Crucial mitochondrial impairment upon CDC48 mutation in apoptotic yeast

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Mutation in CDC48 (cdc48S565G), a gene essential in the ER-associated protein degradation (ERAD) pathway, led to the discovery of apoptosis as a mechanism of cell death in the unicellular organism Saccharomyces cerevisiae. Elucidating Cdc48p-mediated apoptosis in yeast is of particular interest because Cdc48p is the highly conserved yeast orthologue of human valosin-containing protein (VCP), a pathological effector for polyglutamine disorders and myopathies. Here we show distinct proteomic alterations in mitochondria in the cdc48S565G yeast strain. These observed molecular alterations can be related to functional impairment of these organelles as suggested by respiratory deficiency of cdc48S565G cells. Mitochondrial dysfunction in the cdc48S565G strain is accompanied by structural damage of mitochondria indicated by the accumulation of cytochrome c in the cytosol and mitochondrial enlargement. We demonstrate accumulation of reactive oxygen species (ROS) produced predominantly by the cytochrome bc1 complex of the mitochondrial respiratory chain as suggested by the use of inhibitors of this complex. Concomitantly, emergence of caspase-like enzymatic activity occurs suggesting a role of caspases in the cell death process. These data strongly point for the first time to a mitochondrial involvement in Cdc48p/VCP-dependent apoptosis.

Introduction

Fundamental cellular processes, such as the formation of organelles (ER; Golgi apparatus; nuclear envelope), or the ubiquitin-dependent ER-associated protein degradation (ERAD) have been linked to the yeast protein Cdc48p and its highly conserved mammalian orthologue VCP (1-4). Mutations in VCP have been associated with “inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia” (IBMPFD), a dominant human disorder (5,6). A genetic screening of a Drosophila model for human polyglutamine diseases, a class of inherited neurodegenerative disorders, identified the Drosophila homologue of Cdc48p/VCP as a modulator of apoptotic cell death (7), leading these authors to propose VCP as a pathological effector for polyglutamine-induced neurodegeneration. However, the cellular mechanisms underlying VCP-mediated cell death in these human disorders remain largely unknown.

Apototic phenotypes in cells expressing mutated Cdc48p/VCP have originally been described in budding yeast (8), and were thereafter confirmed in mammalian cell cultures (9,10), in trypanosomes (11), and in zebrafish (12). Notably, Cdc48p was the first apoptotic mediator found in S. cerevisiae (8). The expression of a point mutated CDC48 gene (cdc48S565G) leads to a characteristic apoptotic phenotype: phosphatidylserine externalization, DNA fragmentation, chromatin condensation, nuclear fragmentation and vacuolization (8,13). These results obtained in the cdc48S565G strain initiated the establishment of yeast as a model to study evolutionary conserved mechanisms of apoptotic regulation (14-16).

Mitochondria play a crucial role in many apoptotic pathways in both mammalian cells and...
in yeast (17-19). In the present study, we therefore tested for mitochondrial impairment and contribution in Cdc48p-mediated apoptosis. We observed mitochondrial enlargement, distinct alterations in the mitochondrial proteome, release of cytochrome c into the cytosol, impairment in the ability of cdc48S565G cells to adapt to respiratory conditions, as well as mitochondrial ROS production paralleled to the emergence of caspase-like enzymatic activity. These data show mitochondrial impairment at morphological, molecular and functional levels. These alterations are associated with apoptotic cell death indicating the activation of a mitochondrial pathway for Cdc48p-mediated apoptosis.

**Experimental Procedures**

**Yeast strains, culture conditions and assay for respiratory deficiency**

*S. cerevisiae* wild-type KFY417 (CDC48) and mutant strain KFY437 (cdc48S565G) (20) were used in this study. For all experiments (except \(\rho^0/\rho^+\) experiments, see below) induction of apoptosis was done as follows (8,13): glucose medium (YPGlc, 1% yeast extract, 2% bacto peptone, 4% glucose, Otto Nordwald, Hamburg, Germany) was inoculated (OD600 = 0.1-0.3) with stationary YPGal pre-cultures (4% galactose). Cells were then grown in baffled flasks at 28°C until early stationary and stationary phase, respectively, and then subjected to heat shock at 37°C.

For analysis of respiratory deficiency, glucose cultures of both wild-type and cdc48S565G strains were plated on YP plates (1% yeast extract, 2% bacto peptone, 1.5% agar) containing (i) 4% glucose (YPGlc, fermentative selective medium) or (ii) 2% lactate (YPLac, respiratory selective medium). Cells were then grown in baffled flasks at 28°C until early stationary and stationary phase, respectively, and then subjected to heat shock at 37°C.

**Electron microscopy (EM)**

EM analysis of mitochondrial samples was carried out as previously described (21). EM analysis of stationary yeast cells to visualize membrane structures was done essentially according to (22): Cells were harvested and incubated for 8 min in fixative (4% formaldehyde, 2% gluteraldehyde, 4% sucrose, 2 mM calcium acetate, 50 mM sodium cacodylate, pH 7.2) at RT. Fixed cells were stored in fixative overnight at 4°C and subsequently prepared for cell wall removal by incubation in pretreatment solution (0.2 M Tris/HCl, 100 mM β-mercapto ethanol) for 10 min at RT. Removal of cell wall was done with 30 U lyticase (Sigma) and 0.6 U ariysulfatase (Roche) for 90 min at 30°C in digestion buffer (35 mM potassium phosphate buffer pH 6.8, 0.5 mM MgCl2, 1.2 M sorbitol). Cells were washed in cacodylate buffer (0.1 M sodium cacodylate, 5 mM CaCl2), postfixed (0.5% osmium tetroxide, 0.8% potassium ferrocyanide), washed in distilled water, stained en bloc (1% aqueous uranyl acetate), dehydrated in ascending alcohol series and embedded in Araldite. The preparations were sectioned at 50 nm on an ultramicrotome (Ultrotom III; LKB, Bromma, Sweden) and EM micrographs were obtained on a Zeiss (Oberkochen, Germany) EM 10 electron microscope.

**Cell fractionation**

Mitochondria were isolated by differential centrifugation as described in Zischka et al. (21). Cytosol was obtained by ultracentrifugation (177000 g, 90 min, 4°C) from the supernatant of the first mitochondrial sedimentation.

