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A *Kluyveromyces lactis* mutant in the essential gene *KlLSM4* shows phenotypic markers of apoptosis

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Abstract

We report the study of *Kluyveromyces lactis* cells expressing a truncated form of *KlLSM4*, a gene ortholog to *LSM4* of *Saccharomyces cerevisiae* which encodes an essential protein involved in both pre-mRNA splicing and mRNA decapping. We had previously demonstrated that the first 72 amino acids of the *K. lactis* Lsm4p (KlLsm4Δp) can restore cell growth in both *K. lactis* and *S. cerevisiae* cells not expressing the endogenous protein. However, cells showed a remarkable loss of viability in stationary phase. Here we report that cells expressing KlLsm4Δp presented clear apoptotic markers such as chromatin condensation, DNA fragmentation, accumulation of reactive oxygen species, and showed increased sensitivity to different drugs. RNA analysis revealed that pre-mRNA splicing was almost normal while mRNA degradation was significantly delayed, pointing to this as the possible step responsible for the observed phenotypes.

Keywords: Apoptosis; Sm-like; mRNA turn-over; *Kluyveromyces*

1. Introduction

Apoptosis is a regulated form of cell death crucial for health, homeostasis and embryonic development in metazoa. This phenomenon has also been studied in model organisms such as *Drosophila melanogaster* or *Caenorhabditis elegans*, and recent reports support the notion that apoptosis also occurs in unicellular organisms such as yeasts, when cells are triggered by internal and external signals [1,2]. In *Saccharomyces cerevisiae*, apoptosis has been demonstrated in a particular *cdc48* mutant [3], during perturbation of the vesicular trafficking [4], after cell treatment with high salt [5], acetic acid [6] and osmotin, an antifungal protein from tobacco implicated in host-plant defense [7].

Although not all of the identified mammalian genes involved in apoptosis have their correspondents in *S. cerevisiae*, the basic machinery seems to be conserved in this organism as well, in that cells show chromatin condensation, DNA fragmentation and externalization of phosphatidylserine [1]. Furthermore, the heterologous expression of *Bax*, the mammalian apoptosis inducer gene, triggers cell death and can be counteracted by the simultaneous expression of *Bcl-2* [8]. Recently, evidence for the existence in *S. cerevisiae* of a caspase-related protease regulating apoptosis has been reported, indicating yeast as a useful model for the study of this phenomenon [9]. Programmed cell death can be triggered by different mechanisms, and it has been reported that altered pre-mRNA splicing or mRNA stability are involved in mammalian apoptosis-linked diseases [10,11]. In *S. cerevisiae*, Lsm (Sm-like) proteins are involved in different cellular processes, including tRNA and rRNA maturation, pre-mRNA splicing and mRNA decapping [12–14]. These proteins, which are conserved through evolution from archaebacteria to humans, present a common amino acid motif, called the Sm domain, which is also present in the Sm protein family [15]. The Lsm2p/Lsm8p complex, assembled with the U4/U6·U5 snRNAs, has a fundamental role in pre-mRNA splicing, while the Lsm1p/Lsm7p complex, together with Dcp1p, Dcp2p, Pat1p and Xrn1p, is involved in mRNA

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decapping [16,17]. Some of the *LSM* genes, such as *LSM4*, are essential for cell life [18] and we have recently demonstrated that a truncated form of the gene (*Kllsm4Δ1*) from the yeast *Kluyveromyces lactis*, encoding the first 72 out of 183 amino acids and the Sm-like domains, can restore cell viability in *K. lactis* and in *S. cerevisiae* strains not expressing the wild-type gene [19]. However, the absence of the carboxy-terminal region of the protein resulted in the loss of cell viability during the stationary phase in both yeasts.

