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Expression of an expanded polyglutamine domain in yeast causes death with apoptotic markers

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Abstract

Huntington's disease is caused by specific mutations in huntingtin protein. Expansion of a polyglutamine (polyQ) repeat of huntingtin leads to protein aggregation in neurons followed by cell death with apoptotic markers. The connection between the aggregation and the degeneration of neurons is poorly understood. Here, we show that the physiological consequences of expanded polyQ domain expression in yeast are similar to those in neurons. In particular, expression of expanded polyQ in yeast causes apoptotic changes in mitochondria, caspase activation, nuclear DNA fragmentation and death. Similar to neurons, at the late stages of expression the expanded polyQ accumulates in the nuclei and seems to affect the cell cycle of yeast. Interestingly, nuclear localization of the aggregates is dependent on functional caspase Yca1. We speculate that the aggregates in the nuclei disturb the cell cycle and thus contribute to the development of the cell death process in both systems. Our data show that expression of the polyQ construct in yeast can be used to model patho-physiological effects of polyQ expansion in neurons.

Keywords: Huntingtin; Polyglutamine; Yeast; Mitochondria; Apoptosis

1. Introduction

There are numerous studies showing that the budding yeast *S. cerevisiae* is a useful model of the molecular mechanism of aging (reviewed in [1,2]). Yeast appear to be a useful model to study age-related degeneration of post-mitotic cells (see [3] for review). The most popular model for this is a stationary culture of starving yeast cells ([4,5]). It has been shown that, among other treatments (reviewed in [6]), incubation of yeast in stationary cultures induces apoptosis [4]. Moreover, yeast were successfully used to model death of terminally differentiated neurons during various types of pathologies (see [3] for review). In particular, the yeast system provided certain insights into Parkinson disease [7,8] . While it has been shown that mutant forms of synuclein may form inclusion bodies (IB) and cause apoptotic death of neurons in humans (reviewed in [9]), the mechanism of the disease development is largely unknown. To study the mechanism, the mutant forms of the protein were expressed in yeast and inclusion body (IB) formation followed by cell death were observed. Importantly, similar to neuronal death, the death of yeast was accompanied by the appearance of apoptotic markers [10].

Another example of a yeast-based model of neuronal pathology is expression of expanded poluglutamine (polyQ) fragment of huntingtin (Htt1). The mutant form of Htt1 causes hereditary neuronal degeneration in humans [11]. The degeneration is accompanied by polyQ-mediated aggregation of Htt1 and accumulation of apoptotic markers in the affected neurons (see [12,13] for reviews). When expressed in yeast, the polyQ-fragment also forms IBs and appears to be toxic for cells [14,15]. While yeast factors controlling aggregation of the polyQ fragment were extensively studied [14–17], it is not clear whether the polyQ-triggered cell death pathways are similar for both yeast and humans. Here, we show that, similar to neurons [18,19], in yeast

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expanded polyQ aggregates accumulate in the nucleus and induce mitochondrial fragmentation, caspase activation and cell death.

2. Materials and methods

Saccharomyces cerevisiae strains used in this study are derivatives of W303, Mat *a*. The original strain was transformed with either 25Q-CFP- or 103Q-CFP-expressing integrating constructs described in [14]. For polyQ expression, cells were pre-grown at 30 °C in YP-Raf (yeast extract with peptone [20], supplemented with 2% raffinose) and then transferred into YP-Raf/Gal (YP-Raf supplemented with 2% galactose). Deletion of *YCA1* was performed by replacing the gene with the deletion construct amplified from the EUROSCARF yeast strain collection. GAL-Cdc20 construct was described in [21].

Visualization of DNA, mitochondria and ROS were performed as described in [22], Caspase visualization was performed as described in [23]. FACS analysis and propidium iodide (PI) staining were performed as described in [24]. Alpha-tocopherol was used at 30 μM concentration.

