# Yeast Lacking the *SRO7/SOP1*-encoded Tumor Suppressor Homologue Show Increased Susceptibility to Apoptosis-like Cell Death on Exposure to NaCl Stress

**Ingrid Wadskog,\* Corinna Maldener,† Astrid Proksch,† Frank Madeo,† and Lennart Adler\*‡**

\*Department of Cell and Molecular Biology/Microbiology, Göteborg University, SE-40530 Göteborg, Sweden; and †Institute of Physiological Chemistry, University of Tübingen, 720 76 Tübingen, Germany

Yeast cells deleted for the *SRO7/SOP1* encoded tumor suppressor homologue show increased sensitivity to NaCl stress. On exposure to growth-inhibiting NaCl concentrations, *sro7*Δ mutants display a rapid loss in viability that is associated with markers of apoptosis: accumulation of reactive oxygen species, DNA breakage, and nuclear fragmentation. Additional deletion of the yeast metacaspase gene *YCA1* prevents the primary fast drop in viability and diminishes nuclear fragmentation and DNA breakage. We also observed that NaCl induced loss in viability of wild-type cells is Yca1p dependent. However, a yeast strain deleted for both *SRO7* and its homologue *SRO77* exhibits NaCl-induced cell death that is independent on *YCA1*. Likewise, *sro77*Δ single mutants do not survive better after additional deletion of the *YCA1* gene, and both *sro77*Δ and *sro77*Δ*yca1*Δ mutants display apoptotic characteristics when exposed to growth-inhibiting salinity, suggesting that yeast possesses Yca1p-independent pathway(s) for apoptosis-like cell death. The activity of Yca1p increases with increasing NaCl stress and *sro7*Δ mutants achieve levels that are higher than in wild-type cells. However, mutants lacking *SRO77* do not enhance caspase activity when subject to NaCl stress, suggesting that Sro7p and Sro77p exert opposing effects on the cellular activity of Yca1p.

## INTRODUCTION

The physiological cell death process of apoptosis is a morphologically distinct form of cellular suicide (Kerr, 2002), designed to remove potentially threatening or undesired cells (Vaux and Korsmeyer, 1999; Beers and McDowell, 2001). In animal cells, apoptosis occurs in an ordered series of event and is associated with activation of the programmed cell death machinery (Wyllie *et al.*, 1980; Joza *et al.*, 2002). Inappropriate regulation of apoptosis is linked to the pathogenesis of many human diseases, such as AIDS, cancer, autoimmune, and neurodegenerative disorders (Uren and Vaux, 1996). The process of programmed cell death can be triggered by a vast array of stimuli, and a complex network of regulators and effectors coordinates the process (Beers and McDowell, 2001; Joza *et al.*, 2002). At the end of almost all apoptotic scenarios, a constellation of morphological characteristics emerge, including chromatin condensation, DNA fragmentation, flipping of phosphatidylserine to the outer leaflet of the plasma membrane, and breakage of the cells to small membrane-enclosed vesicles, so called apoptotic bodies (Wyllie *et al.*, 1980). These structural features constitute the cytological hallmarks of apoptosis.

The finding that cellular suicide occurs in most, if not all, multicellular organisms raises the question on how the mechanisms of apoptosis have evolved. Do these mechanisms have a common origin and have these processes first evolved in single-celled organisms? For unicellular organisms exposed to harsh conditions, cellular suicide may serve to propagate the genome to future generations if some of the members of a population are sacrificed to promote survival of others (Engelberg-Kulka and Glaser, 1999; Matsuyama *et al.*, 1999; Fröhlich and Madeo, 2000). In *Saccharomyces cerevisiae*, apoptosis-like cell death was first described for a temperature-sensitive *cdc48* mutant (Madeo *et al.*, 1997). Cdc48p is involved in vesicle fusion associated with secretion and cell division (Latterich *et al.*, 1995). At nonpermissive temperature, the *cdc48* mutant exhibited typical apoptotic markers, i.e., exposition of phosphatidylserine, DNA fragmentation, and chromatin condensation (Madeo *et al.*, 1997). It has also become increasingly clear that there is a connection between ageing and apoptosis-like cell death in yeast, as evidenced by the presence of apoptotic markers in old yeast cells (Fröhlich and Madeo, 2001; Laun *et al.*, 2001). Still, there are arguments against a mechanistic and functional conservation between yeast and mammals, because the yeast genome does not encode obvious homologues to any of the core apoptotic machinery proteins, e.g., the Bcl-2/Bax family of proteins or the caspase family. However, recently, a metacaspase called yeast caspase-1 (Yca1p) was identified in *S. cerevisiae*, and this protein was required for $H_2O_2$ or aging-induced apoptosis in yeast (Madeo *et al.*, 2002). The yeast

**Table 1.** Yeast strains used in the present study

| Yeast strain | Genotype | Source |
|---|---|---|
| BY4741 | *MATa his3Δ leu2Δ met15Δ ura3Δ* | Euroscarf |
| BY4742 | *MATα his3Δ leu2Δ lys2Δ ura3Δ* | Euroscarf |
| Y05451 | BY4741 *sro7*::kanMX4 | Euroscarf |
| Y13134 | BY4742 *sro77*::kanMX4 | Euroscarf |
| Y02453 | BY4741 *ycal*::kanMX4 | Euroscarf |
| Y14387 | BY4742 *ckbl*::kanMX4 | Euroscarf |
| YIW-1 | BY4741 *sro7*::kanMX4 *sro77*::kanMX4 | This study |
| YIW-24 | BY4741 *sro7*::LEU2 *ycal*::kanMX4 | This study |
| YIW-25 | BY4741 *sro77*::HIS3 *ycal*::kanMX4 | This study |
| YIW-26 | BY4741 *sro7*::LEU2 *sro77*::HIS3 *ycal*::kanMX4 | This study |
| YSH6.142-3A | *MATα leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2 mal0 gpdl*::TRP1 | (Ansell *et al.*, 1997) |

metacaspase has also been shown to be involved in a te-lomer-initiated apoptotic pathway that exhibits conserved features with that reported for mammalian cells (Qi *et al.*, 2003). In another recent article, Severin and Hyman (2002) presented evidence that apoptosis in yeast can be induced by a factor that is produced by the yeast cells themselves: the α-factor mating pheromone. The data presented indicate that this mechanism might have evolved to remove cells that fail to mate, suggesting a highly sophisticated death machinery in yeast.