**Two-dimensional gel electrophoresis (2-DE) and image analysis**

2-DE was performed according to Zischka et al. (21). Isoelectric focusing (IEF) was done using immobilized pH-gradient (IPG) strips pH 3-10NL and gradient gels (8-16% T) for SDS-PAGE. Resultant protein patterns were detected by standard staining procedures, either silver (23) for analytical purposes (150 µg protein per gel) or ‘ruthenium II tris bathophenanthroline disulfonate fluorescent dye’ (24) for preparative purposes (400 µg protein per gel). Gels treated with the latter were further
stained with colloidal Coomassie Blue for protein analysis (25). Image analysis of the gels was done with the ProteomWeaver™ image analysis software V.2.2 (Definiens AG, München, Germany). For the analysis of mitochondrial extracts data were determined by taking into account three independent experiments.

**Protein identification via MALDI-TOF mass spectrometry**

Proteins were subjected to a sequence-dependent protease treatment (100 ng trypsin per gel plug; Promega, Mannheim, Germany) as described by Shevchenko et al. (23). Resulting peptides were analyzed by peptide mass fingerprinting with the thinlayer method (26) using a Maldi-TOF Reflectron (Waters, Eschborn, Germany). Database searches for protein identification were done in SwissProt using the ProteinLynx Globalserver 1.1 (PLGS 1.1, Waters).

**SDS polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting analysis**

SDS-PAGE and subsequent immunoblotting on PVDF membranes was carried out according to standard procedures. Immunoblots were incubated with anti-55 kDa cytosolic protein (kind gift of G. Blobel) and anti-cytochrome c (kind gift of F. Sherman), respectively. Immunoreactive bands were visualized by ECL plus (GE Healthcare, Freiburg, Germany) and quantified using QuantityOne® V.4.2 software (Bio-Rad, Munich, Germany).

**Staining for reactive oxygen species (ROS)**

ROS were detected with dihydorhodamine 123 (Sigma-Aldrich, Taufkirchen, Germany) according to Madeo et al. (13) with 30 min staining at 30°C. Cells were embedded in 0.5% agarose in PBS and evaluated for staining by fluorescence microscopy using a rhodamine optical filter (RT, 40x/0.75, Axioskop 2, AxiosCam HRc, AxioVision 4, Zeiss, Göttingen, Germany). In \( \rho^0/\rho^+ \) experiments, ROS were detected with the mitochondrial membrane potential-independent stain dihydroethidium (DHE, Sigma-Aldrich). 5x10⁶ cells were pelleted in 96-well microtiter plates (Micron Fluorotrac 600, Greiner, Austria), washed twice with PBS, resuspended in 250 µL of 2.5 µg/mL DHE in PBS and incubated for 10 min at RT. Relative fluorescence units (RFU) were determined using a fluorescence reader (GENios Pro™, Tecan, Grödig, Austria; excitation 515 nm, emission 595 nm, RT). As blank DHE in PBS was used. Additionally, cells were evaluated for staining by fluorescence microscopy using a rhodamine optical filter.

**Survival plating assay**

Survival plating assays were done as previously described (27). Briefly, an aliquot of the culture was counted with a CASY1 (Schärfe Systems, Germany), diluted 1:10000 in water, and 500 cells were plated on YPGlc plates (4% glucose). The number of colonies (colony forming units, CFU) was determined after incubating the plates for 2-3 days at 28°C. For each experiment three plates per strain and condition were evaluated for growth of colonies.

**Tests for apoptotic markers**

In vivo measurement of caspase-like enzymatic activity by flow cytometric analysis was done as previously described (27). Briefly, cells were harvested, washed in PBS and resuspended in staining solution containing FITC-VAD-FMK (CaspACE™, Promega). After incubation for 20 min at 30°C, cells were washed and resuspended in PBS. Stained cells were counted using a FACS Calibur (BD Biosciences, Heidelberg, Germany) and Cell Quest analysis software. CaspACE™ FITC-VAD-FMK in situ marker is a fluoroisothiocyanate (FITC) conjugate of the cell permeable caspase inhibitor VAD-FMK. This structure allows delivery of the inhibitor into the cell where it binds to activated caspase, serving as an in situ marker for apoptosis. The bound marker is localized by fluorescence detection.

The T4 terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was used to visualize DNA fragmentation, a late marker of apoptosis. Cell wall digestion and cell fixation was done as described by Madeo et al. (8). TUNEL reaction was performed using an in situ cell death detection kit (Roche, Mannheim, Germany) and Chromatide Bodipy™ (Molecular Probes, Invitrogen, Karlsruhe, Germany) as fluorescence-labeled dUTP. Cells were evaluated for stained nuclei by fluorescence microscopy using a FITC optical filter (RT, 40x/0.75, Axioskop 2, AxiosCam HRc, AxioVision 4).

**Results**

Mitochondria in cdc48SS65G cells are enlarged compared to wild-type.
In order to check for mitochondrial impairment in the apoptotic cdc48$^{S565G}$ yeast strain we performed ultrastructural analysis (electron microscopy, EM) of yeast cells. In cdc48$^{S565G}$ cells we observed a significant enlargement of mitochondria compared to wild-type (Fig. 1A, for quantification see Fig. 1B). In the cdc48$^{S565G}$ strain 10% of the cellular area was composed of mitochondria compared to 7% in the wild-type strain. Since the average number of mitochondria per cell was highly similar between the cdc48$^{S565}$ and wild-type strains (1.1 for wild-type and 1.2 for cdc48$^{S565G}$ cells), these data hint towards a swelling of mitochondria in the cdc48$^{S565G}$ strain, which is a known feature in pathophysiological processes (28-31).

**Distinct alterations are observed in the mitochondrial proteome of cdc48$^{S565G}$ cells compared to wild-type.**

We further directed our studies to investigate whether mitochondrial enlargement in the cdc48$^{S565G}$ strain was concomitant with alterations at the molecular level of mitochondria. Therefore, we analyzed the mitochondrial proteome applying differential 2-DE analysis of wild-type and cdc48$^{S565G}$ strains. Additionally, we compared their total cell extracts and their cytosolic proteomes.