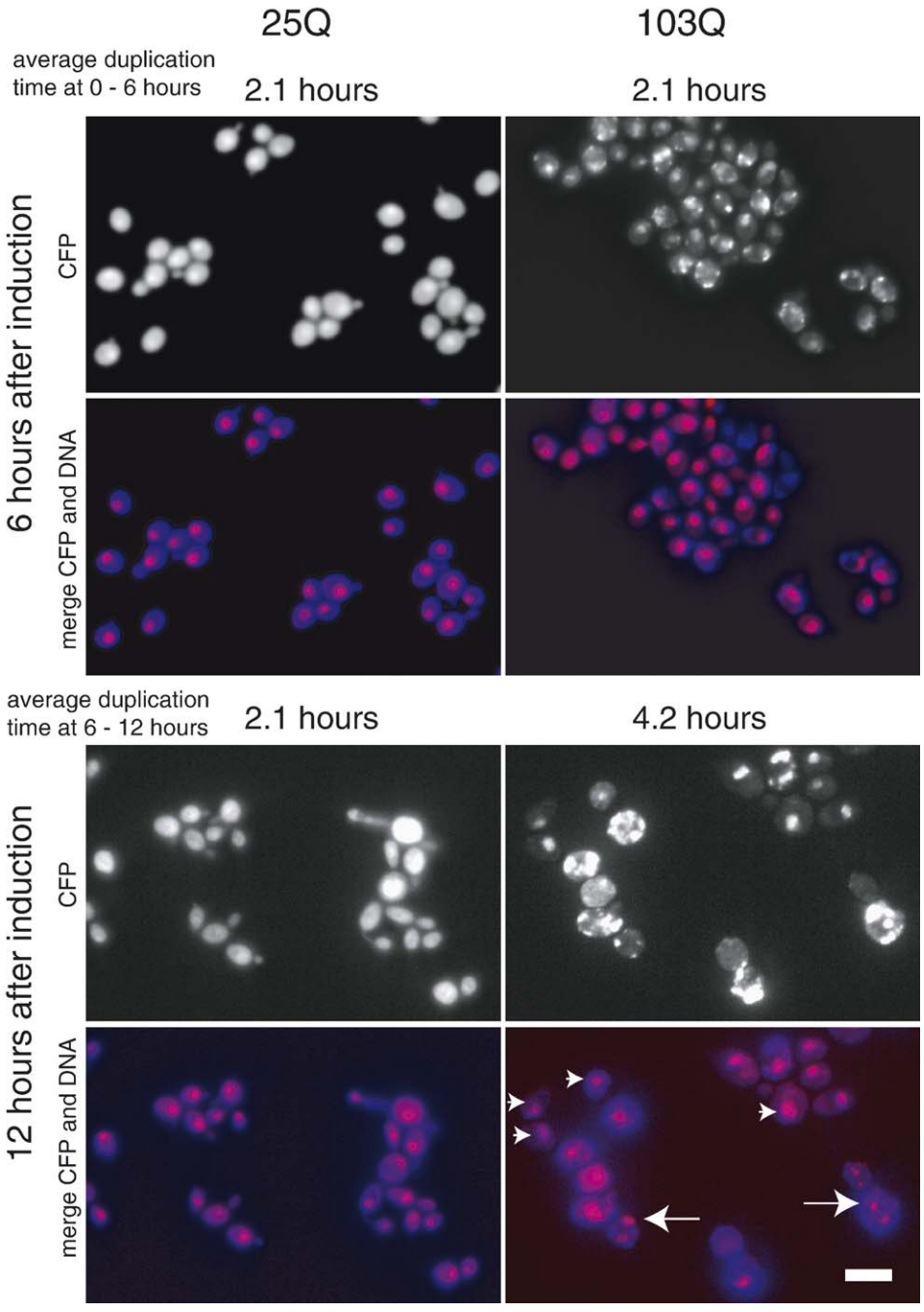

Fig. 1. Toxic effect of the expanded polyQ expression correlates with its accumulation in the nuclei. Liquid cultures expressing either 25Q-CFP or 103Q-CFP for 6 and 12 h were briefly fixed with ethanol and stained with Hoecst 33258 dye (1 μg/ml) to reveal DNA. During the 0–6 h time period, the generation times are the same for both types of cells and both constructs are present in the cytosole. However, during the 6–12 h time period, the average generation time of 103Q-expressing cells doubles its starting value and CFP can be seen in the nuclei (arrowheads). A small but significant percentage of cells expressing 103Q for 12 h show fragmented staining of DNA (arrows). Scale bar — 10 μm.

Colocalization of CFP and DNA (Hoecst 33258) fluorescence was quantified using Colocalization Finder plug-in of ImageJ program. Percentage of colocalization was calculated as the number of the colocalized pixels normalized to Hoecst 33258 self-colocalization. At least 50 cells were analyzed for each strain. Error bars correspond to S.E.M.

3. Results and discussion

In the first series of experiments, we confirmed the data [15,16] on the aggregation and toxicity of a fragment of Htt1 carrying the N-terminal 17-amino-acid stretch followed by expanded polyQ domain (103Q) in yeast. As shown by Fig. 1, the fragment containing normal length polyQ-domain (25Q) accumulates diffusely in the cells. In contrast, 103Q forms distinct aggregates (Fig. 1). Interestingly, while after 6 h of induction these aggregates are found mostly in the cytoplasm, photographs made after 12 h of 103Q induction frequently show distinct nuclear localization of the fragment (Fig. 1, arrow-heads). To estimate the toxicity of the fragment expression, we measured the optical density (OD). The