Recently, evidence has also been presented that exposure of yeast to high salinity induces apoptosis (Huh *et al.*, 2002). Several observations suggested that the death was induced by intracellular ion disequilibria rather than by osmotic imbalance. For example, ectopic expression of the antiapoptotic protein Bcl-2 enhanced NaCl tolerance of wild-type cells and a calcineurin-defective *cnb1Δ* mutant that is defective for ion homeostasis (Huh *et al.*, 2002). However, over-expression of Bcl-2 did not suppress the salt-sensitive phenotype of a yeast *hog1Δ* mutant, which has decreased capacity to osmoregulate, but seems to maintain ion homeostasis. We have observed that deletion of the *SOP1/SRO7* gene (hereafter referred to as *SRO7*) brings about increased sensitivity to NaCl stress and a defective intracellular ion homeostasis (Larsson *et al.*, 1998). *SRO7* is a yeast homologue of the *Drosophila l(2)gl* tumor suppressor gene. This gene is required for development of epithelial cell polarity (Bilder *et al.*, 2000), and mutations in *l(2)gl* leads to formation of epithelial derived tumors in fly larvae (Gateff, 1978). The *l(2)gl* gene is also required for the development of cell polarity associated with asymmetric cell division of neuroblasts (Ohshiro *et al.*, 2000; Peng *et al.*, 2000), and the defective polarity acquisition in *l(2)gl* mutants may give rise to the neoplastic transformation of optic neuroblasts observed in mutant larvae (Gateff, 1978). The salt-sensitive phenotype of the yeast *sro7Δ* mutant results from defective targeting of the *ENA1* encoded sodium extruding ATPase to the plasma membrane (Wadskog, 2003). Instead, Ena1p is delivered to the vacuole where it is degraded. Deletion of *SRO7* together with its iso-gene *SRO77* results in hypersensitivity to NaCl stress (Larsson *et al.*, 1998), and a cold-sensitive phenotype (Lehman *et al.*, 1999). At nonpermissive temperature, the *sro7Δsro77Δ* mutant shows defective secretion and accumulation of post-Golgi vesicles (Lehman *et al.*, 1999). In agreement with these phenotypes, the Sro proteins have been shown to interact physically with Sec9p, a target-soluble *N*-ethylmaleimide-sensitive factor attachment receptor (t-SNARE) protein for vesicle fusion at the plasma membrane (Lehman *et al.*, 1999). *SRO7* also shows genetic interaction with temperature-sensitive alleles of genes for

the exocyst complex (Lehman *et al.*, 1999; Wadskog, 2003). Together, these results suggest that the l(2)gl family of proteins assists in the targeting of vesicles to the correct polar destinations in the plasma membrane.

Here, we report the involvement of *SRO7* and *SRO77* in apoptosis-like cell death in yeast. The studies were initiated for several reasons. First, the two phenotypes associated with *sro* mutants, salt sensitivity and secretion defects, have both been previously linked to apoptosis in yeast (Madeo *et al.*, 1997; Huh *et al.*, 2002). Second, most of the known tumor suppressor genes are involved in signaling that lead either to cell cycle arrest or apoptosis (Wyllie *et al.*, 1999). Finally, two-hybrid screens have indicated physical interactions between Sro77p and the yeast metacaspase Yca1p (Uetz *et al.*, 2000).

## MATERIALS AND METHODS

### Yeast Strains and Growth Media

Yeast strains used in this study are listed in Table 1. Rich YPD medium (2% glucose) was used for growth and survival assays, whereas minimal YNB media (2% glucose) plus essential amino acids were used for selective growth. For salt stress conditions, YPD containing 2 M NaCl was added to yeast cultures to obtain final NaCl concentrations.

The *yca1Δsro7Δ* mutant was constructed using the *sro7*::LEU2 deletion cassette in pBluescriptKS$^+$ (Larsson *et al.*, 1998). The plasmid was cut with restriction enzymes *Hind*III and *Not*I (Roche Diagnostics, Mannheim, Germany) and the *ycalΔ* mutant transformed with the *sro7*::LEU2 fragment of 3.5-kb subsequent to gel purification (QIAquick gel extraction kit; QIAGEN, Hilden, Germany). Transformants were selected on YNB medium lacking leucine and subsequently verified by polymerase chain reaction (PCR), by using PCR primers upstream and downstream of the *LEU2* insert. The *yca1Δsro77Δ* mutant was constructed as follows: PCR was used to amplify the *sro77*::HIS3 fragment of strain WKL-3A (Larsson *et al.*, 1998) by using *SRO77* forward primer TTCCGCTTCATAGGAGGAGA (300 base pairs upstream of START codon) and *SRO77* backward primer ACGGTTCATCATTCGGAAAA (350 base pairs downstream of STOP codon). PCR products were purified (QIAquick PCR purification kit; QIAGEN) and used to transform the *yca1Δ* mutant. Transformants were selected on YNB plates lacking histidine and verified by PCR by using primers that bind upstream and downstream of the primers used to amplify the *sro77Δ*::HIS3 fragment. The *yca1Δsro7Δsro77Δ* strain was constructed by transforming the *yca1Δsro7Δ*strain with the *sro77*::HIS3 fragment described previously. Transformants were selected and verified. To construct the BY *sro7Δ sro77Δ* strain BY *sro7Δ* Mat a was crossed with BY *sro77Δ* Mat α. Tetrads were analyzed and potential double mutants checked by PCR. PCR was performed using *SRO7* and *SRO77* verification primers described above plus additional primers binding within the kanamycin gene.

