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Yeast caspase 1 links messenger RNA stability to apoptosis in yeast

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During the past years, yeasts have been successfully established as models to study the mechanisms of apoptotic regulation. We recently showed that mutations in the *LSM4* gene, which is involved in messenger RNA decapping, lead to increased mRNA stability and apoptosis in yeast. Here, we show that mitochondrial function and *YCA1*, which encodes a budding yeast metacaspase, are necessary for apoptosis triggered by stabilization of mRNAs. Deletion of *YCA1* in yeast cells mutated in the *LSM4* gene prevents mitochondrial fragmentation and rapid cell death during chronological ageing of the culture, diminishes reactive oxygen species accumulation and DNA breakage, and increases resistance to H_2O_2 and acetic acid. mRNA levels in *lsm4* mutants deleted for *YCA1* are still increased, positioning the Yca1 budding yeast caspase as a downstream executor of cell death induced by mRNA perturbations. In addition, we show that mitochondrial function is necessary for fast death during chronological ageing, as well as in *LSM4* mutated and wild-type cells.

Keywords: *LSM4*; mitochondria; programmed cell death; *Saccharomyces cerevisiae*; *YCA1*

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INTRODUCTION

Apoptosis is a form of cellular suicide, particularly important for medical research, because misregulation of apoptosis can result in many human diseases (Steller, 1995). Recent studies have established yeasts as models to study the mechanisms of apoptotic regulation. In the yeast *Saccharomyces cerevisiae*, we detected cell death, with typical markers of apoptosis, such as DNA fragmentation, phosphatidylserine externalization and chromatin condensation, in a strain carrying a mutation in the AAA-ATPase gene *CDC48* (Madeo *et al*, 1997). Its mammalian orthologue VCP was subsequently linked to the regulation of apoptosis (Shirogane *et al*, 1999). In addition, several other pathways crucial for mammalian apoptosis are conserved in yeast; for example, a newly discovered proteasomal apoptotic pathway leading to the destruction of Cdc6 (Blanchard *et al*, 2002). Exposure to low doses of H_2O_2 or acetic acid, which are known to increase reactive oxygen species (ROS) production, induces apoptosis in wild-type yeast cells, indicating that, as in metazoans, ROS are key regulators of yeast apoptosis (Madeo *et al*, 1999; Ludovico *et al*, 2001).

RNA metabolism and turnover is an important regulator of mammalian apoptotic events (Bellacosa & Moss, 2003). In a previous study, we showed that a truncated form of the essential gene *LSM4*, a component of a complex promoting pre-messenger RNA splicing and mRNA decapping (Cooper *et al*, 1995; He & Parker, 2000), still supports growth in an Lsm4 devoid strain (Mazzoni & Falcone, 2001). In the stationary phase, expression of *Kllsm4Δ1*, the truncated form of *LSM4*, leads to an accumulation of mRNAs and the phenotypic markers of apoptosis. We also showed that other mutations in the yeast mRNA-decapping pathway, such as those in the *LSM1*, *LSM6*, *DCP1* and *DCP2* genes, show increased mRNA stability and undergo apoptosis (Mazzoni *et al*, 2003b). Control of mRNA stability has been described as an important checkpoint during apoptosis, and, in addition, it has been reported that Lsm proteins are involved in autoimmune diseases and cancer (Schweinfest *et al*, 1997; Stinton *et al*, 2004). However, a mechanistic link between mRNA stability and apoptosis has not yet been described either in yeasts or in mammals.

It was previously shown that chronological ageing leads to apoptosis in yeast (Herker *et al*, 2004) and that a caspase-like protease (Yca1) mediates cell death triggered by oxygen stress, salt stress or chronological ageing (Madeo *et al*, 2002; Wadskog *et al*, 2004). Here, we show that the yeast caspase Yca1 is also required for apoptosis induced by increased mRNA stability.

The disruption of *YCA1* does not affect mRNA stability but rescues cells from apoptotic death induced by mutation of *LSM4*, a master regulator of mRNA degradation.

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RESULTS AND DISCUSSION

Apoptosis in the *Kllsm4Δ1* mutant requires Yca1

Wild-type yeast cells lose viability during long-term cultivation (Longo *et al*, 1997), a process that is accompanied by apoptotic markers (Herker *et al*, 2004). As shown in Fig 1A, a mutant in the mRNA-decapping protein Lsm4 (Kllsm4Δ1) shows a much higher and more rapid loss of viability than the wild-type strain.

