely unknown (Martinelli and Rugarli, 2010). Here, we demonstrated the direct interaction of defective COX1 proteins with the AFG3L2 protease in human mitochondria (Figure 6F). Interestingly, WT subunits including the WT COX1 and other mitochondrially encoded proteins of CI and CV were also associated with the *m*-AAA protease. This may further imply that in WT cells, a significantly large pool of unassembled subunits exists, which are either available for assembly or are degraded. The existence of excessive COX1 and other mitochondrially encoded RC subunits that could constitute a back-up may also explain the observed high threshold needed to result in severe impairment of mitochondrial function (Figure 1). An alternative, but not exclusive explanation for the interaction of AFG3L2 with WT COX1 and other mitochondrially encoded RC subunits could be that AFG3L2 participates in the folding and correct assembly of its substrates, as AFG3L2 may also exhibit chaperone-like activity. This idea is supported by the observation that inhibition of AFG3L2 also affected cell vitality and induced cell death in WT cells (Ehse *et al*, 2009) (Figure 7D and E).

Although inhibition of AFG3L2 activity could rescue the severe phenotype in A7339G 90% mutant cells, it did not restore activity or the steady-state levels of CIV in A7339G 100% cells (Figure 7B and C). This indicates that stabilization of the mutant subunit itself is not sufficient for stabilization of CIV and other complexes in mutant cells, as assembled complexes and supercomplexes containing the truncated proteins remained unstable (Supplementary Figure S9). Despite our efforts to detect the truncated proteins after inhibition of AFG3L2, using immunoprecipitation with anti-

bodies directed against the N-terminus of COX1 and the holocomplex followed by LC-MS analysis, no truncated COX1 could be found in A7339G 100% cells (data not shown). Thus, it is likely that the –35 AA truncated protein does not accumulate to steady-state levels even upon over-expression of the dominant negative AFG3L2 variant. These data are in agreement with our observation that degradation was only decelerated, but not completely arrested under these conditions (Figure 7A). However, the question how the slower degradation rate can dramatically reduce cell viability and induce cell death remains to be resolved (Figure 7D and E).

Despite the considerable amount of data demonstrating that supercomplexes indeed exist and even can respire (Heinemeyer *et al*, 2007; Acin-Perez *et al*, 2008), the interdependence of individual complexes is still a field of debate. Acin-Perez *et al* (2008) showed that the absence of CIII leads to absence of CI. The destabilization of (sub)complexes and supercomplexes containing the truncated COX1 protein not only results in a loss of CIV, but also leads to the depletion of CI and CIII, present in the assemblies SC1 (CIII₂ + CIV) and SC3 (CI + CIII₂ + CIV_(1–4); Figure 3). The enhanced instability is shown by an accelerated degradation rate (Figure 6A and B) and results in a depleted pool of RC subunits (Figure 2A), reduced steady-state levels of CI and CIII (Figure 2B) and in a significant decline of enzyme activities (Figures 1B and 2C). The finding that nonsense mutations of COX1 can cause destabilization of multiple RC complexes is also supported by the analysis of patients with combined CI + CIII deficiencies, in which either CI (Budde *et al*, 2000; Scacco *et al*, 2003) or CIII (Schagger *et al*, 2004) were primarily affected. CI stability has been shown to depend on the assembled level of CIII in bacteria (Stroh *et al*, 2004), yeast and mammalian cells (Acin-Perez *et al*, 2004; Blakely *et al*, 2005). In the absence of CIV, CI was also reduced in mouse COX10 knockout fibroblasts (Diaz *et al*, 2006) and in human cybrid cells (D'Aurelio *et al*, 2006). In addition, inhibition of COX4 expression led to decreased steady-state levels and activities of CI in mouse cell lines (Li *et al*, 2007).

Some investigators have proposed that the biogenesis of CI (Diaz *et al*, 2006) or the formation of supercomplexes is impaired in the absence of CIV (Suthamarak *et al*, 2009). However, the biogenesis of OXPHOS complexes and the formation of supercomplexes *per se* were not disturbed in A7339G mutant cells (Figure 5). Moreover, we detected a faster-migrating CI + CIII₂ supercomplex lacking the NADH dehydrogenase module, suggesting that it could be either a breakdown product or an assembly intermediate of the normal CI + CIII₂ supercomplex. The same supercomplex was found in patients with mutations in *NDUFV1* and *NDUFS4* genes (Ogilvie *et al*, 2005), in which the assembly of CI was stalled. We therefore conclude that the association of CIII with CI is not sufficient to stabilize this supercomplex and that CIV may function as a stabilizer.