### Growth and Survival Tests

Growth was monitored by plate assays. Yeast strains were grown overnight in YPD medium, adjusted to identical OD$_{610}$ and diluted $10^0$, $10^{-1}$, $10^{-2}$, $10^{-3}$, and $10^{-4}$. Each dilution was spotted in aliquots of 5 μl onto YPD plates with or without NaCl supplementation. For cell survival experiments, yeast cells were grown until they reached exponential phase. NaCl was added to the desired concentration, and samples containing a defined number of cells were plated onto YPD plates after various periods of salt exposure. The number of
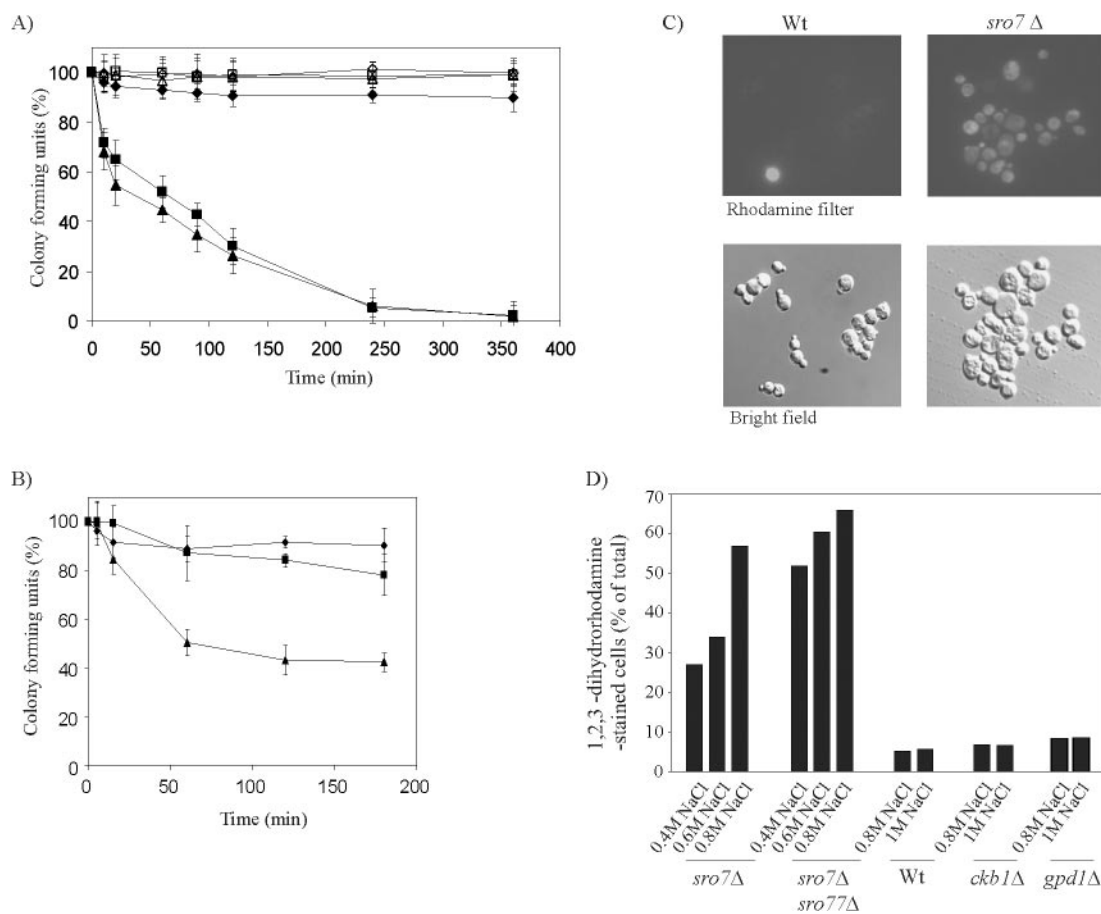
**Figure 1.** Cells lacking *SRO7* accumulate ROS and exhibit reduced survival in saline environments. (A) Survival of wild-type, *sro7Δ*, and *sro7Δsro77Δ* mutants on exposure to 0.7 M NaCl for various periods. Data represent the mean of four different experiments. Wild-type (◆), *sro7Δ* (■), and *sro7Δsro77Δ* (▲). Open symbols represent 0 M NaCl. (B) Survival of wild-type, *sro7Δ*, and *sro7Δsro77Δ* mutants on exposure to 0.4 M NaCl for various periods. Data represent the mean of four different experiments. Wild-type (◆), *sro7Δ* (■), and *sro7Δsro77Δ* (▲). (C) Bright field (bottom row) and fluorescence (top row) photomicrographs of dihydrorhodamine 123 stained wild-type (Wt) and *sro7Δ* cells exposed to 0.8 M NaCl. (D) Diagram showing the percentage of dihydrorhodamine-stained cells of *sro7Δ*, *sro7Δsro77Δ*, Wt, *ckb1Δ*, and *gpd1Δ* strains after exposure to various concentrations of NaCl. Each data bar is based on the evaluation of 500 cells in several independent experiments. For further details, see MATERIAL AND METHODS.

cells was determined (~20 cells/μl, 1:1000 dilution) for each culture by using a Helber counting chamber and a total of 600 cells, divided into three aliquots, were spread onto YPD plates. The number of surviving colonies was determined after 2 d of incubation at 30°C.

### Cytological Analysis

For determination of reactive oxygen species (ROS) accumulation, cells were grown into exponential phase ($OD_{610}$ ~0.5) and exposed to NaCl for 1 h. Dihydrorhodamine 123 (Sigma-Aldrich, St. Louis, MO) was added (5 μg/ml cell culture) and cultivation continued for another 2 h. Stained cells were viewed using a Leica DMRXA fluorescence microscope equipped with a rhodamine optical filter. To determine the frequency of stained cells, at least 500 cells of three independent experiments were evaluated.

