Functional Mitochondria Are Required for α-Synuclein
Toxicity in Aging Yeast

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α-Synuclein is one of the principal toxic triggers of Parkinson disease, an age-associated neurodegenerative disease. Using old yeast as a model of α-synuclein expression in post-mitotic cells, we show that α-synuclein toxicity depends on chronological aging and results in apoptosis as well as necrosis. Neither disruption of key components of the unfolded protein response nor deletion of proapoptotic key players (including the yeast caspase Yca1p, the apoptosis-inducing factor Aif1p, or the serine protease Omi) did prevent α-synuclein-induced cell killing. However, abrogation of mitochondrial DNA (rho0) inhibited α-synuclein-induced reactive oxygen species formation and subsequent apoptotic cell death. Thus, introducing an aging yeast model of α-synuclein toxicity, we demonstrate a strict requirement of functional mitochondria.

Parkinson disease (PD) is the second most frequent age-associated neurodegenerative disease. Several observations suggested malfunctioning of the protein α-synuclein to be a toxic trigger of the neurodegenerative process during PD (1). Fibrillary α-synuclein constitutes the major protein component of Lewy bodies in the brain of sporadic PD patients. Furthermore, three missense mutations (A53T, A30P, and E46K) in the α-synuclein gene are linked to early onset dominant familial PD (2–4). Recently, overexpression of wild type α-synuclein due to gene duplication or triplication was found to be sufficient to cause a familial form of PD (5). In mammalian cell culture, α-synuclein has been reported to have polymorphic, dopamine-dependent neurotoxic effects as well as neuroprotective and antiapoptotic functions, depending on the specific type of neurons or mutants (6, 7). The biochemistry and pathogenicity of α-synuclein has been studied extensively in non-human model organisms including flies, nematodes, and transgenic mice, suggesting a role for α-synuclein in lipid and vesicle dynamics, proteasomal dysfunction, or oxidative stress (8–10). Still, the downstream events or cell death executors required for α-synuclein-mediated death remain elusive.

Most recently, the yeast Saccharomyces cerevisiae has been shown to succumb to human α-synuclein expression. Using this new model, a conserved link between endoplasmic reticulum-Golgi traffic and α-synuclein toxicity has been established (11, 12). In addition, yeast is increasingly recognized as a model organism for the study of apoptosis and necrosis (13, 14) as the basic molecular machinery executing cell death is phylogenetically conserved. Orthologues of caspases (Yca1p), the apoptosis-inducing factor (Aif1p), the serine protease Omi (Nma111p), and endonuclease G (Nuc1p) have been described (15–18). In addition, yeast apoptotic death occurs in dependence of complex apoptotic scenarios such as mitochondrial fragmentation (19), cytochrome c release (20), cytoskeletal pertubations (21), and histone H2B phosphorylation (22). Finally, chronologically aged yeast cells are currently used as a valuable model to study oxidative damage and mitochondrial apoptosis in mammalian cells, and recent studies have demonstrated that aged yeast cells die exhibiting an apoptotic phenotype (23–25).

In this work, we show that both chronological aging and α-synuclein toxicity are strictly dependent on functional mitochondria. Using this new model, we demonstrate that mitochondria function is required for the deleterious consequences of α-synuclein expression.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—Experiments were carried out in BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) and respective null mutants, obtained from Euroscarf. All
strains were grown on SC medium containing 0.17% yeast nitrogen base (BD Diagnostics), 0.5% (NH₄)₂SO₄, and 30 mg/liter of all amino acids (except 80 mg/liter histidine and 200 mg/liter leucine), 30 mg/liter adenine, and 320 mg/liter uracil with 2% glucose or 2% galactose for induction of expression of α-synuclein-FLAG constructs. Induction of α-synuclein expression in rho⁰ strains was achieved using SC medium containing 1.5% galactose and 0.5% glucose since BY4741 rho⁰ cells are unable to grow on pure galactose medium. To construct WT-αSyn and A⁵³T constructs, inserts were amplified by PCR with primers 3'-ATC TAC TAG TAT GGA TGT ATT CAT GAA AGG ACT TTC-5' and 3'-ATC TAT CGA TGT GGC TTC AGG TTC GTA GTC TTG-5', using previously described YEp181-synuclein or YEp181-A⁵³T as template (26), cut with SpeI and ClaI and ligated into pESC-His (Stratagene). Fluorescence microscopy to detect protein aggregation was performed using previously published pUG23-WT-αSynEGFP and pUG23-A⁵³TEGFP constructs (26). For abrogation of the mtDNA (rho⁰), BY4741 wild type cells were grown in full medium containing 10 μg/ml ethidium bromide for 3 days. The resulting respiratory deficiency was confirmed by complete lack of growth on obligatory respiratory medium (glycerol).