In this paper we report the study of *K. lactis* cells expressing Kllsm4Δ1p, the truncated form of the protein, and we demonstrate that the loss of cell viability is accompanied by chromatin condensation, DNA fragmentation and by the accumulation of reactive oxygen species (ROS), all of which are characteristic markers of apoptosis. In addition, we have found that cells expressing the truncated protein showed a significant delay in the degradation of mRNAs which, in turn, could be the basis of the onset of apoptosis.

2. Materials and methods

2.1. Strains and culture conditions

The yeast strains used in this study were the following: CMK40 (Mat a/α, ade/ade, lac4-8/lac4-8, trp/TRP, ura3/ura3), CMK42 (Mat a/α, ade/ade, lac4-8/lac4-8, trp/TRP, ura3/ura3, leu2/LEU2 Kllsm4Δ1/Kllsm4Δ1) [19].

Cells were grown in YP (1% yeast extract, 2% peptone) supplemented with 2% glucose (YPD), or glycerol (YPGly) at 28°C (unless indicated otherwise). Solid media were supplemented with 2% Bactoagar (Difco, Detroit, MI, USA).

2.2. Fluorescence microscopy

For DAPI staining, cells were harvested, resuspended in 70% (v/v) ethanol, stained with 4,6-diamino-2-phenylindole (DAPI) at the concentration of 1 μg ml^{-1} and observed by fluorescence microscopy.

The presence of free intracellular radicals or strongly oxidizing molecules (ROS) was detected with dihydrorhodamine 123 (DHR; Sigma D1054) as described previously [20].

Free 3′-OH were detected by TUNEL [3] using the Roche Diagnostic (Mannheim, Germany) in situ cell detection kit peroxidase and observed with an Axioskop microscope (Carl Zeiss, Jena, Germany).

2.3. Electron microscopy

Exponentially growing cells were fixed with 2% glutaraldehyde in distilled water for 1 h at room temperature and washed with water. To reveal cellular membranes without removing the cell wall, cells were post-fixed with freshly prepared 4% $KMnO_4$ in H_2O for 2 h at 4°C [21]. After washes, cells were incubated with 2% uranyl acetate for 2 h at room temperature, washed and dehydrated in increasing (30–100%) concentrations of ethanol. The samples were infiltrated overnight at 4°C in a 1:1 mixture of ethanol with Epon 812 embedding medium. The mixture was replaced with pure Epon 812 and the samples allowed to polymerize at 60°C for 24 h. Ultrathin sections were stained with lead citrate before examination with an electron microscope.

2.4. RNA isolation and analysis

Total RNA was prepared as previously described [22] from exponential cultures grown on glycerol and 5, 10, 20 and 40 min after addition of ethanol. After spectrophotometric determination of the amount present in each sample, 10 μg of RNA was loaded onto 1.2% agarose–MOPS gels containing formaldehyde and ethidium bromide. Northern analysis was performed by standard procedures [23].

The *KlADH3* probe was a 600 bp SacI–DdeI fragment containing 530 nucleotides of the 5′-upstream region and 75 bp of the leader peptide-coding region [22]. The actin probe was a 1300-bp *Hin*dIII fragment containing part of the intron and the exon 2 of the *KlACT1* gene [24].

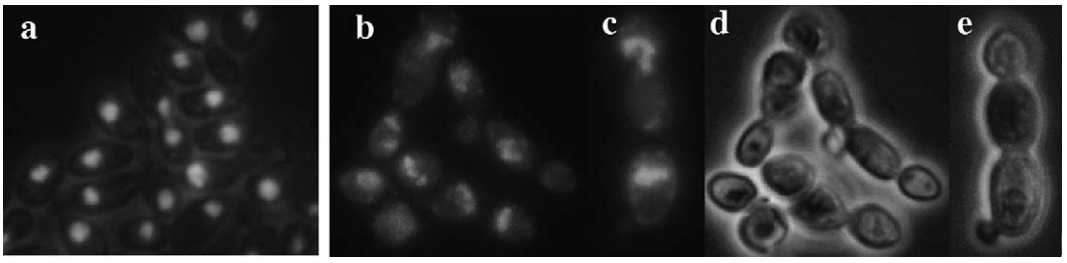

Fig. 1. Chromatin fragmentation. DAPI staining and phase contrast of the wild-type strain CMK40 (panel a) and the mutant strain CMK42 (panels b–e) grown on YPD.