Differential 2-DE analysis of mitochondria resulted in 32 significant protein spot variations between wild-type and cdc48$^{S565G}$ strains (Fig. 2A, compare gels 1 and 2, Table 1). In contrast, only minimal differences were observed in cytosolic fractions (Fig. 2A, compare gels 1 and 2), and the overall cellular proteome remained unchanged (data not shown).

Mass spectrometry (MS) analysis of the 32 altered protein spots in mitochondria identified 23 unique proteins (Table 1), seven of which were established as mitochondrial proteins. Increased (“enrichment”) and decreased (“depletion”) amounts of mitochondrial proteins in mitochondrial extracts of cdc48$^{S565G}$ cells were observed (e.g., YGR086c Fig. 2B, panel 2 and ‘maintenance of mitochondrial function 1’ (MMF1) Fig. 2B, panel 1, respectively; for quantification of protein spot alterations see Table 1).

The observed depletion of MMF1 (Fig. 2B, panel 1) and ‘ketol-acid reductoisomerase’ (I1v5p, Table 1), two mitochondrial proteins fundamental for the stability of mitochondrial DNA (32,33), suggest reduced mitochondrial functionality upon CDC48 mutation. We found depletion of mitochondrial cyclophilin C (Cyp. C) (Fig. 2B, panel 1) and enrichment of ‘mitochondrial 40S ribosomal protein’ (MRP8) (Table 1) in mitochondrial extracts. Cyclophilins are enzymes that catalyze cis-trans isomerization of proline-containing peptides to ensure accurate protein folding (34). MRP8 is a component of the mitochondrial protein translation machinery (35). Alterations in the amount of cyclophilin C and MRP8 therefore may suggest an altered protein turnover in mitochondria. Discrete changes of mitochondrial proteins in mitochondrial extracts suggest that mitochondria are altered upon CDC48 mutation possibly leading to mitochondrial dysfunction.

In addition to the alterations in mitochondrial proteins, we also observed enrichment of four cytoplasmic proteins, three proteins linked to the cytoskeleton and two proteins with unknown localization (Table 1) in mitochondrial fractions of cdc48$^{S565G}$ cells. We observed accumulation ‘F-actin capping protein alpha subunit’ (CAPA, Fig. 2B, panel 1) and of the ‘ARP2/3 complex 20 kDa subunit’ (Fig. 2B, panel 4). The ARP2/3 complex is associated with the actin cytoskeleton and is involved in mitochondrial motility in yeast (36). Accumulation of these proteins may suggest an altered mitochondrial motility in cdc48$^{S565G}$ cells.

Interestingly, we found seven other proteins associated with the NE-ER network, a continuous membrane system consisting of the endoplasmic reticulum (ER) and the ER-related nuclear envelope (NE), to show altered levels in the mitochondrial fraction of the cdc48$^{S565G}$ strain (Table 1). In this context, ER luminal proteins, proteins integrated in or associated with the NE-ER membrane, and nuclear proteins are referred to as “NE-ER-associated”. In fact, the majority (six out of seven) were clearly enriched in mitochondrial extracts (Table 1). Most importantly, Cdc48p-S565G itself was found to be the NE-ER protein demonstrating the strongest enrichment in mitochondrial fractions of cdc48$^{S565G}$ cells compared to wild-type (i.e., 5.8 fold; Fig. 2B, panel 6, Table 1). Accumulation of these NE-ER-associated proteins could be a result of the ER expansion.
and the dysfunction in ERAD earlier described in the cdc48<sup>S56SG</sup> strain (8,39).

**In the cdc48<sup>S56SG</sup> strain cytochrome c accumulates in the cytosol.**

Cytochrome c is a mitochondrial protein essential for the transfer of electrons from the cytochrome bc<sub>1</sub> complex to the cytochrome c oxidase complex of the respiratory chain. Depletion of cytochrome c leads to respiratory chain dysfunction and accumulation of ROS in yeast (40). It is a comparatively small (12 kDa) and basic protein (isoelectric point of 9.5) and therefore hardly analyzable by the applied 2-DE analysis. Hence, we looked for accumulation of cytochrome c in the cytosol using immunoblotting analysis. We found a 2.3 fold enrichment of cytochrome c in the cytosol of cdc48<sup>S56SG</sup> cells compared to wild-type cells (Fig. 3A immunoblots, for quantification see Fig. 3B). Accumulation of the soluble intermembrane protein cytochrome c in the cytosol, as well as depletion of the soluble matrix proteins ARG5,6, MMF1 and cyclophilin C in mitochondrial extracts as evidenced by 2-DE analysis (Table 1), suggest that mitochondrial membranes are more fragile in cdc48<sup>S56SG</sup> cells compared to wild-type cells possibly resulting in the release of mitochondrial proteins into the cytosol.

These observed alterations at the mitochondrial molecular level obtained by 2-DE and immunoblot analyses of cytochrome c consequently propose mitochondrial dysfunction in the cdc48<sup>S56SG</sup> strain.

**Cdc48<sup>S56SG</sup> cells show respiratory deficiency.**

In order to test for loss of mitochondrial functionality in the cdc48<sup>S56SG</sup> strain, we investigated the adaptability of both wild-type and cdc48<sup>S56SG</sup> cells to conditions, which challenge the respiratory capacity of their mitochondria. Only respiratory sufficient *S. cerevisiae* cells, in contrast to respiratory deficient cells, form colonies on media containing a principal carbon and energy source, which is obligatory aerobic (lactate) for growth (41). Consequently, cells with respiratory incompetent mitochondria cannot metabolize lactate, i.e., they are unable to proliferate and do not form colonies. Therefore, a differential plating assay was conducted (41), in which proliferation on agar plates of wild-type and cdc48<sup>S56SG</sup> cultures was analyzed. YPLac (lactate) plates were used as selective respiratory medium and YPGlc (glucose) plates as selective fermentative medium. Cultures were spotted on agar plates in dilution series, clockwise on six distinct sections (Fig. 4, e.g., plate 1), and the proliferation of the plated cultures was subsequently evaluated.

Cdc48<sup>S56SG</sup> cells showed a markedly reduced proliferation on YPGlc compared to wild-type cells (Fig. 4, compare plates 1 and 2) demonstrating the important cellular role of Cdc48p impaired by the mutation. However, the lowest level of proliferation was found on YPLac (Fig. 4, plate 4). The almost complete absence of proliferation on YPLac (Fig. 4, compare plates 2 and 4) suggests respiratory deficiency of cdc48<sup>S56SG</sup> cells probably due to their progressed state of impaired mitochondrial functionality. Such impairment was not detectable in wild-type cells under the same growth conditions (Fig 4, compare plates 1 and 3).