To investigate whether the loss of viability observed in the mutant was mediated by caspases, which are prominent cell executioners in mammalian cells, we assessed the cell viability of a *Kllsm4Δ1/yca1Δ* double-mutant strain.

Deletion of the yeast caspase gene *YCA1* reduced the marked loss of viability observed in the *Kllsm4Δ1* mutant (Fig 1A). The deletion of *YCA1* by itself also increased the fraction of viable cells compared with the wild type, similar to results of a previous study that used strains in a different genetic background (Madeo *et al*, 2002). The absence of the caspase only delays but does not completely prevent cell death in the *Kllsm4Δ1* mutant, indicating that alternative pathways are also involved in this process. The introduction of the *YCA1* and the *KlLSM4* genes into the *Kllsm4Δ1/yca1Δ* double mutant led to cell viability levels similar to those observed for the *Kllsm4Δ1* and *yca1Δ* mutants, respectively (Fig 1B).

Although mitochondria are required for induction of apoptosis in many death scenarios observed in mammalian cells (Danial & Korsmeyer, 2004), it is not yet known whether the apoptotic phenotypes induced by mRNA perturbations in either yeasts or mammals depend on mitochondrial function. To address this question, we created a series of isogenic *rho*$^{\circ}$ strains and measured cell viability during the growth and stationary phases (Fig 1C). We observed that, as in *rho*$^{+}$ cells, the loss of viability was much more pronounced in the *Kllsm4Δ1* mutant and that the deletion of *YCA1* suppressed this phenotype. Moreover, *rho*$^{\circ}$ strains showed significantly slower kinetics of death, probably reflecting a reduced production of stress factors.

Phenotypic markers of apoptosis include chromatin condensation, fragmentation and DNA breakage. To visualize chromatin structure by 4,6-diamidino-2-phenylindole (DAPI) staining, we used *rho*$^{\circ}$ derivative strains to eliminate the mitochondrial DNA background fluorescence. As shown in Fig 2A (second column from the left), wild-type cells had a normal nuclear morphology, whereas, in the *Kllsm4Δ1* mutant, nuclei appeared fragmented and DNA condensed, phenotypes that are much less pronounced in the double mutant. The absence of Yca1 in the *Kllsm4Δ1* mutant also had an effect on cell morphology (Fig 2A, first column to the left). Cells of the double mutant appeared almost normal in shape and size compared with the abnormal morphology of the *Kllsm4Δ1* mutant. A similar aberrant cell morphology has also been observed in apoptotic *cdc48* mutant strains (Madeo *et al*, 1997).

Another hallmark of apoptosis is DNA breakage, which can be detected by the TdT-mediated dUTP nick end labelling (TUNEL) assay (Gavrieli *et al*, 1992; Gorczyca *et al*, 1993). We previously reported that *Kllsm4Δ1* cells contain a high percentage of free 3′-OH ends generated by fragmentation of chromosomes (Mazzoni *et al*, 2003a,b). As shown in Fig 2A (third column from the left), *Kllsm4Δ1* cells showed DNA fragmentation that was almost completely suppressed in cells with the *YCA1* gene deleted.

Another key event in triggering apoptosis in yeast is the production of ROS, which accumulate in senescent cells