average rates of growth between 0 and 6 h of induction were the same for 25Q- and 103Q-expressing cells, whereas during the time period of 6–12 h the increase in OD of 103Q-expressing cells was 2-fold lower compared to that of 25Q-expressing cells (Fig. 1). Moreover, cultures expressing 103Q for 12 h contain a fraction of abnormally large dumbbell-shaped cells, suggesting a cell cycle delay. Thus the expression of 103Q seems to affect the cell cycle, and its detrimental effect correlates in time with its nuclear localization. Importantly, while normal huntingtin (Htt1) in neurons is localized to the cytoplasm, the form carrying pathologically expanded polyQ domain accumulates in the nucleus (reviewed in [12]).

In the same set of experiments we noticed that a fraction of cells expressing 103Q for 12 h was showing abnormal morphology of nuclear DNA (Fig. 1, arrows). DNA in these cells appears to be fragmented and scattered throughout the cytoplasm. It was shown that, similar to higher model systems, DNA fragmentation (reviewed in [7]) and loss of nuclear envelope integrity [25] are markers of late apoptotic events in yeast. To estimate a percentage of cells undergoing DNA cleavage as a consequence of polyQ expression, we performed cellular DNA measurement by FACS. Fig. 2A (top row) shows cells carrying 25Q construct at time points 0, 6 and 12 h after the induction. Two peaks correspond to the cells with unreplicated (before S-phase) and replicated (after S-phase) DNA. While cells expressing 103Q show similar FACS profiles at time points 0 and 6 h, after 12 h following the induction the culture starts to accumulate a significant proportion of cells with DNA content less than 1C (Fig. 2A, arrow). Thus, 103Q expression leads to DNA degradation, suggesting the induction of an apoptotic pathway.