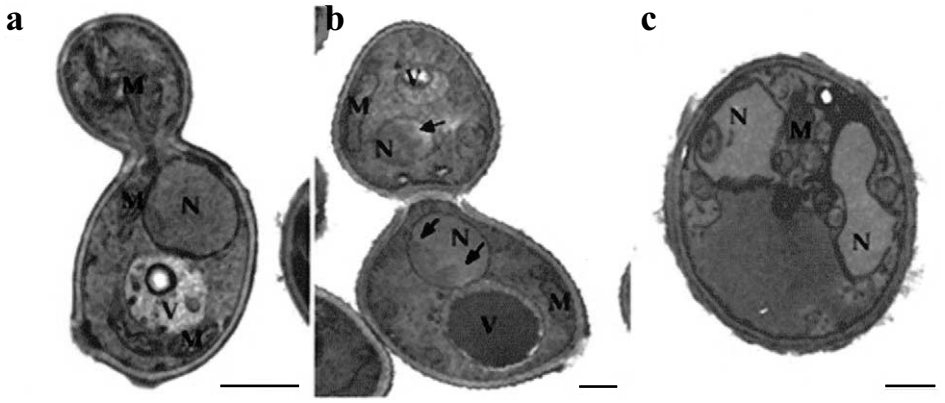

Fig. 2. Electron micrographs of the wild-type strain CMK40 (panel a) and the mutant strain CMK42 (b and c) grown on YPD. The arrows indicate the condensed and marginated chromatin. N, nucleus; M, mitochondria; V, vacuole. Bar, 1 μm.

3. Results

3.1. Detection of apoptotic phenotypes in cells expressing *Kllsm4Δ1p*

We have previously reported that the deletion of the carboxy-terminal region of KlLsm4p resulted in the loss of viability in stationary phase cells of *K. lactis*. We wondered if this phenomenon was related to apoptotic events and, to this purpose, we analyzed the cellular and the nuclear morphology in the wild-type strain CMK40 and in CMK42, a homozygous mutant expressing Kllsm4Δ1p.

As shown in Fig. 1, CMK42 cells frequently presented an elongated morphology and the presence of multiple buds that did not separate from mother cells after division (Fig. 1d,e). DAPI staining revealed a single round-shaped nucleus in wild-type cells (Fig. 1a), whereas nuclei of cells expressing Kllsm4Δ1p showed evident fragmentation (Fig. 1b) or tubular shape (Fig. 1c), as also observed in apoptotic cells of other organisms [25]. Electron microscopy of CMK42 cells revealed an evident condensation and margination of chromatin along the inner part of the nuclear envelope (Fig. 2b), and confirmed the occurrence of nuclei fragmentation (Fig. 2c). As a control, in Fig. 2a a representative cell is reported of the wild-type strain CMK40.

Cells were also analyzed in situ by TUNEL, a staining technique that reveals free 3′-OH ends originated by DNA breaks [26]. As can be seen in Fig. 3, cells expressing Kllsm4Δ1p showed an intense staining, compared to the wild-type strain, indicating a significant presence of breaks in the DNA strands.

We also isolated chromosomal DNA from exponential – and stationary – phase cultures to test the presence of the DNA ladder observed in cells of other organisms, but not all, undergoing apoptosis [27]. Gel electrophoresis analysis did not reveal the DNA ladder (data not shown) and, as also reported by other authors, this can be explained by the fact that the *S. cerevisiae* chromatin structure has short or no linker DNA between nucleosomes [20].