**Accumulating ROS in cdc48<sup>S56SG</sup> cells are predominantly produced by the mitochondrial cytochrome bc<sub>1</sub> complex.**

Mitochondrial impairment (Fig. 1), the alterations at the mitochondrial proteome level (Fig. 2) and the observed respiratory deficiency of cdc48<sup>S56SG</sup> cells (Fig. 4) suggest mitochondrial dysfunction upon CDC48 mutation. Further, the observed release of cytochrome c into the cytosol (Fig. 3) and the protein spot alterations of the ubiquinol-cytochrome c reductase iron-sulfur subunit (UCRI, Table 1), a component of the cytochrome bc<sub>1</sub> complex of the inner mitochondrial membrane, suggest a disturbance of the respiratory chain. It is known that the cytochrome bc<sub>1</sub> complex, upon dysfunction, is a major cellular producer of reactive oxygen species (ROS) (42). We therefore tested whether the emergence of ROS paralleled the observed mitochondrial impairment. In fact, we found a significantly higher number of cdc48<sup>S56SG</sup> cells (2.1 fold), which accumulated ROS compared to wild-type cells (Fig. 5A). In the cdc48<sup>S56SG</sup> strain 52% of the cells were ROS-positive compared to 25% in the wild-type strain.

In order to show that the cytochrome bc<sub>1</sub> complex is a major producer of ROS in the cdc48<sup>S56SG</sup> strain, we used myxothiazol and stigmatellin as inhibitors of this complex (42-44). Both inhibitors interrupt the electron transfer within the cytochrome bc<sub>1</sub> complex but on two different sites (42,43). Applying these inhibitors, we found a significant reduction in the number of cells showing ROS accumulation. In the cdc48<sup>S56SG</sup> strain the proportion of ROS-
positive cells was reduced from 52% to 24% and 22% for myxothiazol and stigmatellin, respectively (Fig. 5B). These data suggest that the mitochondrial cytochrome bc1 complex is a major site of ROS production in the cdc48<sup>S665G</sup> strain. Quenching of ROS production was also observed in the wild-type strain treated with inhibitors of the cytochrome bc1 complex. However, the significant higher number of ROS-positive cells in the cdc48<sup>S665G</sup> strain compared to the wild-type strain, point to a higher susceptibility of mitochondria in the cdc48<sup>S665G</sup> strain to produce the detrimental ROS.

ρ<sup>0</sup> strains generated from wild-type and cdc48<sup>S665G</sup> strains show very low levels of ROS production and highly similar viability.

Mitochondrial contribution to the accumulation of ROS in the cdc48<sup>S665G</sup> strain suggests that the observed impairment of mitochondria may lead to cellular damage. In order to validate such a destructive role of mitochondria, we converted the CDC48 wild-type and the cdc48<sup>S665G</sup> mutant strains (ρ<sup>+</sup> strains) into yeast strains lacking functional mitochondria (ρ<sup>0</sup> strains). Both strains were grown overnight on media containing ethidium bromide resulting in the loss of mitochondrial DNA. Lack of mitochondrial functionality was confirmed by complete lack of growth on media containing obligatory respiratory carbon sources (glycerol).

ρ<sup>0</sup> and ρ<sup>+</sup> strains were evaluated for the emergence of ROS. In both ρ<sup>0</sup> strains (wild-type and cdc48<sup>S665G</sup> mutant), cells accumulating ROS were present only sporadically (Fig. 6A). Further analysis revealed a significant decrease in the production of ROS in both ρ<sup>0</sup> strains compared to the respective ρ<sup>+</sup> strains (Fig. 6B), i.e., 88% and 62% reduction of ROS production in cdc48<sup>S665G</sup> in wild-type, respectively. These data confirm the considerable involvement of mitochondria in both wild-type and cdc48<sup>S665G</sup> strains in the production of ROS as was already suggested by the decrease of ROS production via inhibition of the cytochrome bc1 complex of the respiratory chain (Fig. 5B). Notably, ROS production between the wild-type ρ<sup>0</sup> and the cdc48<sup>S665G</sup> ρ<sup>0</sup> strains assimilated at very low levels (Fig. 6B), further arguing that in the cdc48<sup>S665G</sup> strain impaired mitochondria are responsible for the elevated levels of ROS.

In order to assess the viability of both ρ<sup>+</sup> and ρ<sup>0</sup> cultures, we applied a survival plating assay. In this assay equal numbers of cells were plated onto YPGA plates and the numbers of formed colonies were determined. The cdc48<sup>S665G</sup> ρ<sup>0</sup> strain showed a significant lower viability (30%) than the wild-type ρ<sup>0</sup> strain (Fig. 6C), as evidenced by the decreased number of formed colonies. In contrast, the viabilities of the cdc48<sup>S665G</sup> ρ<sup>+</sup> and the wild-type ρ<sup>0</sup> strains assimilated (Fig. 6C). Notably, the viability of the cdc48<sup>S665G</sup> ρ<sup>0</sup> strain lacking functional mitochondria was slightly higher (16%) than the viability of the cdc48<sup>S665G</sup> ρ<sup>+</sup> strain. These data hint to a deleterious role of the impaired mitochondria in the mutant cdc48<sup>S665G</sup> strain.

Caspase-like enzymatic activity and DNA fragmentation emerge in the cdc48<sup>S665G</sup> strain.

Recently, a yeast protein demonstrating caspase-like enzymatic activity upon applied oxidative stress has been described (27). Since we demonstrated accumulation of ROS (Fig. 5 and 6), we tested for caspase-like enzymatic activity in vivo in the wild-type and the cdc48<sup>S665G</sup> strains. Cells were labeled for active caspase with the fluorescence-tagged and cell permeable caspase inhibitor FITC-VAD-FMK and analyzed by flow cytometry. A significantly higher portion of cdc48<sup>S665G</sup> than of wild-type cells (2.2 fold) demonstrated caspase-like enzymatic activity (Fig. 7A, for quantification see Fig. 7B). Using DNA fragmentation as a marker of apoptosis (TUNEL assay), consistently to previous results (8), cell death was observed in 50% of cdc48<sup>S665G</sup> cells whereas cell death in wild-type cells did not exceed 20% (Sfig.1A micrographs, for quantification see Sfig.1B). Thus, the observed mitochondrial impairment due to CDC48 mutation is paralleled by the emergence of apoptotic cell death as indicated by caspase-like enzymatic activity and DNA fragmentation.