We also noticed that the polyQ expression alters the ratios of 1C and 2C DNA peaks. With the most of the non-expressing cells containing 1C DNA, the polyQ expression leads to the increase of the 2C peak (Fig. 2A). This is consistent with the cell cycle delay phenotype suggested by the presence of large dumbbell cells in 103Q-expressing cultures (Fig. 1). What is the nature of the 103Q-induced alteration of the cell cycle? It is

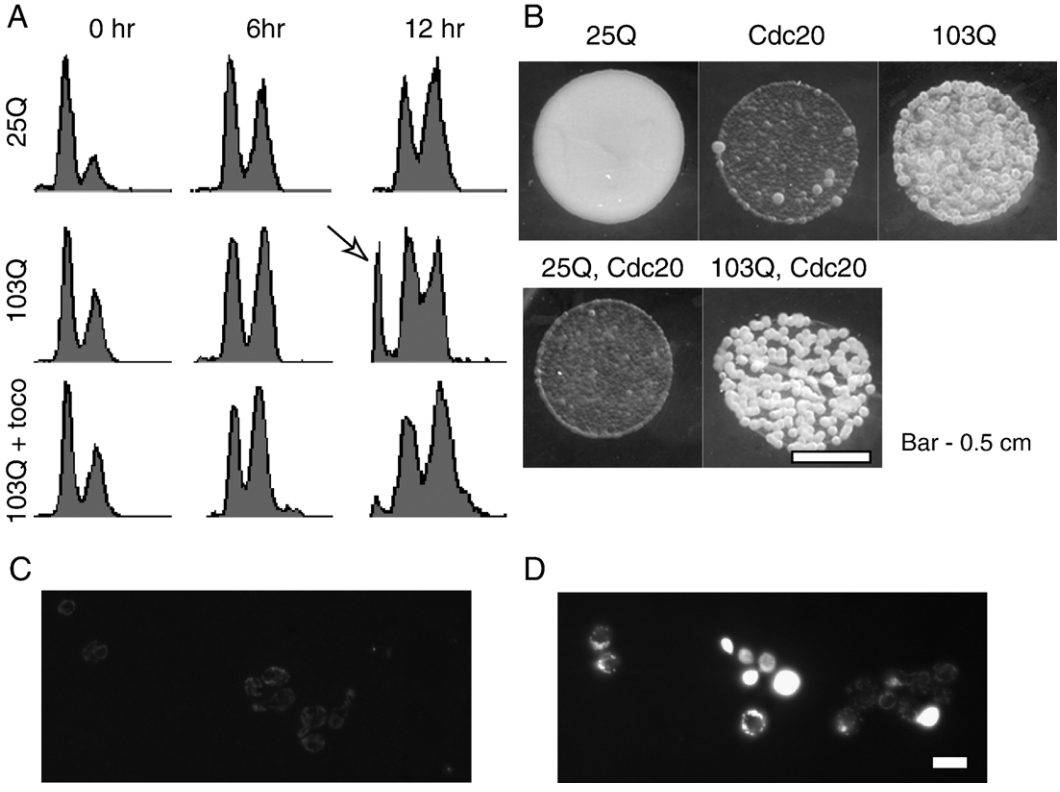

Fig. 2. The length of the polyQ-domain and alpha-tocopherol affect DNA content, cell cycle and mitochondrial morphology in polyQ-expressing cells. FACS profiles (A) show that after 12 h of 103Q (but not 25Q) expression, there is a significant fraction of cells with less than 1C DNA content (arrow). Tocopherol addition prevents the formation of this fraction of the cells (103Q+toco). (B) 103Q alleviates the toxicity of Cdc20 overexpression. Mitochondrial morphologies and Mitotracker Orange staining intensities are different in 25Q- (C) and 103Q-expressing cells (D). Dead cells show strong non-specific Mitotracker staining (D). Scale bar — 10 μm.