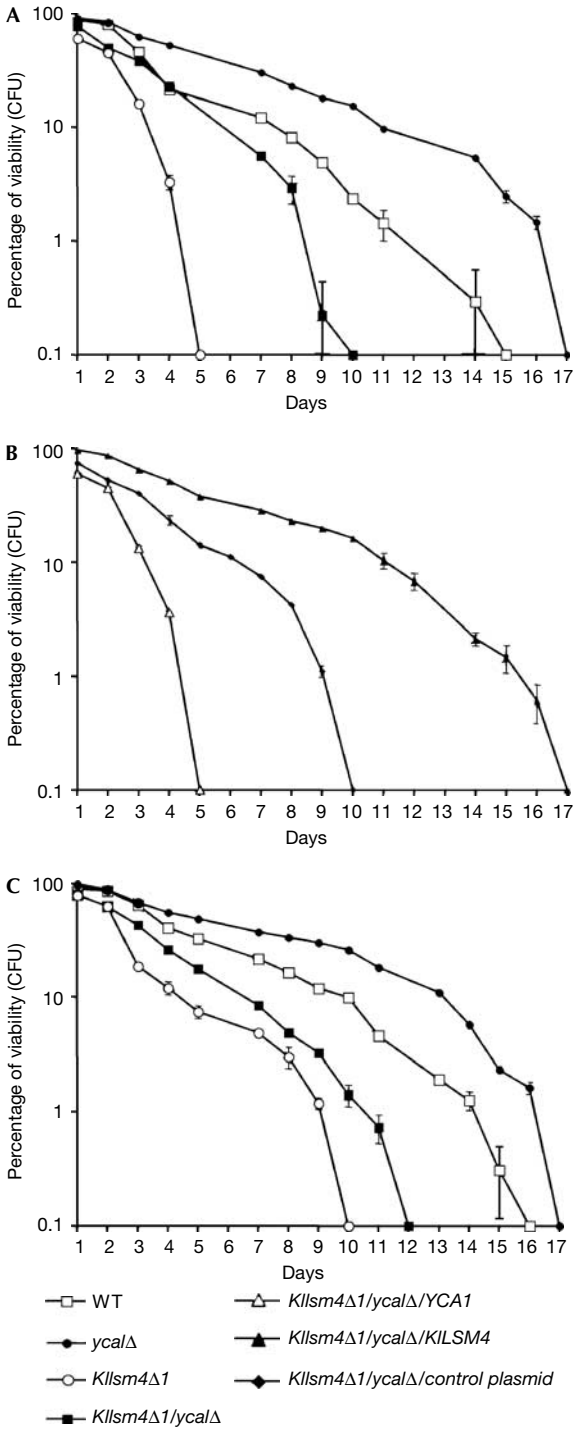

Fig 1 | Cell death in *lsm4* mutants during chronological ageing is attenuated by deletion of the yeast caspase *YCA1*. Cells of wild-type (WT), *yca1Δ*, *Kllsm4Δ1*, *Klsm4Δ1/yca1Δ* (**A**) and their derivative *rho*$^{\circ}$ strains (**C**) were grown in SD medium. Viability is expressed as a percentage of micro-colony-forming units (CFU). (**B**) Cell viability of *Klsm4Δ1/yca1Δ* mutants after the reintroduction of the *YCA1* and *KlLSM4* genes. Average and standard deviation of three independent experiments are shown.

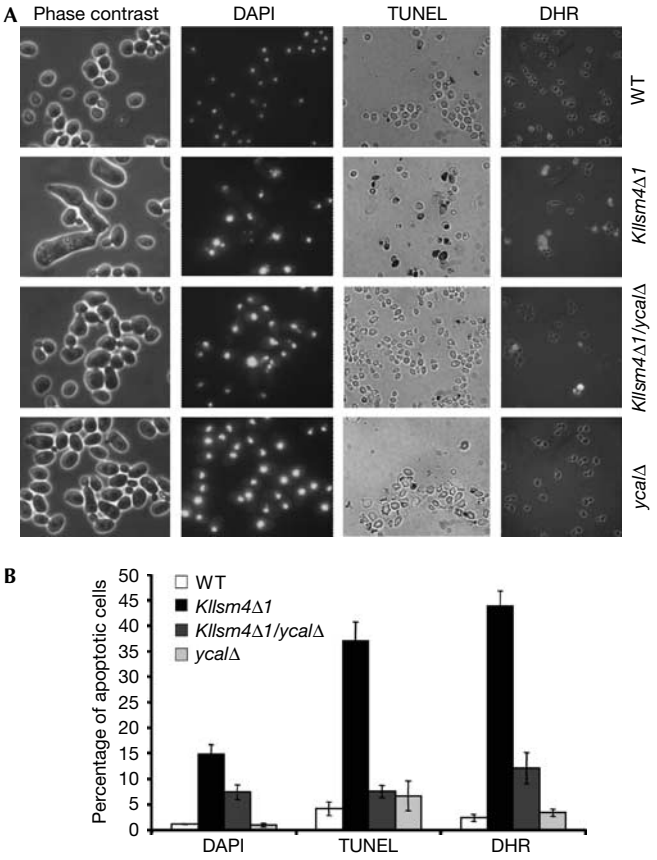

Fig 2 | The absence of Yca1 attenuates the apoptotic phenotypes of *lsm4* mutants. (**A**) Wild-type (WT), *yca1*Δ, *Kllsm4*Δ*1* and *Klsm4*Δ*1/yca1*Δ cells were analysed for nuclear fragmentation (4,6-diamidino-2-phenylindole (DAPI) staining), DNA strand breaks (dark cells in TdT-mediated dUTP nick end labelling test) and reactive oxygen species production (bright cells after dihydrorhodamine 123 (DHR) staining). Left column: phase-contrast pictures corresponding to DAPI staining. (**B**) Quantification of cell population showing apoptotic phenotypes.