Discussion

Mitochondria are crucially impaired in apoptotic cdc48<sup>S665G</sup> cells.

In this study we addressed the issue whether mitochondria are affected at the molecular and functional level and whether they participate in apoptosis in a yeast strain upon CDC48 mutation (cdc48<sup>S665G</sup>). Our data demonstrate mitochondrial impairment in cdc48<sup>S665G</sup> cells:

First, mitochondria are a specific site for qualitative as well as quantitative protein alterations in cdc48<sup>S665G</sup> cells (Fig. 2). Both “enrichment” and “depletion” of distinct proteins were seen (Table 1). In particular, we observed
the depletion of two proteins, MMF1 and Ilv5p (Fig. 2), which are necessary for mitochondrial DNA stability and mitochondrial functionality (32,33). A recent transcriptome analysis of cdc48S565G cells demonstrated nuclear genes coding for mitochondrial proteins to be the largest group of differentially regulated genes (45). Thus, the observed distinct alterations at the mitochondrial protein level suggest that mitochondria are a pivotal site of changes on the protein level associated with CDC48 mutation. Second, we demonstrated mitochondrial enlargement (Fig. 1) and release of cytochrome c into the cytosol (Fig. 3) in the cdc48S565G strain compared to wild-type hinting to a facilitated mitochondrial rupture. Third, the deficit of cdc48S565G cells to adapt to respiratory growth conditions (Fig. 4) as well as accumulation of ROS produced by mitochondria (Fig. 5, 6) suggest dysfunction of the mitochondrial respiratory chain.

Mitochondrial damage and dysfunction, release of cytochrome c into the cytosol and emergence of ROS are characteristic features of most mitochondria-dependent apoptotic pathways in both mammalian cells and in yeast (17-19). Consistently to previous studies (8,13,45), we observed apoptotic cell death in the cdc48S565G strain as evidenced by DNA fragmentation (SFig. 1). Moreover, we revealed the emergence of caspase-like enzymatic activity in the cdc48S565G strain (Fig. 7) concomitantly to the accumulation of ROS (Fig. 5). In yeast, the caspase Yca1p is activated upon exogenously applied oxidative stress (27). Thus, it is likely that endogenously accumulating ROS in the cdc48S565G strain induce caspase activity that precedes and subsequently triggers DNA fragmentation and cell death. In a previous study, ROS have been demonstrated to be essential for the progression of cell death in the cdc48G strain (13). Therefore, the increased production of ROS by the mitochondrial cytochrome bc1 complex suggests a mitochondrial contribution in apoptotic cell death in the cdc48S565G strain. Consistently, generation of yeast strains lacking functional mitochondria (ρ0 strains) revealed that the cdc48S565G ρ0 strain was found to be highly similar to the wild-type ρ0 strain in both cell viability (Fig. 6) and growth rates (data not shown). In contrast, the cdc48S565G ρ− strain showed significantly lower cell viability (Fig. 6) and a markedly decreased growth rate (data not shown) compared to the wild-type ρ− strain. These data indicate that mitochondria play a detrimental role during cell death in the cdc48S565G strain.

Single protein spot alterations in 2-DE of mitochondrial extracts sustain mitochondrial involvement in apoptotic cell death. We found depletion of cyclophilin C (Cyp. C) in mitochondrial extracts of apoptotic yeast (Fig. 2). Mitochondrial cyclophilin in mammalian cells has been described as a repressor of mitochondria-dependent apoptosis (46). Depletion of its homologue during apoptosis suggests a similar role in yeast. We observed accumulation of the actin cytoskeleton proteins ‘ARP2/3 complex 20 kDa subunit’ (Fig. 2) and ‘F-actin capping protein alpha subunit’ (CAPA, Fig. 2). Recently, a connection between yeast apoptosis and actin dynamics has been made (47,48). These authors demonstrated that decreased actin dynamics caused depolarization of the mitochondrial membrane and an increase in ROS production resulting in cell death, highly similar features we observed in this study.

We found several proteins associated with the NE-ER to be enriched in mitochondrial extracts in the cdc48S565G strain (Table 1) suggesting an increased NE-ER content in mitochondrial fractions. Interestingly, the strongest accumulation was observed for Cdc48p-S565G itself (Fig. 2). Previous studies revealed deficiency of the ER-associated protein degradation (ERAD) pathway (39) and expansion of the ER (8) in the cdc48S565G strain. Thus, enhanced co-purification of NE-ER-associated proteins might be a result of ERAD dysfunction upon CDC48 mutation. Notably, we found that ERAD deficiency in the cdc48S565G strain is paralleled with an increased co-purification of NE-ER-derived microsomes with mitochondria (Zischka et al., submitted). Interestingly, Haynes et al. have shown that in an ERAD-deficient yeast strain, overexpression of a single misfolded model protein leads to ER stress, accumulation of ROS and ultimately apoptotic cell death (49). These authors demonstrated contribution of mitochondria to ROS accumulation arising from inhibition of ERAD. Thus, the mitochondrial impairment and contribution in apoptotic cell death in the cdc48S565G strain observed in our study might be a consequence of the described ERAD dysfunction in this strain (39).

Cdc48p/VCP-mediated apoptosis and human disease.
Cdc48p/VCP is a highly conserved protein essential for cellular function (for review: (4)). Upon mutation impairment of Cdc48p/VCP-mediated functions increase the risk for apoptotic cell death in different species. Classical morphological apoptotic characteristics, e.g., DNA fragmentation, chromatin condensation, nuclear fragmentation and membrane blebbing, were observed in cells expressing mutated Cdc48p/VCP homologues in mammalian cell cultures (9,10), in trypanosomes (11), in zebrafish (12), and in budding yeast (8), although the molecular mechanisms how impairment of Cdc48p/VCP relates to apoptotic cell death remain largely unknown. Especially mitochondrial contribution to cell death has not been demonstrated yet.