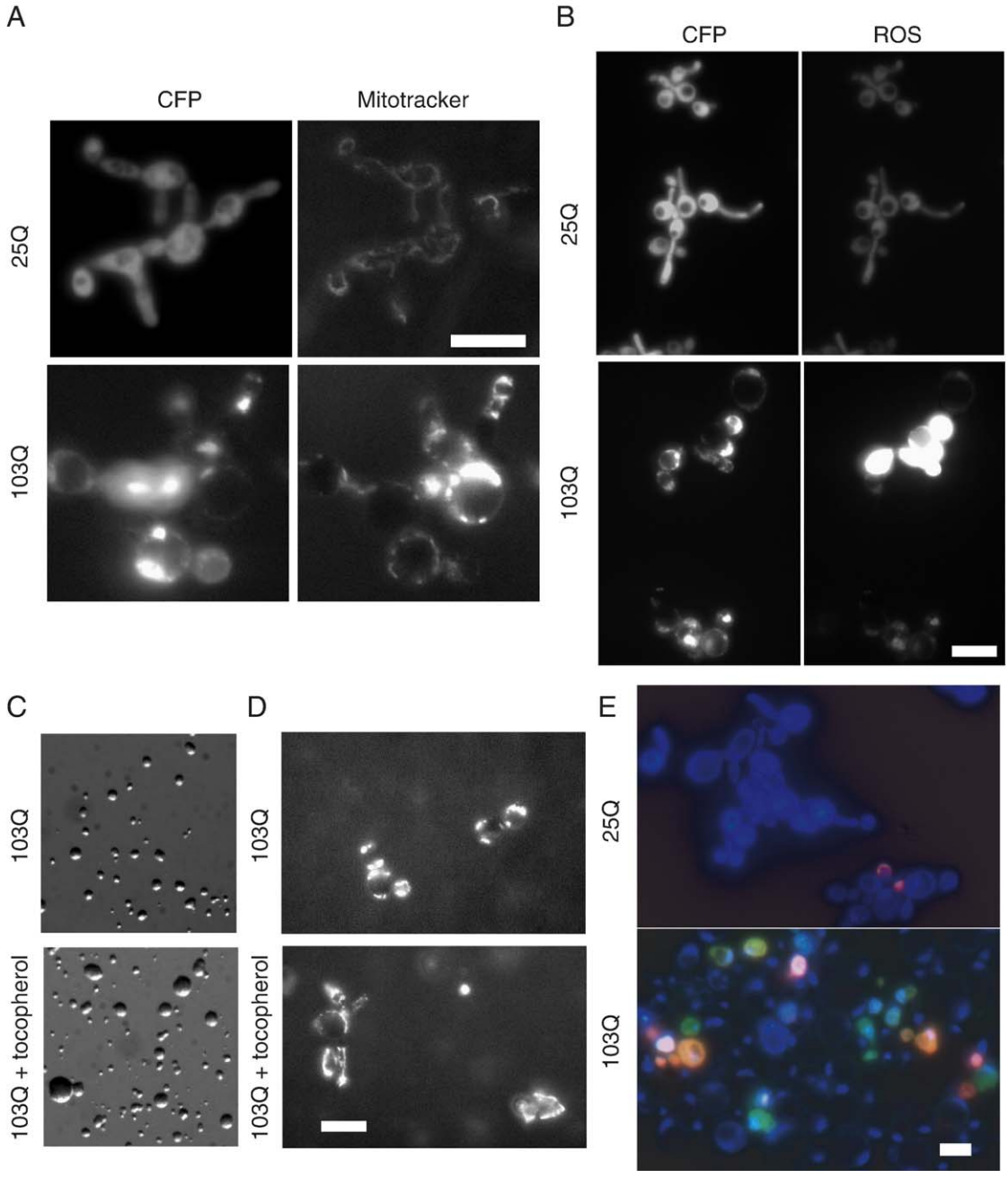

Fig. 3. The polyQ-expressing cells grown on solid media for 24 h display apoptotic markers. (A) Similar to the liquid-grown cultures, 25Q accumulates diffusely in the cytoplasm and mitochondria in these cells are filamentous and relatively weakly stained with Mitotracker; 103Q-expressing cells accumulate 103Q in the nuclei (DNA staining not shown) and frequently contain bright and fragmented mitochondria. (B) While 25Q-expressing cells are ROS-negative, expression of 103Q drives ROS formation in a fraction of the cells. (C, D) Effects of alpha-tocopherol (30 μM) addition on the colony-forming properties of (C) and mitochondrial morphology in (D) the 103Q-expressing cells. (E) Caspase activation in 103Q-expressing cells. Merged-color photographs show PolyQ constructs in blue, PI — in red, FITC-VAD-fmk — in green. A minor fraction of the 25Q-expressing cells accumulate PI, but these cells are FITC-VAD-fmk negative (purple, top panel). Expression of 103Q leads to formation of FITC-VAD-fmk — positive/PI-negative (dark green), FITC-VAD-fmk — positive/PI-positive (orange) and FITC-VAD-fmk — negative/PI-positive cell types (bottom panel). Scale bars — 10 μm.

possible that the 103Q expression inhibits APC (anaphase promoting complex), thus delaying the exit from mitosis. To address this possibility we tested the effect of polyQ expression in cells with elevated APC level. The elevation of APC level was achieved by expression of APC subunit Cdc20 using GAL1 promoter. These cells are unable to grow on YP-Raf/Gal due to tight APC-induced arrest in G1 phase of the cell cycle [21]. Interestingly, unlike the 25Q construct, 103Q expression did allow a minor fraction of the cells to grow and form micro-colonies (Fig. 2B). This result is consistent with the idea that the 103Q expression delays the cell cycle and suggests that the delay is achieved via lowering the activity of APC.

It was previously shown that, similar to the mammalian cells, yeast apoptosis is sometimes accompanied by the sharp transient