Analysis of nuclear fragmentation (4,6-diamidino-2-phenylindole [DAPI] staining) and DNA strand breaks (terminal deoxynucleotidyl transferase dUTP nick-end labeling [TUNEL] analysis) was performed on fixed cells. Exponentially growing cells were fixed by the addition of 1:10 volumes of formaldehyde (leading to a final concentration of 3.7%) followed by incubation for 1 h in 30°C. The cells were washed twice in apoptosis buffer 1 (35 mM potassium phosphate and 0.5 mM $MgCl_2$, pH 6.8), resuspended in apoptosis buffer 2 (apoptosis buffer 1 + 1.2 M sorbitol), and treated with lyticase (50 U/ml) for 30 min at 30°C. After careful washing in apoptosis buffer 2 spheroplasts were bound on poly-lysine–coated slides. For DAPI staining, cells were incubated with diaminophenylindole (1 μg/ml; Sigma-Aldrich) for 2 min, washed twice with apoptosis buffer 2, and examined under a Leica DMRXA fluorescence microscope. TUNEL analysis was performed as described previously (Madeo *et al.*, 1997, 1999).

### Caspase Activity Measurements

Caspase activity was measured by using fluorescein isothiocyanate (FITC)-VAD-fmk (CaspACE; Promega, Madison, WI), which binds at the catalytic center of active caspases. NaCl was added to stationary overnight cultures of wild-type and *yca1Δ* cells at various concentration, and the cultures were incubated for 1 or 4 h at 28°C. After incubation, cells were harvested (5 × 10$^6$ cells, washed in 1 ml phosphate-buffered saline, and stained for 20 min in 200 μl FITC-VAD-fmk diluted 1:1000 in the same buffer. For flow cytometric analysis, stained cells were counted using FACSCalibur (BD Biosciences, San Jose, CA) and CellQuest analysis software with excitation and emission settings of 488 nm and 525–550 nm (filter FL1), respectively.

## RESULTS

### NaCl Stress Reduces Survival of sro7Δ and sro7Δsro77Δ Mutants and Induces Intracellular Accumulation of Reactive Oxygen Species

Survival tests demonstrated that *sro7Δ* and *sro7Δsro77Δ* mutants are highly sensitive to NaCl stress. Already, a 10-min exposure to 0.7 M NaCl killed ~30% of either population (Figure 1A), and <5% remained viable after 6 h. Equivalent treatment of wild-type cells resulted in <5% loss in viability. These results demonstrate that *sro7Δ* and *sro7Δsro77Δ* mutants not only exhibit reduced ability to grow at moderate
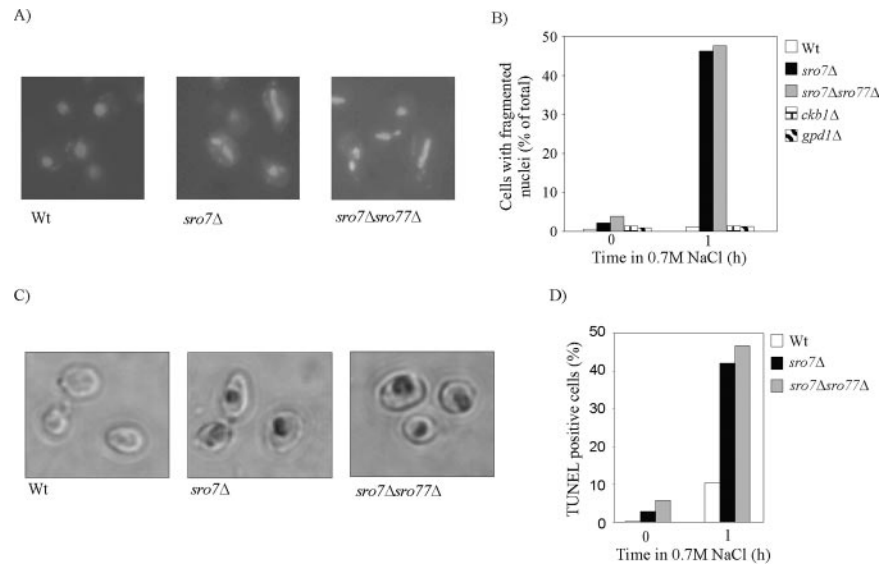
**Figure 2.** *sro7Δ* and *sro7Δsro77Δ* mutants display fragmented nuclei and DNA strand breaks after NaCl treatment. (A) Photomicrograph of DAPI-stained wild-type (Wt), *sro7Δ*, and *sro7Δsro77Δ* cells exposed to YPD containing 0.7 M NaCl for 1 h. (B) Percentage of wild-type (Wt), *sro7Δ*, *sro7Δsro77Δ*, *ckb1Δ*, and *gpd1Δ* cells containing fragmented nuclei after 0 and 1 h in YPD containing 0.7 M NaCl. (C) TUNEL staining of wild-type, *sro7Δ*, and *sro7Δsro77Δ* cells after 30 min of incubation in YPD containing 0.7 M NaCl. (D) Quantification of TUNEL-positive staining of wild-type (Wt), *sro7Δ*, and *sro7Δsro77Δ* after 1-h exposure to 0.7 M NaCl.

salt stress, as has been reported previously (Larsson *et al.*, 1998) but also that the cells are actually killed by the stress. The observations prompted us to analyze the cells for the presence of ROS, which are considered a key stimulus for cell death by apoptosis (Madeo *et al.*, 1999). Using dihydrorhodamine 123 as ROS indicator, accumulation of ROS was observed in ~60% of *sro7Δ* or *sro7Δsro77Δ* mutants exposed to 0.8 M NaCl (Figure 1, C and D). Control wild-type cells showed little dihydrorhodamine staining, even when exposed to 1 M NaCl (Figure 1D). At lower salinities (0.4 and 0.6 M NaCl), the proportion of stained *sro7Δ* cells decreased to become about one-half of that of the *sro7Δsro77Δ* mutants. In agreement with this pattern the double mutant is more efficiently killed by moderate salt stress than the *sro7Δ* single mutant (Figure 1B). We also examined ROS mediated staining of two other yeast mutants, *gpd1Δ* and *ckb1Δ*, that exhibit a salt-sensitive growth phenotype similar to that of *sro7Δ*. Interestingly, the accumulation of ROS in the *gpd1Δ* and the *ckb1Δ* mutants remained similar to that of wild-type cells, even at 1 M NaCl (Figure 1D).