(Laun *et al*, 2001) or after oxidizing treatment, such as exposure to H_2O_2 (Fahrenkrog *et al*, 2004). Therefore, we determined the amount of ROS by incubation with dihydrorhodamine 123 (DHR; Schulz *et al*, 1996). Wild-type and *yca1*Δ strains showed about 1% of ROS-positive cells (Fig 2A). In contrast, about 40% of *Kllsm4*Δ*1* cells showed intense intracellular staining with DHR. However, the fraction of DHR-positive cells was reduced to about 10% in the double mutant that had the *YCA1* gene deleted. These results are summarized in Fig 2B.

The double mutant is more resistant to apoptotic stress

Low doses of H_2O_2 and acetic acid are established triggers of apoptosis in yeast (Madeo *et al*, 1999; Ludovico *et al*, 2001). Therefore, we measured the cell viability of strains after exposure to different concentrations of H_2O_2. As already reported (Madeo *et al*, 2002), the *yca1*Δ strain survived better than the wild-type cells, and, in addition, the double mutant *Kllsm4*Δ*1/yca1*Δ showed a higher percentage of viable cells than the *Kllsm4*Δ*1* mutant (Fig 3A). The increased resistance of the double mutant to oxygen peroxide could also be visualized through the halo test (Fig 3B), showing that the halo size of the *Kllsm4*Δ*1* mutant was about one-third that of the double mutant.

Similarly, the *Kllsm4*Δ*1* mutant showed a higher sensitivity to acetic acid, as it was not able to grow on YPD plates containing 70 mM of the compound. Again, the observed sensitivity was strongly reduced when the *YCA1* gene was also inactivated (Fig 3C).

In a previous work, we observed a pleiotropic phenotype in *Kllsm4*Δ*1* cells of the related yeast *Kluyveromyces lactis* as growth was impaired by several drugs (Mazzoni *et al*, 2003a). These included caffeine, a drug that affects the protein kinase C (PKC)–mitogen-activated protein kinase (MAPK) pathway.

Caffeine is also known to induce apoptosis in mammalian cells, and this event is mediated by the p53, Bax and caspase 3 pathways (He *et al*, 2003).

As can be seen in Fig 3C, *S. cerevisiae* cells expressing *Kllsm4*Δ*1* also showed sensitivity to caffeine, which could be fully suppressed by inactivation of *YCA1*. This result suggests that in yeast, in which the homologues of p53 and Bax have not yet been identified, caffeine could trigger cell death through a caspase-dependent pathway (supplementary Fig 1 online). Interestingly, the overexpression of *SLT2/MPK1*, a member of the PKC–MAPK pathway, suppressed the caffeine sensitivity of the *Kllsm4*Δ*1* cells (data not shown).

*Kllsm4*Δ*1* cells show aberrant mitochondrial morphology

In wild-type yeast strains, mitochondria are arranged as a tubular network that is the product of an equilibrium between fusion and fission events.

Excessive mitochondrial fission and/or lack of fusion result in the breakdown of the mitochondrial network, leading to the fragmentation of mitochondria, respiratory deficiency, ROS generation and apoptosis in mammalian cells (Yaffe, 1999; Frank *et al*, 2001; Karbowski & Youle, 2003).

Similarly, *Kllsm4*Δ*1* cells showed an accumulation of ROS (Mazzoni *et al*, 2003b) and growth arrest on respiratory carbon sources (Fig 3D; supplementary Fig 1 online). Again, the inactivation of *YCA1* in this strain could rescue respiratory deficiency (Fig 3D). We analysed the mitochondrial morphology in the *Kllsm4*Δ*1* and *Kllsm4*Δ*1/yca1*Δ mutants using mitochondria-targeted green fluorescent protein (mitoGFP), a protein that specifically targets the mitochondrial matrix compartment (Westermann & Neupert, 2000). A total of 87% of the *Kllsm4*Δ*1* cells showed an aberrant mitochondrial morphology with a punctuate distribution instead of the wild-type tubular shape (Fig 3E). This could be the result of increased fission activity as well as of fusion deficiency (Mozdy *et al*, 2000; Westermann, 2002). Intriguingly, inactivation of *YCA1* restored the tubular structures in 53% of cells, which suggested that the signals for mitochondrial fission in the *Kllsm4*Δ*1* mutant are at least partially caspase dependent.