increase in mitochondrial membrane potential ([22] and references within). To test whether 103Q expression causes such an increase, we stained the cells that had expressed either 25Q or 103Q for 12 h with the dye Mitotracker Orange. Mitotracker Orange was shown to accumulate in mitochondria, dependent on the membrane potential and was successfully used in yeast to estimate the extent of mitochondrial hyperpolarization [22]. As shown by Fig. 2C and D, mitochondria in 25Q-expressing cells are relatively weakly stained, whereas 103Q-expressing cells tend to accumulate more of the dye in their mitochondria. The morphology of mitochondria also seems to be different for 25Q- and 103Q-expressing cells. While 25Q-expressing cells contain filamentous mitochondria, the brightly stained 103Q-expressing cells accumulate the dye in separate round organelles. As mitochondrial fragmentation was shown to be a hallmark of apoptosis in yeast and higher cells ([26], also see [27] for review), the effect of 103Q on mitochondrial morphology is another indication of the pro-apoptotic action of 103Q construct induction.

Increase of mitochondrial membrane potential can directly cause accumulation of reactive oxygen species (ROS) in apoptotic yeast cells [22]. It is known that ROS are an important intermediate in the progression of an apoptotic cascade ([28], also see [27] for review). To test whether quenching of ROS will affect the detrimental effects of 103Q expression, we added antioxidant alpha-tocopherol to the cells expressing 103Q construct. While the addition had no or very small effect on the rate of the OD increase (data not shown), quenching of ROS inhibited the DNA cleavage as shown by FACS (Fig. 2A, 103Q+toco). Thus, while not relieving the detrimental effect of 103Q on cell growth, alpha-tocopherol seems to inhibit the final stages of cell death in the 103Q-expressing cells. It suggests that in liquid yeast cultures, 103Q does not trigger apoptosis directly but interferes with the normal functioning of the cells upstream of the ROS production. In other words, apoptotic death of 103Q-expressing yeast might be an indirect consequence of its toxic activity. Nuclear localization of 103Q suggests that, similar to mammalian systems, it may disturb the normal function of yeast cells by acting as a transcription-influencing factor.