Data obtained from patients with an isolated COX deficiency in muscle, which however show close to normal activities of other RC complexes (Tiranti *et al*, 2000; Kollberg *et al*, 2005; Valente *et al*, 2009; Supplementary Figure S3), seem to be controversial to our findings. It is likely that tissue-specific effects in COX biogenesis or maintenance could compensate for the defects, resulting in a tissue-specific decrease of COX activity only (Stiburek *et al*, 2005). As shown

In Figure 1, a CIV defect only leads to a combined OXPHOS deficiency when it has surpassed a certain threshold, since even small levels of the fully assembled CIV is sufficient for the stabilization of other RC complexes (Figure 2B). In contrast, concomitant reductions in activities of other complexes have recently been noted in some patients and mice with mutations in the *SCO2* gene (Tarnopolsky *et al*, 2004; Mobley *et al*, 2009; Joost *et al*, 2010; Yang *et al*, 2010). Mutations in nuclear-encoded assembly factors lead to strongly reduced activities of CIV (Diaz, 2010) and may thus better reveal the destabilization effects. Indeed, the reduced activities of other complexes observed in a patient with a COX10 mutation (Ogier *et al*, 1988) are probably due to lower levels of assembled CI, CII and CIII in addition to CIV (Supplementary Figure S3).

In summary, our results indicate that mutations in an individual subunit not only lead to an isolated complex defect, but also to combined OXPHOS deficiency when the mutation load has surpassed a certain threshold, so that not sufficient WT subunits are present for assembly. Therefore, slowing down their turnover by inhibition of the m-AAA protease can rescue the assembly defect to some extent.

Materials and methods

Cell culture

Cybid cell lines were constructed by fusion of platelets from a patient with osteosarcoma 143B cells lacking mtDNA as described before (Broker *et al*, 1998), and the degree of A7339G heteroplasmy of different clones was routinely surveyed during long-term culture by RFLP analysis. Cells were cultivated in DMEM with Glutamax supplemented with 10% FCS, 200 μ M Uridine, 2.5 mM sodium pyruvate, 100 μ g/ml Streptomycin and 100 U/ml Penicillin at 37°C and 5% CO₂.

Oxygen consumption measurements

Respiration was recorded in intact cells and then, after permeabilization with digitonin, oxidation rates for pyruvate + malate (CI), succinate (CII) and glycerol-3-phosphate (CII + CIII) as substrates were determined. Spectrophotometric measurements were performed as described by Rustin *et al* (1994) to determine the activities of RC complexes and citrate synthase.

In vitro pulse labelling of mitochondrial translation products

Cells were labelled with ³⁵S-methionine (0.2 mCi/plate) for 2 h in methionine- and cysteine-free DMEM supplemented with 100 μ g/ml of the cytoplasmic translation inhibitor emetine and 5% dialysed FBS (Chomyn, 1996). In all, 25 μ g protein was electrophoresed through a 15% denaturing gel. Band intensities corresponding to mitochondrial translation products were quantified densitometrically. In chase experiments, cells were incubated for 24 h in 40 μ g/ml chloramphenicol prior to labelling, and emetine was replaced with 100 μ g/ml cycloheximide (Supplementary Figure S6A). Instead of drying the gels, proteins were transferred to PVDF membranes before autoradiography, and sequentially probed with antibodies. Band intensities of HSP60, VDAC and β -actin were used to ensure equal loading of the solubilized mitochondria and whole cell lysates, respectively.

Assembly assays and enzymatic in-gel-activity

Mitochondria from cells were isolated by superparamagnetic microbeads (Hornig-Do *et al*, 2009), whereas mitochondria from muscle tissues of patients were purified by differential centrifugation. Mitochondria were solubilized by dodecylmaltoside at 2 g/g protein and digitonin at 4 g/g, respectively, and incubated for 20 min on ice. After 20 min centrifugation at 25 000 g, the supernatant was collected, and to resolve individual complexes and smaller supercomplexes, DDM-treated mitochondrial membrane proteins (7.5 μ g) were run on 4.5–16% gels. Digitonin-treated mitochondrial membrane proteins (7.5 μ g) were separated though

3–13% gradient BNGE gels to resolve supercomplexes (Wittig *et al*, 2006). For 2D analysis, the first-dimension lane was cut, incubated for 15 min in 1% SDS and 1% β -mercaptoethanol, and run in a 15% second-dimension denaturing gel. After electrophoresis, the complexes were blotted to PVDF filters and sequentially probed with antibodies. In-gel enzyme activity assays were carried out to estimate the activity of CI, CII and CIV (Calvaruso *et al*, 2008).