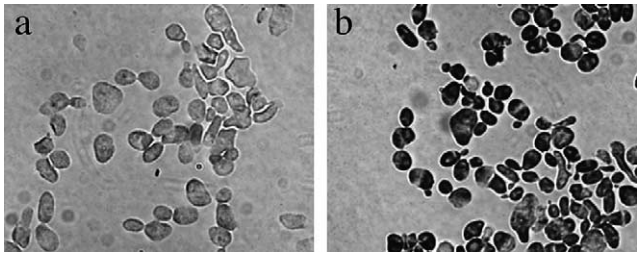

Fig. 3. DNA strand breakage detection. Wild-type CMK40 (panel a) and mutant CMK42 (panel b) strains were grown on YPD. After cell fixing and digestion of cell walls, strand breaks in DNA were detected according to the TUNEL protocol.

3.2. Sensitivity to hydrogen peroxide and ROS production

Cells expressing Kllsm4Δ1p showed a reduced viability in the presence of hydrogen peroxide (data not shown) and an increased sensitivity to this compound, as revealed by halo test (Fig. 4).

Hydrogen peroxide treatment generates the production of ROS which are naturally produced in aging cells [28] and are considered an important event in triggering apoptosis [20].

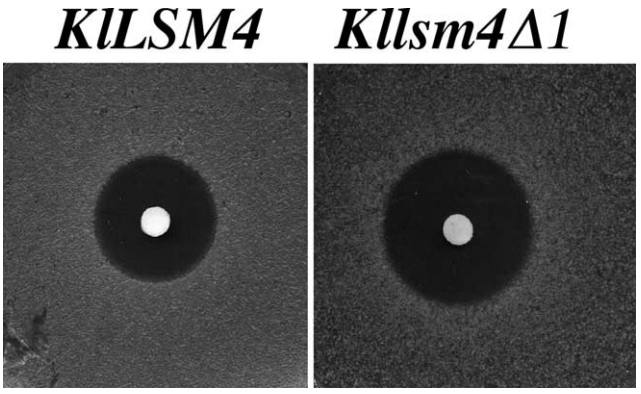

Fig. 4. *K. lactis* strains expressing *Kllsm4Δ1* are sensitive to H_2O_2. 10^8 cells of CMK40 (*KlLSM4*) and CMK42 (*Kllsm4Δ1*) strains were spread onto YDP plates. Whatman 003 paper disks were soaked with 10 μl of 30% H_2O_2 and placed on the surface of the plates. Halo formation was recorded after 2 days of growth at 28°C.

To verify the occurrence of this phenomenon, strains CMK40 and CMK42 were tested for the endogenous production of ROS during their growth by incubation with DHR, a compound that is oxidized to the fluorescent chromophore rhodamine 123 by the presence of ROS [29].

As already reported [20], less than 1% of wild-type cells treated with DHR showed a marginal fluorescence (data not shown), whereas about 30% of cells expressing Kllsm4Δ1p showed an intense intracellular staining (Fig. 5). The DHR-positive phenotype was observed both in normal and abnormal looking cells, suggesting that the morphological abnormalities could be functionally unrelated to the apoptotic phenotypes. In any case, the high level of ROS production in CMK42 could explain the increased sensitivity to hydrogen peroxide and the occurrence of apoptosis in this strain.

3.3. Increased mRNA stability in cells expressing Kllsm4Δ1p

In *S. cerevisiae* it has been demonstrated that mutations in the *LSM4* gene result in decapping defects, a crucial step for mRNA degradation, and in pre-mRNA splicing defects [14].

To verify if mRNA degradation was delayed in the CMK42 mutant, we followed the stability of the transcript of *KlADH3*, a nuclear gene encoding an alcohol dehydrogenase activity localized within mitochondria [30]. The gene shows a peculiar regulation in that it is highly expressed in the presence of glycerol and other respiratory carbon sources except ethanol, which, on the contrary, rapidly represses the expression of *KlADH3* at the transcriptional level [22].