Mitochondrial morphology also changed in the wild-type cells after H_2O_2 treatment. In this case, 73% and 92% of cells showed the punctuate morphology after 20 and 60 min of incubation with 3 mM H_2O_2, respectively. Under the same conditions, 42% and 78% of *yca1*Δ cells showed the punctuate morphology, suggesting that an increase in mitochondrial fission is a general feature of yeast apoptosis. In accordance with this, it has been published recently that mitochondrial fission increases after cell treatment

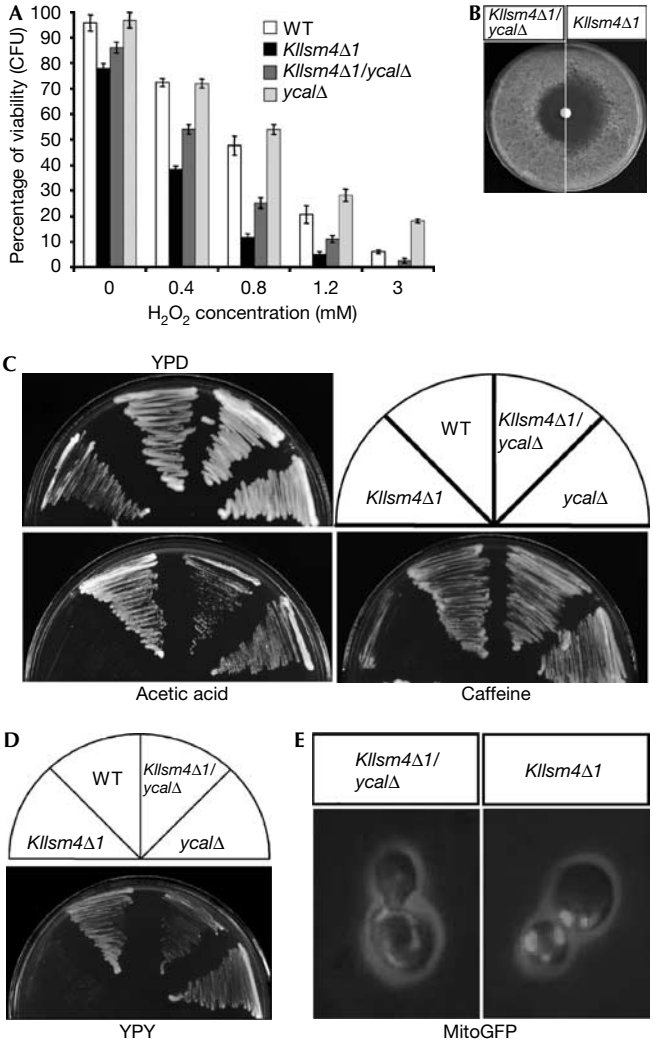

Fig 3 | Deletion of *YCA1* in the *Klsm4Δ1* mutant suppresses the sensitivity to H_2O_2, recovers the lack of growth on acetic acid, caffeine and glycerol and relieves mitochondrial fragmentation. (**A**) Cell viability was measured after exposure of cells to H_2O_2 at the indicated concentration for 4 h. CFU, colony-forming unit; WT, wild type. (**B**) Halo test: about 10^8 cells of *Kllsm4Δ1* and *Klsm4Δ1/yca1Δ* strains were spread onto YPD plates. Whatman 003 paper discs were soaked with 10 μl of 30% H_2O_2 and placed on the surface of the plates. Halo formation was recorded after 2 days of growth at 28 °C. (**C**) Cells were streaked on YPD, YPD + 0.25% caffeine and YPD + 70 mM acetic acid, and plates were incubated at 28 °C for 3 days. (**D**) Cells were streaked on YP + 3% glycerol (YPY) plates and incubated at 28 °C for 2 days. (**E**) Mitochondrial morphology in *Kllsm4Δ1/yca1Δ* and *Kllsm4Δ1* cells shown by mitochondria-targeted green fluorescent protein (mitoGFP).

with acetic acid (Fannjiang *et al*, 2004). Moreover, our data suggest that mitochondrial fragmentation after apoptotic stimuli is partially dependent on the presence of Yca1.