Expression of a dominant negative AFG3L2 mutant (AFG3L2^{E408Q}) and cell functional assays

After introducing a mutation into the WB motif (E408Q), human AFG3L2 was cloned into pcDNATM5/FRT/TO. Cell lines were transiently transfected with AFG3L2^{E408Q} using Lipofectamin 2000 according to the manufacturer's instructions (Invitrogen). The number of living cells was evaluated by automated counting and neutral red assay was performed according to Repetto *et al* (2008). Annexin V staining was used to determine the translocation of phosphatidylserine. Cells were harvested by trypsinization, including floating cells, stained using a human Annexin V-FITC kit according to the manufacturer's instructions (Bender MedSystems) and were analysed by flow cytometry using a FACSCalibur (BD Biosciences).

His-tagged protein purification and pull down assays

Mitochondria (0.5 mg protein) were isolated from A7339G WT and 100% mutant cells by differential centrifugation and solubilized at 2 mg/ml in buffer A (1% digitonin, 1 mM ATP, 1 mM PMSF, EDTA-free protease inhibitor cocktail in 1 $\times$ PBS) for 20 min at 4°C under gentle mixing. Insoluble material was removed by centrifugation for 20 min at 25 000 g at 4°C. His-tagged AFG3L2^{E408Q} was purified by metal chelating chromatography using Dynabeads[®] TALONTM. Bound proteins were washed extensively with 80 mM imidazole in buffer B (0.5% digitonin, 1 mM ATP in 1 $\times$ PBS). Proteins eluted with 300 mM imidazole in buffer C (0.5% digitonin in 1 $\times$ PBS) were TCA precipitated, analysed by SDS-PAGE and transferred to a PVDF membrane before autoradiography. After exposition, membrane was probed with antibodies directed against AFG3L2 and HSP60.

For native immunoprecipitation, mitochondria were solubilized in DDM (2 g/g protein) and clarified lysates (100 μ g) were precleared with an irrelevant antibody bound to Protein G Dynalbeads (Invitrogen), prior to incubation with the indicated antibodies bound to Protein G Dynalbeads for 15 min. Beads were washed four times with solubilization buffer, resuspended in 2 $\times$ Laemmli sample buffer and boiled for 5 min. Preclearings and immunoprecipitations were separated by SDS-PAGE and transferred to PVDF for autoradiography.

Depletion of AAA proteases

Transient siRNA-mediated knockdown was performed using stealth RNAs (AFG3L2HSS116886(3_RNAI), YME1L1HSS116547(3_RNAI); Invitrogen). Cells were transfected with 20 nM of RNAi using LipofectamineTMRNAiMAX (Invitrogen). Transfection was repeated once within 16 h and knockdown efficiency was tested 48 h after second knockdown by western blot analysis.

Antibodies

Rabbit antisera had been raised against recombinant human TFAM (Weber *et al*, 2002), against human m-AAA AFG3L2 protease subunits (Koppen *et al*, 2007) and against human holo-COX (Nijtmans, unpublished data). The following commercially available antibodies were used: against different RC subunits (Molecular Probes), against β -actin (Sigma Life Science), against Cyt c (BD Biosciences), against HSP60 (BD Biosciences), against LONP1 (Sigma Life Science), YME1L1 (Proteintech Group), VDAC (Cell Signaling) and against mouse or against rabbit horseradish peroxidase-conjugated antibodies (Dianova). Signals were detected using electrochemiluminescence reagents (Amersham) and captured on films (Amersham).

Statistics

Thresholds of defects (dotted lines, Figure 1) were calculated by means of a linear Bayesian change point model with boundary condition, and outliers removed beforehand. The model was implemented and simulated in OpenBUGS. In case the simulation failed, mean profiles with standard errors were calculated and *t*-tests against the baseline value were performed. In all other

figures, results are given as mean values $\pm$ s.d. and were compared using *t*-tests assuming unequal distribution. A significance level of $P < 0.05$ was considered to be statistically significant.

Supplementary data

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

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Author contributions: RJW, LN, TT and HH conceived and designed the research. HH, TT, AB, MB, GK and AR performed the experiments. MH analysed threshold defect and significance of the data. RJW, LN and HH wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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