Strains expressing the wild-type and truncated forms of *KlLSM4* were grown on glycerol and, after the addition of ethanol, RNA samples were prepared over a 40 min interval and probed with *KlADH3* DNA in Northern analysis.

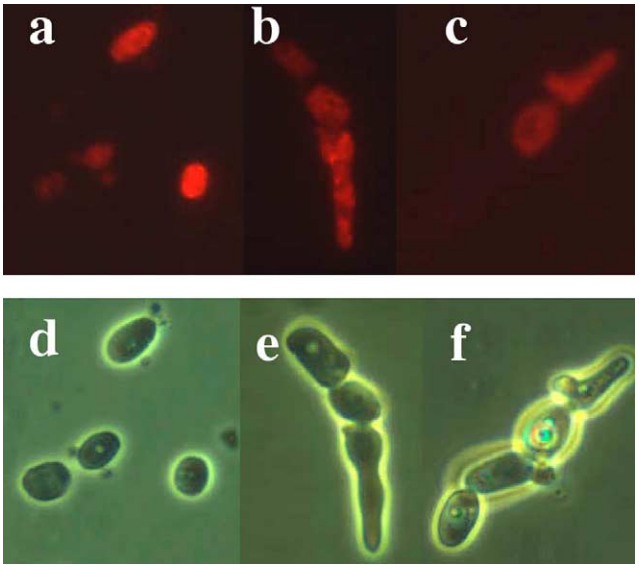

Fig. 5. CMK42 expressing *Kllsm4Δ1* accumulates ROS. Cell fluorescence (panels a–c) and phase contrast (panels d–f) of cells after 2 h incubation with DHR.

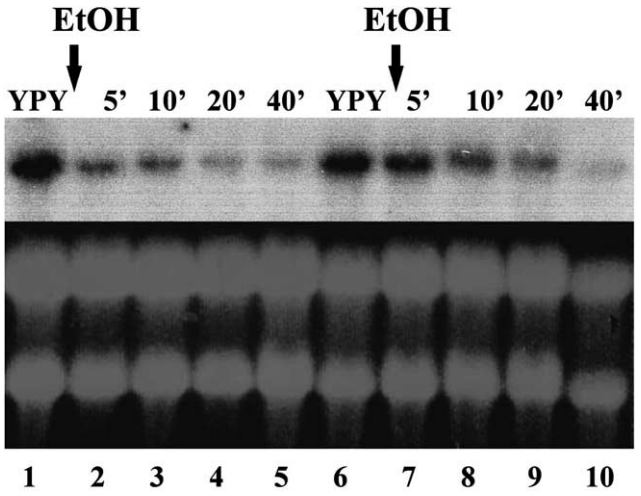

Fig. 6. mRNA stabilization in CMK42 cells. CMK40 and CMK42 were grown overnight on YPGly to induce *KlADH3*. 2% Ethanol was added to each culture and total RNAs were prepared from cell samples before (lanes 1–6) and 5, 10, 20 and 40 min after the addition of ethanol (lanes 2–5 and 7–10) and probed with *KlADH3* gene. In the bottom part are shown the ribosomal RNAs 25S and 18S of each sample.

As shown in Fig. 6, the amount of *KlADH3* mRNA was dramatically reduced in the wild-type 5 min after the addition of ethanol and became almost undetectable after 20 min (lanes 1–5). On the contrary, in CMK42 cells the degradation of the transcript followed a slower time course; in fact, 5 min after the addition of ethanol the amount of *KlADH3* mRNA was still about half of the control and only after 40 min reached the same level as observed in the wild-type after 20 min (lanes 6–10).

To investigate the effect of the truncation of KlLsm4p on pre-mRNA maturation, we analyzed the splicing efficiency by probing Northern blots with *KlACT1*, the actin-encoding gene of *K. lactis* which contains a single intron of 778 bp. After prolonged autoradiographic exposure, we could detect a faint band corresponding to the *KlACT1* mRNA precursor in CMK42, indicating that the truncation of KlLsm4p had little influence on this process (data not shown).