### *sro7Δ* and *sro7Δsro77Δ* Mutants Display Chromatin Condensation and DNA Fragmentation

Having established increased ROS occurrence in salt-stressed *sro* mutants, we wanted to examine the cells for nuclear fragmentation, a well-established cytological hallmark of apoptosis. For the *sro7Δ* mutant, cells with irregularly shaped and fragmented nuclei were evident 30 min after exposure to 0.7 M NaCl (Figure 2A). In contrast, virtually no cells of the similarly treated wild-type strain showed abnormal nuclei. After 1 h at 0.7 M NaCl, about one-half of the *sro7Δ* and *sro7Δsro77Δ* mutants displayed fragmented nuclei (Figure 2B). At lower salinities, the proportion of aberrant nuclei decreased, and this tendency was, as expected, more pronounced for *sro7Δ* mutants than for *sro7Δsro77Δ* mutants (our unpublished data). The salt-sensitive *gpd1Δ* and *ckb1Δ*, mutants showed little chromatin condensation and fragmentation on exposure to NaCl stress similar to wild-type cells (Figure 2B). These mutants show that sensitivity to NaCl is not generally associated with apoptotic features such as ROS accumulation (Figure 1D) and nuclear fragmentation.

Another familiar apoptotic feature is fragmentation of DNA in the nucleus. DNA strand breaks can be visualized

by TUNEL tests, in which fluorescent nucleotides are added to the 3'-OH end of the DNA fragment, making the phenomenon visible by fluorescent microscopy. Both *sro7Δ* and *sro7Δsro77Δ* mutants display TUNEL-positive nuclei after 30 min of exposure to 0.7 M NaCl (Figure 2C), whereas the nuclei of control wild-type cells remained unstained or only slightly stained. After 1 h at 0.7 M NaCl, about one-half of the *sro7Δ* and *sro7Δsro77Δ* population showed a clear TUNEL-positive staining, whereas ~10% of the wild-type cells exhibited this characteristic (Figure 2D). We conclude that NaCl stress that does not markedly affect viability of wild-type cells, induces cell death in *sro7Δ* and *sro7Δ sro77Δ* mutants that bears the structural attributes of apoptosis with respect to ROS accumulation, nuclear fragmentation, and DNA strand breakage.

### *Yca1p Is Involved in the NaCl-induced Lethality of sro7Δ Mutants*

To investigate whether the yeast metacaspase Yca1p has a role in NaCl-mediated cell death, we first examined whether salt stressed cells show increased caspase activity. To this end, cells were incubated with the FITC-labeled pan-caspase inhibitor VAD-fmk that binds to the active site of the caspase, allowing for flow cytometric determination of cells with active enzyme (Madeo *et al.*, 2002). This assay revealed a significant increase in the proportion of cells with active caspase after exposure to NaCl stress for 4 h (Figure 3). Compared with nonstressed conditions, wild-type cells exposed to 1.0 M NaCl increase their caspase activity more than sevenfold. Cells lacking *YCA1* exhibit basal level caspase activity both at 0 and 0.5 M NaCl, whereas a modest increase was apparent at 1 M NaCl. We next examined the involvement of Yca1p in the NaCl induced lethality by deleting the *YCA1* gene in the *sro7Δ* background. If the salt sensitivity of the *sro7Δ* strain is caused by Yca1p-mediated cell death, the *sro7Δyca1Δ* double mutant would display higher salt tolerance than the *sro7Δ* single mutant. This simple prediction was, however, not supported by testing serial dilutions for growth on YPD plates (Figure 4A), which showed that the sensitivity of *sro7Δ* mutants to 0.7 M NaCl is very similar to that of *sro7Δyca1Δ* mutants. In fact, the two strains grew equally well at all NaCl concentrations tested, showing inhibited growth by salt concentrations of ≥0.5 M
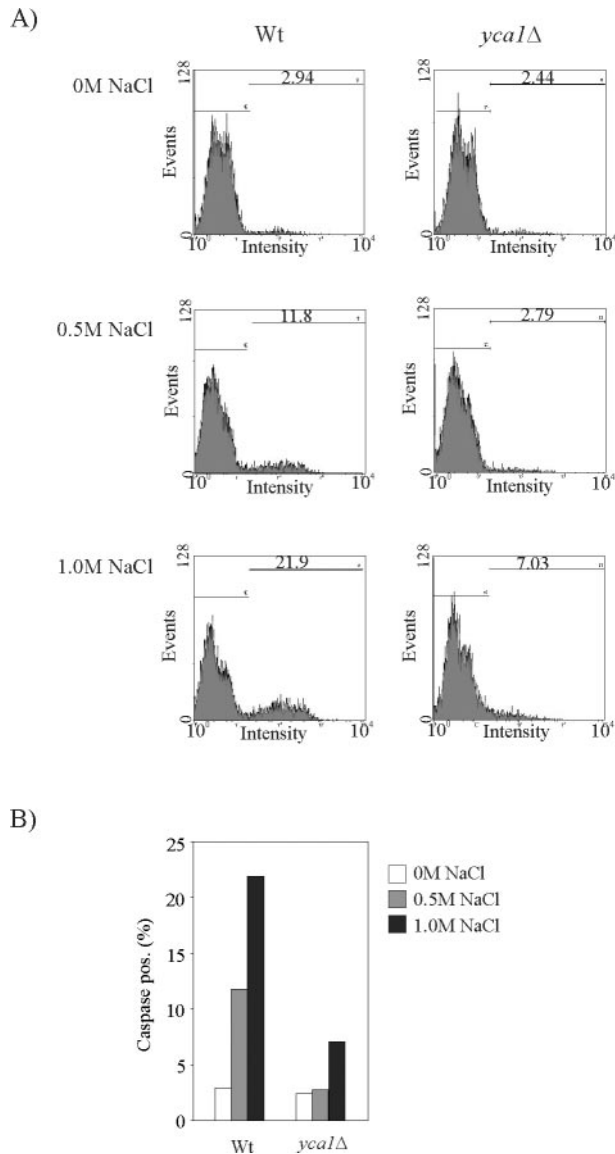
A)