This study revealed crucial mitochondrial impairment in the cdc48<sup>S565G</sup> yeast strain associated with apoptosis. Yeast Cdc48p and its orthologues, such as mammalian VCP show very high sequence and functional conservation (50). Therefore, we suggest mitochondria as being involved in apoptotic cell death in other species expressing mutant variants of Cdc48p/VCP.

Mutant VCP is an inductor of IBMPFD, a dominant human disorder (5,6). Wild-type VCP has been described as a pathological mediator for human polyglutamine diseases (7,10,51). In these disorders and in particular Huntington’s disease, typical features of mitochondria-dependent cell death have been noted: depolarization of mitochondria, emergence of ROS and cytochrome c release (52,53). Thus, our finding of a mitochondrial contribution to cell death in cdc48<sup>S565G</sup> yeast is compatible with the role of both Cdc48p/VCP and mitochondria in these human disorders. Based on this study we propose cdc48<sup>S565G</sup> yeast as a model to elucidate the remaining unknown processes of VCP-mediated apoptosis in human degenerative diseases.
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Figure Legends

Figure 1:
**Mitochondria are enlarged in cdc48<sup>S565G</sup> cells.**

(A) EM analysis of wild-type and cdc48<sup>S565G</sup> cells. Wild-type cells (1) show intact nuclei (arrows: nuclear envelope) with mitochondria predominantly distributed near the plasma membrane. Cdc48<sup>S565G</sup> cells (2) frequently demonstrate chromatin condensation (arrowheads), nuclear fragmentation and enlarged mitochondria. Mt: mitochondria, N: nucleus, arrows: nuclear envelope, arrowheads: chromatin condensation.

(B) Quantification of mitochondrial enlargement. Mitochondrial and total cellular area was determined using AxioVision Software LE V.4.2 (Zeiss). In order to exclude artifacts due to the fixation procedure, mitochondrial area was normalized to total cellular area. The obtained percentage of the mitochondrial area within cells was significantly increased in cdc48<sup>S565G</sup> (10%) compared to wild-type cells (7%) (p<0.02, Student’s t-test). These figures mean enlargement of mitochondria in cdc48<sup>S565G</sup> cells, since the average number of mitochondria within 1 µm² of cellular area remained unchanged (1.1 for wild-type and 1.2 for cdc48<sup>S565G</sup> cells). For quantification and statistics 62 and 128 mitochondria for wild-type and cdc48<sup>S565G</sup> strain, respectively, were evaluated.

Figure 2:
**Differential 2-DE analysis of mitochondrial and cytosolic fractions from wild-type and cdc48<sup>S565G</sup> cells.**

(A) 2-DE comparison of cytosolic extracts (wild-type vs. cdc48<sup>S565G</sup>, gels 1 and 2, respectively): 6 reproducible differences (arrows) out of 1600 protein spots per gel were found (Proteom Weaver™). (n=6)

Comparison of mitochondrial extracts (gels 3 and 4): 32 reproducible differences (arrows) out of 1400 protein spots per gel were found; identified proteins and results of quantification (ProteomWeaver™) are listed in Table 1. (n=7)

(B) Representative differences between wild-type and cdc48<sup>S565G</sup> strains in mitochondrial extracts.

Figure 3:
**Release of cytochrome c into the cytosol of cdc48<sup>S565G</sup> cells.**

Increased amounts of cytochrome c were found in the cytosol in cdc48<sup>S565G</sup> compared to wild-type cells.

(A) Representative immunoblot of cytochrome c (10 µg protein load per lane). The 55 kDa cytosolic protein was used as loading control.

(B) Histogram showing levels of cytochrome c in the cytosol. Note that yeast cultures grown on (fermentative) glucose medium contain mitochondria with a certain tendency for disruption resulting in marked amounts of cytochrome c in the cytosol of wild-type strain upon cell fractionation. However, the significant higher amounts of cytochrome c levels in the cytosol of cdc48<sup>S565G</sup> cells suggest for a pronounced higher fragility of mitochondria compared to wild-type. The level of cytochrome c in the cytosol of cdc48<sup>S565G</sup> cells was set to 100% in every single experiment. A 2.3 fold increase in cytochrome c amount was observed in the cytosol of cdc48<sup>S565G</sup> compared to wild-type cells (**p<0.01, Student’s t-test). The data shown here are percent change values of six independent experiments. Error bars: s.d.

Figure 4:
**Respiratory deficiency of cdc48<sup>S565G</sup> cells.**

Wild-type and cdc48<sup>S565G</sup> cultures were plated on YPLac (respiratory selective medium) and YPGlc (fermentative selective medium). Cultures were spotted on agar plates in logarithmic dilution series clockwise on six distinct sections: Section 1: 5x10<sup>6</sup>, section 2: 5x10<sup>5</sup>, section 3: 5x10<sup>4</sup>, section 4: 5x10<sup>3</sup>, section 5: 5x10<sup>2</sup>, section 5: 5x10<sup>1</sup> cells plated. Treated sections were evaluated for growth. Proliferation of cdc48<sup>S565G</sup> cells (YPGlc) was low on YPGlc plates (plate 2) and almost completely eliminated on YPLac plates (plate 4); (n=3).

Figure 5:
**Enhanced mitochondrial ROS production in cdc48<sup>S565G</sup> cells.**
Mitochondrial impairment in cdc48SS65G yeast

(A) Accumulation of ROS. A significant higher number of cdc48SS65G than wild-type cells showed ROS accumulation (2.1 fold, n=8, p<0.002, Student’s t-test). Representative micrographs of wild-type and cdc48SS65G cells stained with dihydrorhodamine 123. For quantification >1000 cells per strain and experiment were evaluated.

(B) Quenching of ROS accumulation. Cultures were grown in the presence of inhibitors of the cytochrome bc1 complex (myxothiazol and stigmatellin, respectively, 1 µM) and tested for accumulation of ROS (n=4 for myxothiazol, n=3 for stigmatellin). In the case of the cdc48SS65G strain, the number of ROS accumulating cells decreased from 52% to 24% (**p<0.005) and 22% (***p<0.005), respectively. The number of wild-type cells showing ROS accumulation was reduced from 25% to 11% (p<0.02) and 10% (p<0.03), respectively. For quantification >1000 cells per strain and experiment were evaluated. p-values: Student’s t-test. Error bars: s.d.