3.4. Pleiotropic phenotypes of cells expressing Kllsm4Δ1p

It has been reported that mRNAs of genes involved in cellular proliferation present a rapid turnover and, consequently, an alteration of this process would result in the production of some proteins at the wrong moment of the cell cycle, perturbing the delicate equilibrium between cellular factors.

For this reason, we have also analyzed the growth of strains CMK40 and CMK42 under different conditions and in the presence of drugs.

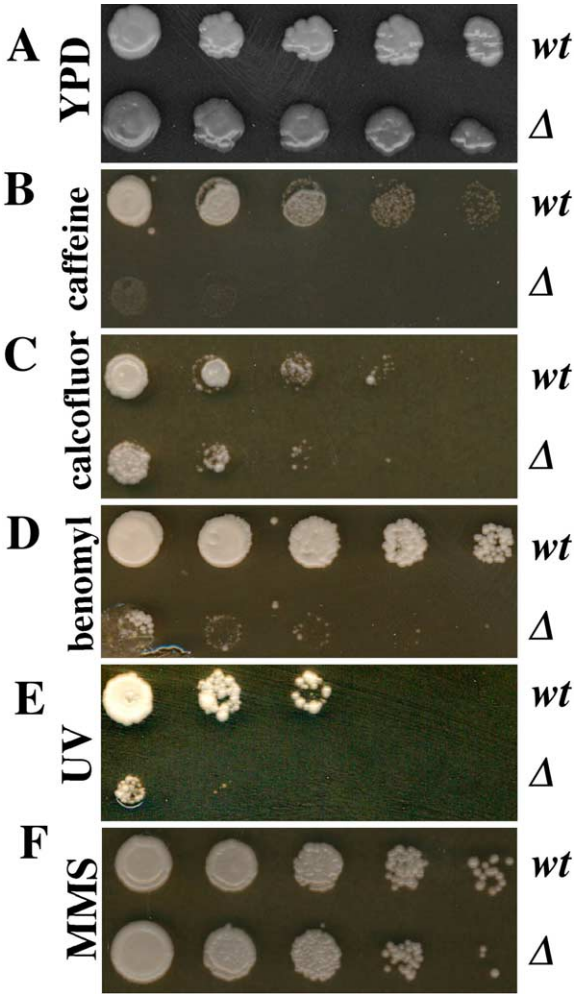

Fig. 7. CMK42 cells show a pleiotropic phenotype. CMK40 (wt) and CMK42 (Δ) cells were grown on YPD to saturation and 5 μl of 10-fold dilutions were spotted onto agar medium and incubated at 28°C for 3 days (panel A). Cells were spotted onto YPD plates containing caffeine 0.2% (panel B), 10 μg ml^{-1} calcofluor (panel C), 250 μM benomyl (panel D), and incubated at 28°C for 3 days. Cells plates on YPD were exposed to UV light for 30 s and incubated in the dark for 3 days at 28°C (panel E). Cells were grown for 5 days at 28°C on YPD plates containing 0.02% MMS (panel F).

The results of this analysis, reported in Fig. 7, revealed that CMK42 cells were sensitive to caffeine, a phenotype often associated with defects in the protein kinase C (PKC)-mitogen-activated protein (MAP) kinase pathway, indicating possible defects in the synthesis of the cell wall. This was also confirmed by a slight but significant sensitivity of CMK42 to calcofluor white, a compound that specifically binds to chitin and interferes with yeast cell wall deposition [31].

CMK42 cells also showed sensitivity to benomyl, a microtubule-destabilizing compound, indicating defects in nuclei division and migration [32].

As far as DNA metabolism is concerned, cells were also sensitive to UV treatment, whereas the alkylating agent methyl methanesulfonate (MMS) had no effect.