B)



**Figure 3.** Salt-stressed cells exhibit increased caspase activity. (A) Caspase activity measurement by flow cytometric analysis of wild-type and *yca1Δ* mutant cells exposed to NaCl (0, 0.5, or 1.0 M) for 4 h. (B) Percentage of FITC-VAD-fmk–positive cells in fluorescence-activated cell sorting measurements of wild-type (Wt) and *yca1Δ* mutant strains exposed to different NaCl concentrations.

NaCl (our unpublished data). Similar result was obtained with the *sro7Δsro77Δ* double mutant. This mutant experience growth inhibition at ≥0.2 M NaCl, and additional deletion of *YCA1* did not improve NaCl tolerance as revealed by growth tests on YPD plates containing various salt concentrations (our unpublished data). However, similar growth inhibition in the presence of NaCl does not necessarily mean that the strains survive equally well on exposure to salt stress. Actually, there was a significant difference between the survival traits of *sro7Δ* and *sro7Δyca1Δ* mutants (Figure 4B). The *sro7Δ* mutants exhibit an immediate decrease in viability after exposure to 0.7 M NaCl, leading to a rapid elimination of ~30% of the population. Similar stress exposure of the *sro7Δyca1Δ* mutants resulted only in a slight decrease in cell viability (~5%). However, after the rapid

phase of cell demise, the death rate for the *sro7Δ* population changed to a pace that was similar to that shown by the *sro7Δ yca1Δ* mutants (Figure 4B). These results suggest that Yca1p is required for the primary, fast drop in viability of the *sro7Δ* mutants. The better survival of the *sro7Δ yca1Δ* strain is consistent with markedly decreased DNA breakage (Figure 4, C and D). A similar tendency was apparent for the control *yca1Δ* and wild-type strains, although the salt concentration is too low to produce strong effects in these strains.

### Deletion of YCA1 Improves Survival of Wild-Type Cells but Has Little Effect on Survival of sro77Δ Mutants

Yca1p has previously been shown to mediate apoptosis-like cell death in aged yeast cells and in yeasts treated with $H_2O_2$ or acetic acid (Madeo *et al.*, 2002). For example, cells lacking *YCA1* survive high levels of $H_2O_2$ much longer than wild-type cells. Yca1p obviously also has a role in mediating demise of wild-type cells exposed to high salinity. About 50% of wild-type yeast survives exposure to 1.2 M NaCl for 4 h, whereas ~85% of the cells survives the same treatment if deleted for the *YCA1* gene (Figure 5B). This observation agrees with the decreased DNA cleavage seen for the *yca1Δ* mutant (Figure 5, C and D). Hence, survival assays and caspase activity measurements demonstrate that the apoptotic-like response seen for wild-type cells during salt stress is strongly dependent on Yca1p activity.