Levels of *YCA1* transcript are higher in *Kllsm4Δ1*

We previously suggested that, in the *Kllsm4Δ1* mutant, the stabilization of mRNAs could lead to the abnormal production

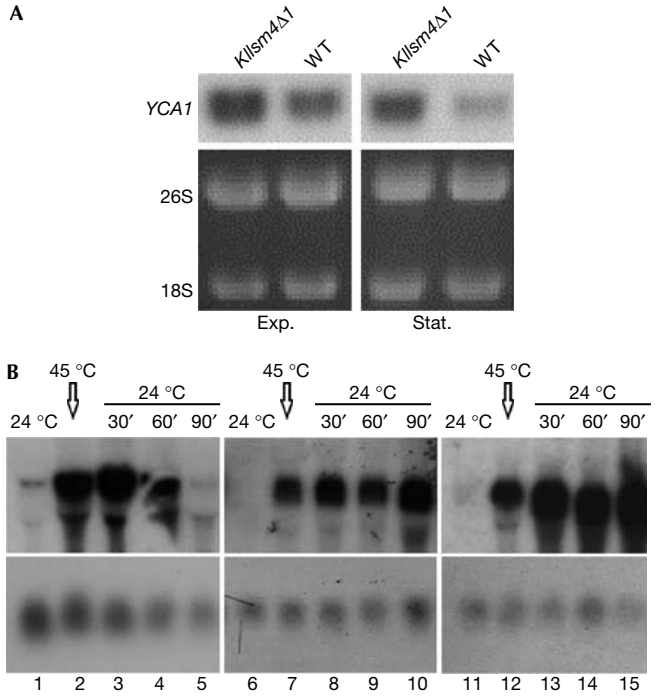

Fig 4 | The *Kllsm4Δ1* mutant shows a higher *YCA1* transcript level, and the deletion of *YCA1* in *Kllsm4Δ1* does not affect messenger RNA turnover rate. (**A**) Total RNAs were prepared from exponentially (Exp.) growing and stationary (Stat.) cells and analysed for *YCA1* expression. Ribosomal 26S and 18S were used as internal standards. WT, wild type. (**B**) Total RNAs were prepared from WT (lanes 1–5), *Kllsm4Δ1* (lanes 6–10) and *Kllsm4Δ1/yca1Δ* (lanes 11–15) cells grown overnight in YPD at 24 °C. *SSA4* expression was induced by incubating cells for 15 min at 45 °C. Thereafter, cultures were back-shifted at 24 °C and RNA samples, prepared after 30, 60 and 90 min, were probed with the *SSA4* gene (upper panels). Ribosomal RNA 5S internal was used as a control (lower panels).

of some proteins that trigger apoptosis (Mazzoni *et al*, 2003b). In fact, the rate at which a specific mRNA is degraded can be a significant contributing factor to the overall expression levels of the encoded protein, and it has been recently reported that mRNA decay mutants show defects in translational control (Holmes *et al*, 2004).

As the absence of *YCA1* could suppress most of the apoptotic phenotypes in *Kllsm4Δ1* cells, we investigated whether *YCA1* mRNA was also stabilized in this mutant. We prepared total RNA from exponentially growing and stationary cultures of wild-type and *Kllsm4Δ1* cells and analysed the amount of *YCA1* transcript. As shown in Fig 4A, we observed that the amount of *YCA1* mRNA was more abundant in the mutant than in the wild type, both in exponential and stationary cells, suggesting the possibility that the stabilization of the transcript in the mutant could result in the progressive accumulation of Yca1 and trigger apoptosis.

Yca1 is not involved in mRNA degradation defects

As the *Kllsm4Δ1* mutant shows a delayed mRNA degradation (Mazzoni *et al*, 2003b), we investigated whether the increased survival and suppression of apoptotic phenotypes observed in the

double mutant *Kllsm4Δ1/yca1Δ* were due to a restoration of mRNA degradation rate to the wild-type level.

We previously detected in *Kllsm4Δ1* cells a stabilization of the transcript of the *SSA4* gene (Mazzoni *et al*, 2003b), the transcription of which can be switched on and off at 45 and 24 °C, respectively (Boorstein & Craig, 1990).

Therefore, we determined *SSA4* mRNA levels in wild-type and mutant strains.