Figure 6: Wild-type ρ0 and cdc48SS65G ρ0 strains showed very low levels of ROS production and highly similar viability.

CDC48 wild-type (KFY417) and cdc48SS65G mutant (KFY437) strains (ρ0 strains) were converted into yeast strains lacking functional mitochondria (ρ0 strains) as described in Experimental Procedures.

(A) ρ0 strains are unable to accumulate ROS. Representative micrographs of wild-type ρ0 and cdc48SS65G ρ0 cells stained with dihydroethidium.

(B) Quantification of ROS accumulation in ρ0 and ρ+ strains. ROS accumulation was measured in a fluorescence reader after staining with dihydroethidium. In the case of the cdc48SS65G strains, ROS accumulation was decreased by 88% in the ρ0 strain compared to the ρ+ strain (from 35200 RFU to 4100 RFU, ***p<0.001). ROS accumulation in the wild-type ρ0 strain was found to be reduced by 62% compared to the wild-type ρ+ strain (from 7100 RFU to 2700 RFU, ****p<0.0001). Note that ROS production in the cdc48SS65G ρ0 and wild-type ρ0 strains assimilated at very low levels. In contrast, the cdc48SS65G ρ+ strain showed significant higher levels of ROS compared to the wild-type ρ+ strain. The data shown here are mean values of three independent experiments. p-values: Student’s t-test. Error bars: s.d.

(C) Cdc48SS65G ρ0 strain shows highly similar viability compared to the wild-type ρ0 strain. For each culture, ρ0 and ρ+, 500 cells were plated on YPGlc plates and the number of formed colonies (colony forming units, CFU) was determined. The viability of the cdc48SS65G ρ0 strain was highly similar when compared to the wild-type ρ0 strain (8% lower viability of the cdc48SS65G ρ0 strain compared to the wild-type ρ0 strain, p=0.42). In contrast, the cdc48SS65G ρ+ strain revealed a significant decreased viability compared to the wild-type ρ+ strain (30% decrease, p<0.01). Notably, the viability of the cdc48SS65G ρ0 strain was found to be increased compared to the cdc48SS65G ρ+ strain. The data shown here are mean values of three independent experiments. p-values: Student’s t-test. Error bars: s.d.

Figure 7: Emergence of caspase-like enzymatic activity in the cdc48SS65G strain.

Wild-type and cdc48SS65G cells were labeled for active caspase by the cell permeable fluorescence-labeled caspase inhibitor FITC-VAD-FMK and analyzed by flow cytometry as described in Experimental Procedures.

(A) Representative flow cytometric diagrams of wild-type and cdc48SS65G strain. The nature of the second peak in the flow cytometric diagram of the cdc48SS65G strain remained unknown.

(B) Quantification of caspase activity. A 2.2 fold increase in caspase activity was observed in the cdc48SS65G compared to the wild-type strain (n=3, *p<0.05, Student’s t-test). Error bars: s.d.

Supplementary Figure 1: DNA fragmentation can be induced in cdc48SS65G cells.

(A) DNA fragmentation, visualized by TUNEL staining, is increased in cdc48SS65G cells compared to wild-type cells. Representative micrographs of wild-type and cdc48SS65G cells.

(B) Quantification reveals 2.6 fold increase in cdc48SS65G cells showing DNA fragmentation compared to wild-type cells (n=4, ***p<0.001, Student’s t-test). Error bars: s.d.
Table 1
Identified proteins differentially found in mitochondrial extracts

Maldi-TOF Mass Spectrometry:
Protein spots were subjected to trypsin treatment. Resulting peptides were analyzed by peptide mass fingerprinting using a Maldi-TOF Reflectron (Waters). Spectra were annotated applying MassLynx software (Waters). Subsequent database searches in SwissProt were done using the ProteinLynx Globalserver 1.1 software (PLGS 1.1, Waters) with the following search parameters:
- Organisms: Unrestricted
- Fixed modifications: Carbamidomethyl (C)
- Variable modifications: Oxidations (M)
- Mass values: Monoisotopic
- Protein Mass: Unrestricted
- Peptide Mass Tolerance: ± 150 ppm
- Peptide Charge State: 1+
- Max Missed Cleavages: 1

2-DE Analysis of Mitochondria:
Image analysis of the gels was performed by ProteomWeaver™ image analysis software V.2.2 (Definiens). For the analysis of mitochondrial extracts data were determined by taking into account three independent experiments. In total seven 2D gels for wild-type and seven 2D gels for cdc48S565G were considered for quantification and statistics.