Survival Plating and Test for Apoptotic Markers—Chronological aging experiments were performed as described (16, 24), and representative aging experiments are shown. Notably, at least three different clones were tested for the survival plating to rule out clonogenic variation of the observed effects. Dihydroethidium (DHE) staining, annexin V/propidium iodide (PI) costaining, and TUNEL staining were performed and quantified using FACSAria or a fluorescence reader as described (15). To calculate p values, a one-way analysis of variance followed by a post hoc test (Bonferroni) was used.

Immunoblotting—Preparation of cell extracts and immunoblotting were performed as described (16). Blots were probed with murine monoclonal antibodies against FLAG-epitope (Sigma), murine monoclonal antibodies against glyceraldehyde-3-phosphate dehydrogenase (Sigma), and the respective peroxidase-conjugated affinity-purified secondary antibody (Sigma).

FIGURE 1. Age-dependent α-synuclein-mediated death is accompanied by phenotypic manifestations of apoptosis and necrosis. A, survival determined by clonogenicity of WT cells expressing human α-synuclein (WT-αSyn) or the point mutant A⁵³T or harboring the corresponding empty vector during chronological aging on 2% galactose synthetic media. A representative aging experiment is shown, with data representing mean ± S.E. of four independent experiments performed at the same time. CFU, colony-forming unit. 8, quantification (fluorescence reader) of ROS accumulation using DHE staining at days 1, 3, and 5 of the aging experiment shown in A. Data represent mean ± S.E. of four independent experiments. In each experiment, 5 × 10⁶ cells were evaluated. **, p < 0.01, ***, p < 0.001. RFU, relative fluorescence units. C, quantification (FACS analysis) of DNA fragmentation using TUNEL staining and of phosphatidylinositol externalization and loss of membrane integrity using annexin V/PI costaining at day 3 of the aging experiment shown in A. In every experiment, 30,000 cells were evaluated. DIC, differential interference contrast. D, fluorescence microscopy of DHE, TUNEL, and annexin V/PI staining of wild type cells expressing WT-αSyn or A⁵³T or harboring the empty vector at day 3 of aging.
RESULTS

Age-dependent \( \alpha \)-Synuclein-mediated Death Is Accompanied by Phenotypic Manifestations of Apoptosis and Necrosis—Although most neurodegenerative disorders only manifest well beyond adulthood, the relationship between cellular aging and eventual cellular demise has not been elucidated. To approach this question, we investigated toxicity mediated by human \( \alpha \)-synuclein in the context of yeast chronological aging, an established model for regulation of aging in postmitotic mammalian cells (23, 24). Expression of wild type \( \alpha \)-synuclein (WT-\( \alpha \)Syn) and the hereditary toxic point mutant A53T under the control of an inducible galactose promoter accelerated chronological aging of BY4741 wild type yeast. After 3 days of aging, cells expressing WT-\( \alpha \)Syn or A53T exhibited only \(~ 20\%\) survival compared with \(~ 70\%\) of the cells harboring the empty vector, as determined by clonogenicity (Fig. 1A).

We next investigated whether \( \alpha \)-synuclein-mediated death of aged yeast cells is of apoptotic nature. To quantify the generation of reactive oxygen species (ROS), the superoxide-driven conversion of non-fluorescent DHE into fluorescent ethidium was assessed in an automated assay. The prolonged expression of WT-\( \alpha \)Syn or A53T caused massive generation of ROS (Fig. 1, B and D). Moreover, the excessive death of cells expressing WT-\( \alpha \)Syn or A53T was accompanied by a major increase (3-fold) in apoptotic DNA fragmentation as detectable by TUNEL staining (Fig. 1, C and D). The simultaneous detection of phosphatidylserine externalization and loss of membrane
integrity with fluorescein isothiocyanate-conjugated annexin V and PI, respectively, discriminates between early apoptosis (annexin V<sup>+</sup>/PI<sup>−</sup>), late apoptosis eventually leading to secondary necrosis (annexin V<sup>+</sup>/PI<sup>+</sup>), and primary necrosis (PI<sup>+</sup>). As shown in Fig. 1C, expression of WT-αSyn or A53T stimulated the apoptotic externalization of phosphatidylserine and simultaneously led to an increase in cells only positive for PI indicative of necrosis (Fig. 1, C and D). Similar results were obtained using the wild type W303 transformed with constructs allowing expression of α-synuclein from the constitutive TPI1 promoter (data not shown). We conclude that α-synuclein triggers both apoptosis and necrosis during chronological aging of yeast.