At the same time, in mammalian cell models of polyQ-mediated diseases it was shown that the cell death is accompanied by accumulation of apoptotic markers [29]. We wanted to see whether, similar to mammalian cells, it is possible to detect an apoptosis-dependent cell death effect of 103Q expression in yeast. To do that, instead of liquid culture we grew yeast cells on solid media. Consistent with [15,16], we found that after 24 h of growth, the colonies formed by 103Q-expressing cells were much smaller compared to the colonies of 25Q-expressing cells (data not shown). Mitotracker Orange staining of yeast cells grown on solid media showed that, similar to liquid culture, 103Q-expressing cells contained hyperpolarized and fragmented mitochondria, while mitochondria of the 25Q-expressing cells were filamentous and relatively weakly stained (Fig. 3A).

Does the increase of the membrane potential drive ROS formation in the cells grown on solid media? To test this we added ROS-sensitive fluorescent dye H_2-DCF-DA to the plate-grown 25Q- and 103Q-expressing cells. While 25Q-expressing cells were ROS-negative, a small (1–5%) and not entirely reproducible amount of 103Q-expressing cells showed diffuse ROS staining (Fig. 3B). Thus, our data suggest that high levels of ROS in 103Q-expressing cells are transient and most likely occur shortly before the cell death. The latter is suggested by highly abnormal (swollen) appearance of the ROS-positive cells (Fig. 3B).

Nevertheless, unlike liquid media, addition of alpha-tocopherol had a noticeable effect on the growth of 103Q-expressing yeast cells (Fig. 3C). To estimate the effect of alpha-tocopherol we plated equal amounts (approximately 1000) of yeast cells on YP-Raf/Gal plates, incubated the plates for 24 h at 37 °C, collected the microcolonies (Fig. 3C) by washing them off the plates in equal volumes and then measured the optical density of the suspensions. The number of the cells estimated in this way was 2.6-fold higher from the alpha-tocopherol-containing plate compared to the control one. To confirm that in this experiment alpha-tocopherol inhibited cell death, we estimated the fractions of the dead cells in the suspensions. To do that we added Propidium Iodide (PI) to the suspensions. The addition of alpha-tocopherol lowered the proportion of the PI-positive cells from 15% to 7.5%.

By staining the plate-grown cells with Mitotracker Orange we noticed that the morphology of mitochondria in plate-grown cells seemed to be affected by alpha-tocopherol addition. Fig. 3D shows that the addition of the antioxidant partly prevented fragmentation of the mitochondrial network.

The major feature of apoptotic death, including apoptosis in neurons, is activation of caspases [29]. Yeast caspase Yca1 has been shown to play an essential role during apoptosis triggered by

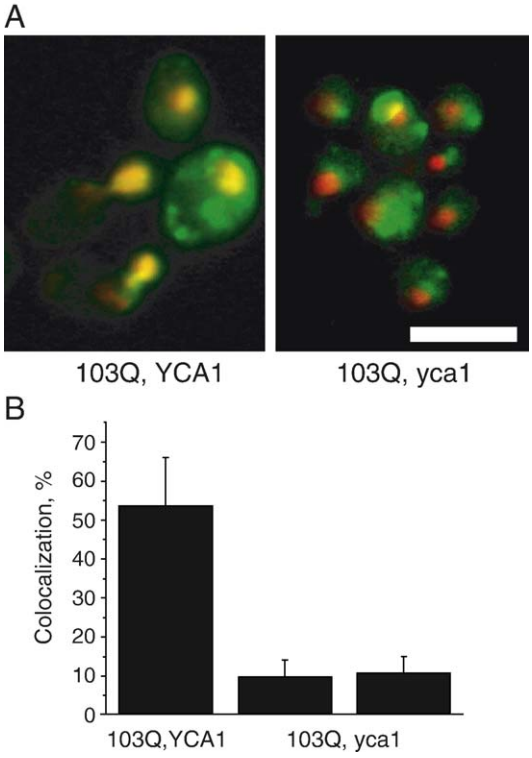

Fig. 4. Deletion of *yca1* affects the intracellular localization of 103Q aggregates. (A) In *yca1*-delta cells, unlike the control *YCA1*, nuclear DNA signal (red channel) does not overlap with 103Q-CFP signal (green channel) Scale bar — 10 μm. (B) Quantification of the colocalization in the control 103Q-expressing strain and in two independent *yca1*-delta 103Q-expressing transformants.