As can be seen in Fig 4B, the exposure to 45 °C activated gene transcription, and 90 min after shifting cells back to 24 °C, the amount of *SSA4* mRNA declined to the basal level in wild-type cells (lanes 3–5). In single and double mutants, the level of *SSA4* transcript remained high 90 min after shifting cells back to 24 °C (lanes 8–10, 13–15). Therefore, the absence of Yca1 did not suppress the defects in transcription degradation observed in the *Kllsm4Δ1* mutant, positioning the budding yeast caspase Yca1 as a downstream executor of cell death induced by mRNA stability.

In conclusion, we showed that the deletion of the yeast caspase gene *YCA1* inhibits mitochondrial fragmentation and most of the apoptotic phenotypes observed in the apoptotic *Kllsm4Δ1* mutant.

It is not yet known how mRNA perturbations lead to apoptosis in mammalian cells (Bellacosa & Moss, 2003). Future studies in yeasts may show more details of the connection between mRNA stability and apoptosis.

METHODS

Strains and plasmids. The Euroscarf *YCA1* (Yor197w) disruption strain (Y02453) was crossed four times with the strain MCY4/313Kllsm4Δ1 (Mat α, ade1-101, his3-Δ1, trp1-289, ura3, LEU2-GAL1-SDB23, pRS313/Kllsm4Δ1) to obtain the isogenic strains CML39-11A (Mat a, ade1-101, his3-Δ1, leu2, ura3, trp1-289), CML39-9A (Mat a, ade1-101, his3-Δ1, leu2, ura3, trp1-289, yor197w::kanMX4) and CML39-8D (Mat a, ade1-101, his3-Δ1, leu2, ura3, trp1-289, LEU2-GAL1-SDB23, yor197w::kanMX4, pRS313/Kllsm4Δ1).

Plasmid pRS313/*Kllsm4Δ1* was obtained by cloning a *Sma*I–*Eco*RV fragment from plasmid pKS/*KlLSM4* (Mazzoni & Falcone, 2001), containing the promoter and gene portion encoding the first 72 amino acids of *KlLSM4*, into the *Sma*I site of pRS313 (Sikorski & Hieter, 1989). This plasmid was transformed into the MCY4 strain to give the strain MCY4/313*Kllsm4Δ1*.

Plasmid pUG36ng/*YCA1* was obtained by cloning a *Hin*dIII fragment from plasmid pKF788 (Madeo *et al*, 2002) into the *Hin*dIII of the pUG36 vector without an *Xba*I fragment containing yeast enhanced green fluorescent protein (yEGFP; pUG36ng).

Cells were grown in YP (1% yeast extract, 2% peptone) supplemented with 2% glucose (YPD) at 28 °C or in minimal medium (0.67% yeast nitrogen base) containing 2% glucose (SD) supplemented with 10 μg/ml of the appropriate nutritional requirements according to the genotype of the strains.

rho° strains were obtained as described by Mazzoni *et al* (2003b).

Fluorescence microscopy. For nuclear morphology, exponentially growing cells were fixed with 70% (v/v) ethanol and stained with DAPI (1 μg/ml). The presence of ROS was detected with DHR (Sigma Aldrich Co. D1054), as described previously (Madeo *et al*, 1999). Free 3′-OH ends were detected by TUNEL on cells grown for 9 days, as described (Madeo *et al*, 1997). For analysis of mitochondria morphology, we used plasmids pVT100U-mtGFP and pYX232-mtGFP (Westermann & Neupert, 2000) that target GFP into mitochondrial matrix. For image acquisition, we used an Axioskop2 fluorescence microscope (Carl Zeiss, Jena, Germany) equipped with a digital camera (micro-CCD).

RNA isolation and analysis. Total RNA (10 μg) was loaded onto 1.2% agarose–MOPS gels containing formaldehyde (Schmitt *et al*, 1990). The *SSA4* probe was obtained by PCR amplification with primers designed in the coding region. The *YCA1* probe was an *Eco*RI–*Not*I fragment derived from plasmid pFM21. Hybridization experiments followed standard procedures (Sambrook *et al*, 1989).

For RNA analysis in the exponential and stationary phases, yeast strains were grown overnight in YPD, cells were diluted to an optical density $(OD)_{600}$ of 0.065–0.07, cultured in YPD medium to an OD_{600} of 0.4–0.5 and an aliquot was taken for isolation of RNA.

The rest of the culture was grown to an OD_{600} of 5–7 and used for isolation of RNA from stationary phase cells.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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