4. Discussion

In a previous work we have reported that a truncated form of the essential gene *KlLSM4*, encoding the first 72 amino acids of the *K. lactis* Lsm4p, was sufficient to allow cell growth and we showed that this mutated allele could also rescue viability in an *S. cerevisiae* strain deprived of the endogenous Lsm4p. Nevertheless, cells of both yeasts expressing the truncated protein showed a remarkable loss of viability in the stationary phase [19]. Here we describe that the loss of viability in a *K. lactis* homozygous mutant expressing the truncated form of the *KlLSM4* gene was due to programmed cell death, as evidenced by the appearance of cytological markers of apoptosis. In fact, fluorescence and electron microscopy clearly revealed that cells expressing Kllsm4Δ1p actually exhibit nuclear fragmentation and chromatin condensation, higher ROS production and increased sensitivity to oxidative stress. These apoptotic phenotypes have also been observed in *S. cerevisiae* cells deprived of the endogenous protein and expressing Kllsm4Δ1p, and a normal situation can be restored in both yeasts after transformation with the wild-type *KlSM4* allele [33]. In *S. cerevisiae*, Lsm4p plays an important role both in mRNA decapping and splicing when assembled in complex with other proteins of the Lsm family. We demonstrated that in *K. lactis* cells expressing *Kllsm4Δ1* mRNA splicing was essentially normal whereas mRNA degradation was significantly delayed, pointing to this as a crucial step in triggering programmed cell death. Interestingly, we had observed that *S. cerevisiae dcp1* and *dcp2* mutants, which lack the mRNA-decapping enzymes, also entered apoptosis [33].

Our results clearly demonstrate that the missing part of the KlLsm4 protein is necessary for normal cell growth. The C-terminal region of eukaryotic Lsm4 proteins is evolutionarily conserved and contains stretches rich in arginine and glycine residues [34] that are supposed to be important for protein–protein interactions. As an example, the human Lsm4p interacts by means of its C-terminus with the spinal muscular atrophy disease gene product (*SMN*) which, in turn, interacts with the anti-apoptotic protein *Bcl2* [35,36]. The C-terminus of the Lsm4 proteins of *S. cerevisiae* and *K. lactis*, which are quite similar, differ from the other eukaryotic counterparts in that arginine and glycine stretches are replaced by asparagine stretches [19,34]. At present, we do not know the exact mechanism responsible for the onset of apoptosis in yeast cells expressing Kllsm4Δ1p.

We can speculate that the absence of the C-terminus of the protein impedes the correct formation of the LSM complex responsible for mRNA decapping, with a consequent stabilization of transcripts. This alteration in RNA metabolism could lead to the production of some proteins at the wrong moment during the cell cycle, thus perturbing the delicate equilibrium between protein factors and, in turn, triggering apoptosis. This picture seems to be con-

firmed by the pleiotropic phenotypes observed in cells expressing Kllsm4Δ1p, such as higher sensitivity to UV and to different drugs such as caffeine, calcofluor and benomyl. Caffeine sensitivity has been associated with defects in the MAP kinase pathway, which is also related to the synthesis of the cell wall. In fact, CMK42 cells also demonstrated an increased sensitivity to calcofluor, which is also associated with cell wall defects. Moreover, caffeine sensitivity, together with UV sensitivity, indicates that DNA metabolism is altered in cells expressing Kllsm4Δ1p, at least in some step, since MMS has no apparent effect.

On the other hand, benomyl sensitivity suggests the presence of cytoskeleton defects, and this phenotype has been associated with mutations that increase the frequency of chromosome loss [37].

All the above phenotypes have also been observed in an *S. cerevisiae* strain expressing Kllsm4Δ1p, indicating a strong conservation of functions between these two yeasts (unpublished data).

Proteome analysis of cells expressing Kllsm4Δ1p could give relevant information on the alteration of the peptide balance and support the identification of factors involved in the onset of apoptosis.

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