various stimuli [23], in particular in aging-related cell death in the stationary culture [4]. To test whether caspase activity increases during 103Q expression in yeast, we stained the cells grown on solid media with FITC-VAD-fmk — a fluorescent caspase substrate analog. We found that while cultures of 25Q-expressing cells contained a small percentage of dead (PI-positive) cells, they did not show any caspase-positive cells (Fig. 3E and data not shown). On the contrary, 2-day old cultures of 103Q-expressing cells contained large fractions of caspase-positive/PI-negative and caspase-positive/PI-positive cells (Fig. 4E). Thus, 103Q expression leads to caspase activation in yeast.

What is the role of caspase in the polyQ-induced death? To test that we introduced the 103Q construct in yeast caspase *yca1* deletion mutant. We found that the absence of Yca1 did not rescue significantly the detrimental effect of 103Q on the colony growth (data not shown). Surprisingly, the deletion of *yca1* strongly affected the intracellular localization of the aggregates. Fig. 4A shows that the absence of Yca1 prevented the accumulation of the aggregates in the nuclei. Using ImageJ program (see Materials and methods) we estimated the extent of the colocalization in the control 103Q-expressing strain and in two independent *yca1* deletion mutants. Fig. 4B shows that the overlap between 103Q and DNA fluorescent signals was approximately 4 times lower in the deletion mutants. Here it is important to mention that the polyQ-expanded Htt1 accumulates in the nuclei in mammalian cells, and this accumulation is thought to be linked to the proteolytic cleavage of Htt1 (reviewed in [12]). In the case of 103Q expression in yeast, we can rule out the cleavage of the fragment. First, Western blots of 103Q-expressing cells show a single band with a molecular weight corresponding to the full-length construct (M. Sherman, unpublished). Second, while CFP is fused to the C-terminus, the polyQ stretch is at the very N-terminus of the yeast-expressed construct [14]. Thus, unlike mammalian cells, translocation of 103Q to the nuclei of yeast cells seems to be proteolysis-independent. At the same time, in both systems nuclear localization of the aggregates seems to be caspase-dependent. It is possible that the expanded polyQ activates caspase, which in turn leads to the loss of the nuclear permeability barrier.

What are the functional consequences of the nuclear translocation of the expanded polyQ-containing fragment? In mammalian cells polyQ was shown to bind CBP [30] — a transcription factor. It was suggested that the IBs trigger neuronal degeneration by sequestering CBP [30]. Recently, it was shown that CBP is a direct binding partner of APC [31]. Thus, it is possible that the toxic effect of the expanded polyQ in yeast and neurons are in part due to the disruption of the cell cycle-regulating machinery. Indeed, while neurons are post-mitotic cells, aging- and disease-related degeneration of neurons is often accompanied by abortive cell cycle initiation (see [32,33] for review). In particular, it was shown that during Huntington's disease neurons start to accumulate cyclin B — an indicator of cell cycle activation [32].

As for yeast, it was shown that deletion of the S-phase checkpoint gene significantly increased apoptotic cell death in the stationary culture [34,35]. Interestingly, 103Q-expressing cells seem to have a delay in the S-phase: after 12 h of expression the peaks of the cells containing 1C and 2C DNA are not as clearly separated as in case of 25Q-expressing cells (Fig. 2A).

To conclude, taken together with the previous observations, our data allow to speculate that in both yeast and neurons the expanded polyQ protein molecules aggregate in the nucleus, causing cell cycle defects. These defects contribute to the pleiotropic toxic effects of the expanded polyQ expression and induce cell death, accompanied by a set of apoptosis-linked changes in mitochondria, caspase activation and DNA cleavage. We suggest that a yeast-based system of the polyQ expression is an appropriate model to study neuronal degeneration triggered by aggregation of proteins with expanded polyQ domains.

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