The *sro77Δ* mutants show a similar tolerance to 1.2 M NaCl as wild-type cells. Interestingly, however, deletion of *YCA1* in *sro77Δ* background does not significantly improve the viability at high salinity, as it does for wild-type cells (Figure 5B). In accordance with these results, the *sro7Δsro77Δ* and the *sro7Δsro77Δyca1Δ* mutants also display similar survival curves after exposure to 0.4 M NaCl (Figure 5A). The observation that NaCl induced lethality is independent of Yca1p in strains lacking *SRO77*, is in agreement with the finding that DNA breakage (Figure 5, C and D) and nuclear fragmentation (our unpublished data) is very similar for *sro77Δ* and *sro77Δyca1Δ* cells. Similarly, nuclear fragmentation and the proportion of TUNEL-positive cells are very similar for NaCl exposed *sro7Δsro77Δ* and the *sro7Δsro77Δyca1Δ* mutants (our unpublished data). These results imply a genetic interaction between *SRO77* and *YCA1*, which encode proteins that have been previously shown to interact in two-hybrid screens (Uetz *et al.*, 2000; Drees *et al.*, 2001). To further study the effect of Sro7p and Sro77p on Yca1p, we examined the proportion of cells with active caspase in wild-type, *yca1Δ*, *sro7Δ*, and *sro77Δ* cultures exposed to NaCl stress for 1 h (Figure 5E). The observed caspase activity remained unaffected by salt stress in *sro77Δ* mutants, similar to what was noted for *yca1Δ* cells, whereas mutants lacking *SRO7* exhibited a generally increased casapase activity, which consistently was kept higher than that of wild-type cells. These results indicate that Sro7p and Sro77p have opposing effects on Yca1p; Sro77p being required for the salt induced activation of Yca1p, whereas Sro7p seems to moderate the activity of the caspase. Furthermore, the observation that *sro77Δyca1Δ* mutants show NaCl induced nuclear fragmentation and strong DNA strand breakage (Figure 5, C and D) suggests the existence of an Yca1p independent apoptotic pathway in yeast.
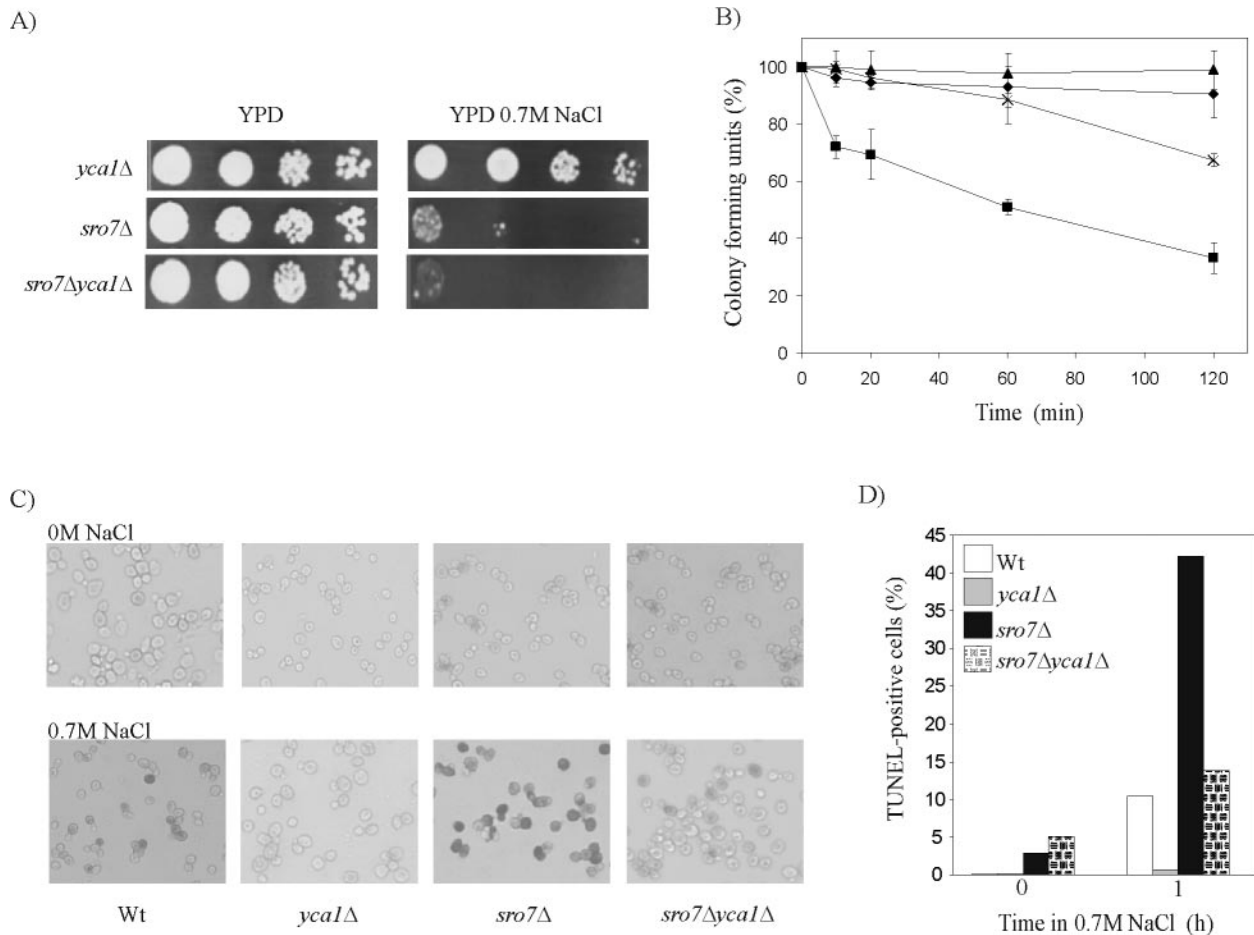
A)



B)



C)



D)



**Figure 4.** Rate of killing of *sro7Δ* cells after exposure to salt stress is *YCA1* dependent, and the level of salt induced double stranded DNA breaks is reduced in the *sro7Δyca1Δ* mutant. (A) Growth of *sro7Δ*, *yca1Δ*, and *sro7Δyca1Δ* mutants at high salinity monitored by drop assays. Serial 10-fold dilutions of cell suspensions were spotted onto YPD medium with and without 0.7 M NaCl. (B) Survival curves for wild-type (♦), *sro7Δ* (■), *yca1Δ* (▲), and *sro7Δyca1Δ* (X) mutants after incubation with 0.7 M NaCl. Samples were taken after incubation with 0.7 M NaCl for various periods of time and plated onto YPD medium. Each data point is based the plating of 200 cells in three independent experiments. (C) Photographs of TUNEL staining showing DNA breakage in the *sro7Δ* mutant after 1 h of incubation in YPD medium containing 0.7 M NaCl. (D) Quantification of TUNEL-positive cells subsequent to 1 h 0.7 M NaCl exposure. To determine the frequency of TUNEL-positive cells, at least 1000 cells were evaluated for each strain and each condition.

## DISCUSSION

### Exposed to Salt Stress, the sro7Δ Mutant Exhibits Hallmarks of Apoptosis

Cells committing active suicide by apoptosis are subject to a series of stereotyped morphological changes that differentiate apoptotic cells from healthy or necrotic cells (Webb *et al.*, 1997; Kerr, 2002). The most typical structural changes occur within the nucleus, where chromatin condenses and aggregates along the margins of the nuclear membrane and DNA is degraded, first into large fragments and later into short oligomers of ~180–200 base pairs. Coincident with these changes are alterations of the nuclear structural framework leading to fragmentation of the nucleus. Here, we demonstrate NaCl-induced cell death associated with the typical nuclear features of apoptosis for yeast cells deleted for the tumor suppressor homologue *SRO7*. NaCl stress is one of the environmental factors that has been demonstrated previously to induce apoptosis-like cell death in yeast and plants (Katsuhara, 1997; Huh *et al.*, 2002). Wild-type cells of *S. cerevisiae* were shown to exhibit apoptotic features after treatment with 1.2 M NaCl (Katsuhara, 1997; Huh *et al.*,