Under chosen conditions (high expression levels due to 2% galactose), WT-αSyn and the point mutant A53T exhibited similar toxicity (Fig. 1). However, at lower expression levels (using 0.5% galactose), A53T exacerbated WT-αSyn toxicity when survival and ROS accumulation was monitored at early time points after induction of expression. Fig. 2A shows that A53T killed a significantly higher portion of the cells when compared with WT-αSyn (24, 36, and 48 h after promoter induction). Consistently, the point mutation A53T enhanced α-synuclein-induced ROS production (Fig. 2B). Western blot analysis was performed to ensure similar expression levels of WT-αSyn and A53T on 0.5% galactose (Fig. 2C). As previously shown (12), WT-αSyn as well as A53T are associated with the plasma membrane and form cytoplasmatic inclusions (Fig. 2D). Thus, in aging yeast, α-synuclein expression leads to cellular toxicity, which is enhanced by introduction of the hereditary point mutation A53T.

α-Synuclein-mediated Death of Aged Cells Does Not Depend on the Apoptotic Key Players Yca1p, Aif1p, or OMI (Nma111p)—Recent studies indicated that the mammalian apoptotic serine-protease OMI (also called HtrA2) and the yeast caspase Yca1p might be involved in α-synuclein-mediated toxicity (27, 28). Therefore, we comparatively analyzed α-synuclein-mediated cell killing during chronological aging in yeast cells lacking YCA1, NMA111, or the mitochondrial apoptosis-inducing factor AIF1. Deletion of YCA1 had no effect on cell survival upon WT-αSyn expression during aging (Fig. 3A). Consistently, α-synuclein-induced DHE-detectable ROS accumulation during chronological aging was not affected by the absence of YCA1 (Fig. 3D). These results were confirmed using another ROS-sensitive probe, dihydrothorhadine (data not shown). Cytofluorometric quantification of DNA fragmentation
\(\alpha\)-Synuclein Kills Aged Yeast

A

B

C

D

E

AnnexinV

AnnexinV/PI

PI

CFU/500

days in culture

CFU/500

days in culture

time after induction of expression

CFU/500

time after induction of expression

stained cells (%)
(TUNEL) and phosphatidylserine externalization and/or membrane permeabilization (annexin V/PI costaining) further confirmed that Yca1p did not influence the α-synuclein-facilitated cell killing (data not shown). Furthermore, neither deletion of AIP1 nor deletion of NMA111 reduced α-synuclein-mediated death (Fig. 3, B and C) or ROS production during chronological aging (Fig. 3D). Similar expression levels of α-synuclein were confirmed in all knock-out strains using Western blot analysis (Fig. 3E).

Autophagy has been implicated in cellular aging and α-synuclein toxicity during PD (29). However, survival of cells expressing WT-αSyn was not affected by disruption of the essential macroautophagy genes ATG5 or ATG6 (data not shown). Thus, we conclude that yeast death induced by α-synuclein is independent from autophagy.

Functional Mitochondria Are Essential for α-Synuclein Toxicity—Oxidative processes mediated by enhanced ROS production are prominent traits of apoptosis and organismal aging throughout phylogeny. ROS are generated mainly from two sources: the mitochondrial respiratory chain and the unfolded protein response (UPR) machinery. The accumulation of misfolded proteins within the endoplasmic reticulum leads to prolonged activation of the UPR, which in turn causes oxidative stress and finally cell death in yeast (30). To test whether α-synuclein-mediated death depends on the UPR-activated cell death pathway, α-synuclein was expressed in the deletion mutants Δire1 and Δhac1, two key players of the UPR. Ire1p initiates the UPR by regulating the synthesis of the transcription factor Hac1p (31). Neither deletion of IRE1 nor deletion of HAC1 affected α-synuclein-mediated toxicity (supplemental Fig. S1), suggesting a pathway in which UPR signaling via Ire1p and Hac1p does not contribute to the loss of viability induced by α-synuclein. As mammalian and yeast apoptosis are under mitochondrial control (17, 19, 20, 32, 33), we next investigated the impact of mitochondrial function on α-synuclein-triggered cell death. Therefore, we generated cells that lack mitochondrial DNA (ρ0) and thus are incapable of oxidative phosphorylation. Contrasting with its marked toxicity on wild type (ρ+) cells, enforced expression of WT-αSyn or A53T failed to provoke the death of ρ0 cultures during chronological aging (Fig. 4, A and B), although ρ0 and wild type cells expressed similar levels of α-synuclein (data not shown). It should be noted that the lack of respiratory function via abrogation of mtDNA compromised overall survival during aging, presumably because these cells cannot switch from fermentation to respiration during the diauxic shift. Thus, we decided to closely monitor the kinetics of early α-synuclein-mediated death in wild type and ρ0 cells. WT-αSyn and A53T expression led to massive cell killing in the wild type at early time points (16–48 h of aging), whereas death was completely inhibited in ρ0 cells (Fig. 4C). Concomitantly, the deletion of mtDNA completely inhibited α-synuclein-induced ROS-generation (as determined by DHE staining, Fig. 4D) and phosphatidylserine externalization (Fig. 4E). Remarkably, at early time points of aging, death caused by WT-αSyn or A53T expression is predominantly due to apoptosis since cells only positive for PI did not increase within the first 48 h (Fig. 4E). Thus, abrogation of mtDNA, and therefore respiratory function, suppresses the apoptotic component of α-synuclein-mediated yeast cell killing.