a) SwissProt database: http://us.expasy.org/sprot/
b) PLGS 1.1 scoring
c) MIPS database: http://mips.gsf.de/projects/fungi
d) Factor: mutant (cdc48S565G) versus wild-type strain: <1 for depletion, >1 for accumulation of protein
e) Mitop2 database: http://ihg.gsf.de/mitop2/start.jsp
f) according to (54), high probability for mitochondrial localization according to Mitop2 database
g) according to (55)
h) according to (56)
i) ribosomal proteins are predominantly NE-ER-associated (57)
IM: inner mitochondrial membrane
| Spot-No. (Fig. 3) | SwissProt Accession-No. (a) | Sequence Coverage [%] | P.LiGS1.1 Score of Identified Protein/Score of Next Yeast Hit (b) | Matched Mass Values | Gene Name (MIPS) (c) | Protein Name | Relative Protein Spot Intensities on 2-DE (cdc48S565G vs. Wild-type) (d) | p-values of 2-DE Image Analysis (Student’s t-test) (e) | Localization (Mitop2) (e) | Function |
|------------------|----------------------------|----------------------|---------------------------------------------------------------|-------------------|---------------------|---------------------|---------------------------------------------------------------|---------------------------------|----------------------|------------|
| 1                | Q01217                     | 42                   | 54/26                                                         | 9/13              | YER069w             | ARG5,6 protein     | 0.5                                                       | 0.00200                          | Mitochondria, matrix          | Amino acid metabolism |
| 2                | P06168                     | 96                   | 201/57                                                        | 13/13             | YLR355c             | Ketol-acid reductoisomerase (Ilv5p) | 0.6                                           | 0.00332                          | Mitochondria                  | Amino acid metabolism |
| 3, 4             | P40185                     | 82                   | 82/-                                                          | 7/9               | YIL051c             | Maintenance of mitochondrial function 1 (MMF1) | 0.4                                           | 0.00361                          | Mitochondria, matrix          | Amino acid metabolism |
| 5                | P25719                     | 65                   | 49/14                                                         | 8/11              | YML078w             | Cyclophilin C (Cyp C) | 0.7                                           | 0.00247                          | Mitochondria, matrix          | Protein folding              |
| 6                | P35719                     | 48                   | 32/34                                                         | 3/6               | YKL142w             | Mitochondrial 40S ribosomal protein (MRP8) | 4.0                                           | 0.01214                          | Mitochondria                  | Protein biosynthesis         |
| 7, 8             | P08067                     | 78                   | 74/47                                                         | 9/17              | YEL024w             | Ubiquinol-cytochrome c reductase iron-sulfur subunit (UCRI) | 0.65/1.2                                         | 0.04284/0.17448                 | Mitochondria, IM             | Energy metabolism            |
| 9                | P53252                     | 69                   | 21/3                                                          | 4/6               | YGR086c             | Hypothetical protein YGR086c/Sphingolipid long chain base-responsive protein PIL1 | 3.5                                           | 0.00007                          | Mitochondria (f), lipid particles | Membrane traffic             |
| 10               | P33204                     | 78                   | 28/10                                                         | 3/6               | YKL013c             | ARP2/3 complex 20 kDa subunit | 3.5                                           | 0.00038                          | Cytoskeleton                  | Mitochondrial motility       |
| Pseudo-Gen | Pseudonym | Molec. Type | Description | Normalised Log Odds | FDR | Functions | Description |
|---|---|---|---|---|---|---|---|
| P28495 | 35 | 31/12 | 7/10 | YKL007w | F-actin capping protein alpha subunit (CAPA) | 2.8 | 0.00020 | Cytoskeleton | Actin cytoskeleton |
| P35691 | 49 | 13/12 | 3/7 | YKL056c | Translocation protein Sec72p | 3.3 | 0.00037 | Cytoskeleton, mitochondria-associated (g) | Microtubule associated, translocates to mitochondria upon oxidative stress (g) |
| P39742 | 55 | 11/7 | 2/5 | YLR292c | Guanine nucleotide binding protein subunit beta like protein | 1.8 | 0.00460 | ER membrane | Secretory pathway |
| P07283 | 53 | 28/20 | 6/8 | YBR072w | Heat shock protein 26 (Hsp26) | 1.7 | 0.00106 | Nucleus, cytoplasm | Protein folding |
| P35176 | 75 | 22/-1 | 7/13 | YDR304c | Cyclophilin D (Cyp D) | 0.5 | 0.00291 | ER lumen | Protein folding |
| P15992 | 53 | 28/20 | 6/8 | YMR116c | Guanine nucleotide binding protein subunit beta like protein | 2.1 | 0.00154 | Ribosome (i) | Protein biosynthesis |
| P01001 | 71 | 70/22 | 6/8 | YKR059w | Eukaryotic initiation factor 4A | 1.6 | 0.05509 | Ribosome (i) | Protein biosynthesis |
| P25694 | 20 | 29/3 | 5/9 | YDL126c | Cell division cycle protein 48 (Cdc48p) | 5.8 | 0.00002 | ER associated, nucleus, cytosol | ERAD, organelle formation, spindle apparatus |
| P06106 | 60 | 32/8 | 9/20 | YLR303w | O-acetylhomoserine sulphydrolase (MET17) | 1.5 | 0.00630 | Cytoplasm | Amino acid metabolism |
| P04173 | 41 | 48/16 | 8/15 | YCL018w | 3-isopropylmalate dehydrogenase | 1.4 | 0.00615 | Cytoplasm | Amino acid metabolism |
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|   |   |   |   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|---|---|---|
| 26, 27 | P00924 | 57 | 23/-2 | 10/14 | YGR254w | Enolase 1 | 2.2 | 0.00199 | Cytoplasm | Energy metabolism |
| 28-30 | P38219 | 70 | 27/1 | 6/9 | YBR025c | Putative GTP binding protein | 2.4 | 0.00803 | Cytoplasm | Unknown |
| 31 | Q05016 | 76 | 22/-6 | 6/9 | YMR226c | Putative oxidoreductase | 2.8 | 0.01019 | Unknown | Unknown |
| 32 | Q12447 | 75 | 38/20 | 5/8 | YDR071c | Hypothetical protein | 3.2 | 0.00070 | Unknown | Unknown |
Figure 1

**A**

Wild-type

Mutant

1 μm

1 μm

**B**

Percentage of Mitochondrial Area within Cells

|       | WT  | M  | StDev | Student's t-Test |
|-------|-----|----|-------|------------------|
| 7%    | 10% |    |       |                  |
| 1%    | 2%  |    |       |                  |
| p=0.02|     |    |       |                  |
Figure 3

Panel A: Western blot analysis showing Cyt. c and 55 kDa cytosolic protein in Wild-type and Mutant samples.

Panel B: Bar graph comparing the relative amount of cytochrome c between Wild-type and Mutant samples. The Mutant sample shows a significantly higher amount of cytochrome c compared to the Wild-type sample (***).
Figure 4

| Fermentation | Respiration |
|--------------|-------------|
| YPGlc plates | YPLac plates |

Wild-type

1. Fermentation (YPGlc plates)
2. Fermentation (YPGlc plates)

Mutant

3. Respiration (YPLac plates)
4. Respiration (YPLac plates)
Figure 5

A

Wild-type  Mutant

Rhodamine

DIC

B

ROS-positive cells [%]

w/o Inhibitor  Myxothiazol  Stigmatellin

Wild-type  Mutant

* *  * *
Figure 6

A

Wild-type
Mutant

Rhodamine

DIC

B

Relative fluorescence units (RFU)

\( \rho^+ \)
\( \rho^0 \)

C

CFU (500)

\( \rho^+ \)
\( \rho^0 \)
Figure 7

A

Wild-type

Counts vs Intensity

87%

13%

Mutant

Counts vs Intensity

77%

23%

B

Cells showing caspase activity [%]

Wild-type

Mutant

*
Supplementary Figure 1

A

Wild-type

Mutant

B

Apoptotic cells [%]

Wild-type

Mutant

***
Crucial mitochondrial impairment upon CDC48 mutation in apoptotic yeast
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