Interestingly, upon deletion of the mtDNA, and therefore prevention of the toxic function of α-synuclein, expression of WT-αSyn but not of A53T even suppressed the onset of apoptotic markers. At early time points, WT-αSyn expressed in ρ0 cells significantly reduced the accumulation of ROS when compared with the corresponding vector control (p = 0.024 at 24 h) (Fig. 4D). Additionally, the externalization of phosphatidylserine and the loss of membrane integrity was diminished upon expression of WT-αSyn but not of A53T (Fig. 4E). These data are consistent with previous findings in mammalian cells showing that α-synuclein can have antiapoptotic functions (6, 7). In yeast, this protective effect becomes apparent only after abrogation of the toxic function via deletion of mtDNA and is absent in the A53T point mutant.

DISCUSSION

In this study, we investigated α-synuclein-mediated toxicity during chronological aging of yeast cells using clonogenic assays combined with the assessment of cell death markers. Our data indicate that expression of WT-αSyn and the early onset PD-associated point mutant A53T dramatically reduces the survival of aging yeast cells due to a marked increase in ROS generation and the induction of apoptotic and necrotic cell death. These data extend previous observations made in yeast showing that α-synuclein-induced toxicity is related to the ability of α-synuclein to interact with the plasma membrane and to form inclusions (12, 26). In contrast to earlier findings using exponentially growing yeast cells expressing α-synuclein (27), our results indicate that α-synuclein toxicity does not depend on the metacaspase Yca1p. Recent observations in mammalian cellular models and histopathological specimens from PD patients indicate enhanced UPR activity to be a primary cause for the loss of viability (34) and the apoptotic serine protease Omi/HtrA2 to be an executor of α-synuclein-mediated cell death (28). However, in yeast, neither key components of the UPR nor Omi (Nma111p) or the apoptosis-inducing factor Aip1p seem to impart α-synuclein toxicity. Deregulation of the N-methyl-D-aspartic acid subtype glutamate receptor, malfunctioning of the ATP-sensitive potassium channels (35), or dopamine metabolites leading to oxidative damage (36) have been suggested to

![Image](228x26 to 255x38)
account for selective dopaminergic degeneration in PD. However, in yeast, α-synuclein must rely on other, more general mechanisms, for the simple reason that yeast lacks analogues of neuron-specific ion channels or neurotransmitters.

Oxidative stress and mitochondrial dysfunction have long been recognized as key factors that operate in the pathogenesis of PD (9). A significant reduction of cardiolipin in mitochondrial membranes concomitant with a drop in complex II and complex III activity was noticed in α-synuclein knock-out mice (37). The recent discovery of the mitochondria-associated proteins Parkin, DJ-1, and PINK1 has further strengthened the importance of mitochondrial functions and placed apoptosis to the forefront of PD biology (38, 39). In this line, our studies identified mitochondrial function and oxidative phosphorylation as central players in α-synuclein-mediated apoptosis and necrosis of yeast cells since deletion of mtDNA completely abrogates the toxic function of WT-α-Syn and A53T. It should be noted that survival of rho0 cells is compromised during aging, especially at later time points (since these cells cannot switch from fermentation to respiration during the diauxic shift). Nonetheless, under conditions where deletion of mtDNA itself does not sensitize cells to apoptosis (early time shift). Nonetheless, under conditions where deletion of mtDNA itself does not sensitize cells to apoptosis (early time points during aging, Fig. 4, C and D), it still clearly protects cells against death mediated by α-synuclein expression. Moreover, under these conditions, a protective function of WT-α-Syn on apoptotic and necrotic markers is observable. Once more, these studies show the potential offered by yeast models for defining novel fundamental mechanisms and factors involved in the pathogenesis of PD.

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