2002). The *sro7Δ* mutant displays a similar response at NaCl concentrations beginning at 0.5 M NaCl. Because the inducing salinity correlates with the NaCl concentration for strong growth inhibition of each strain, a simple interpretation would be that severely growth-inhibiting concentrations suffice to initiate apoptosis-like cell death. In other words, the lack of *SRO7* may simply lower the salt concentration needed for apoptotic induction. However, two other yeast mutants that display NaCl sensitivity similar to that of *sro7Δ*, namely, *ckb1Δ* (de Nadal *et al.*, 1999) and *gpd1Δ* (Larsson *et al.*, 1993; Albertyn *et al.*, 1994), do not show nuclear fragmentation (Figure 2B) and do not seem to accumulate reactive oxygen species (Figure 1D) on exposure to growth preventing NaCl concentrations. The salt sensitivity of the *gpd1Δ* mutant is due to a defective production and intracellular accumulation of glycerol, the primary yeast osmoregulator (Larsson *et al.*, 1993). The reason for the salt-sensitive phenotype of the *ckb1Δ* mutant, which lacks a functional casein kinase II, is less clear, although the mutant seems to maintain intracellular ion homeostasis under salt stress (de Nadal *et al.*, 1999). Hence, the different display of apoptotic
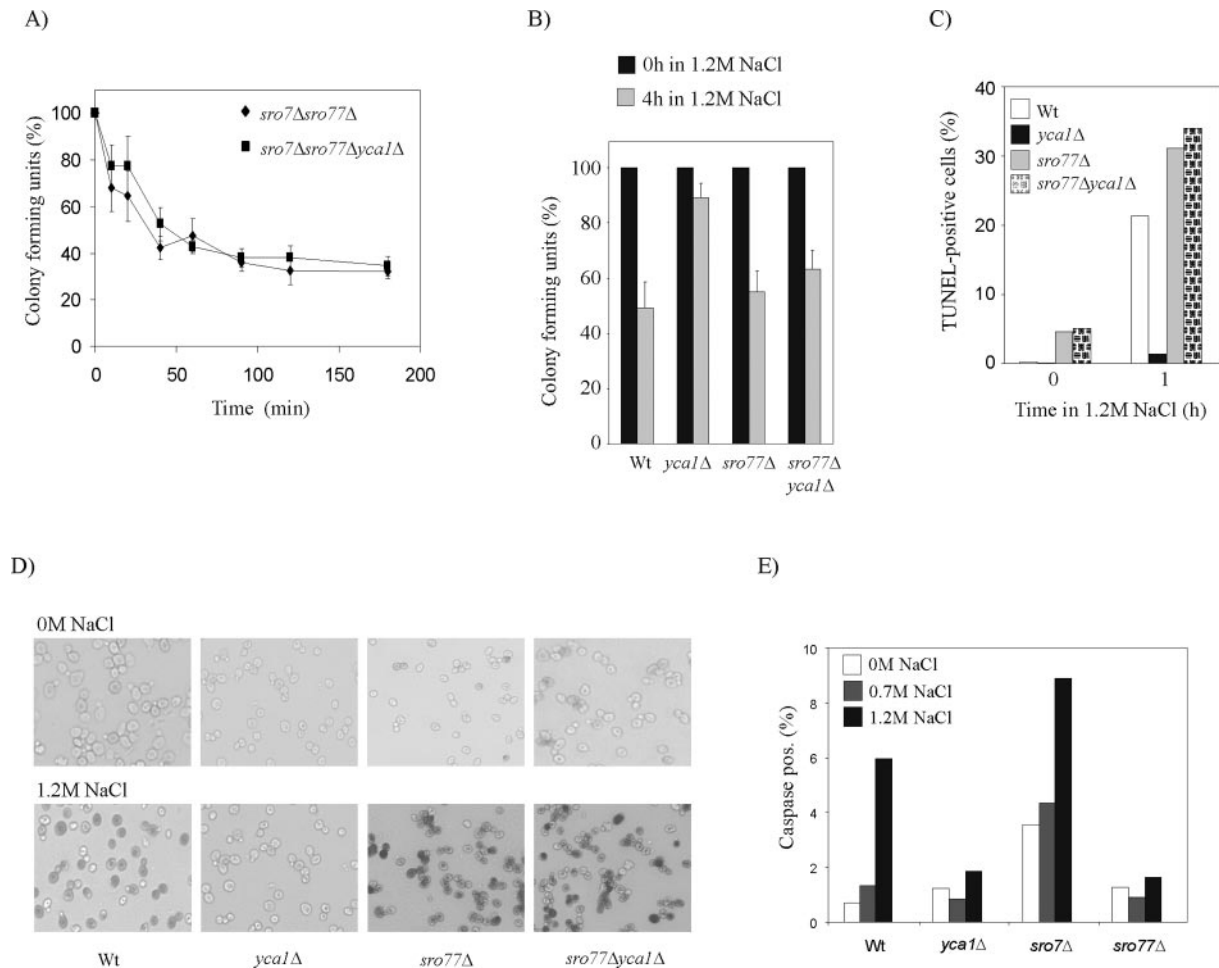
**Figure 5.** Survival at high salinity is *YCA1*dependent for wild-type cells but not for *sro77Δ* mutants. (A) Survival kinetics of *sro7Δsro77Δ* and *sro7Δsro77Δyca1Δ* cells in YPD containing 0.4 M NaCl. (B) Survival of wild-type, *yca1Δ*, *sro77Δ*, and *sro77Δyca1Δ* after incubation for 4 h in YPD containing 1.2 M NaCl. (C) Percentage of TUNEL-positive cells after 1-h exposure to 1.2 M NaCl. For quantification, at least 1000 cells were evaluated for each strain and each condition. (D) Photographs showing examples of TUNEL staining of wild-type (Wt), *yca1Δ*, *sro77Δ*, and *sro77Δyca1Δ* cells before and after 1-h exposure to1.2 M NaCl. (E) Caspase activity, shown as percentage of FITC-VAD–positive cells by fluorescence-activated cell sorting analysis of wild-type, *yca1Δ*, *sro7Δ*, and *sro77Δ* strains, grown overnight, and exposed to NaCl stress for 1 h.

features in response to growth inhibiting NaCl concentrations by *sro7Δ* mutants, on the one hand, and *gpd1Δ* and *ckb1Δ* mutants on the other, reinforces the observations by Huh *et al.* (2002) that disruption of inorganic ion homeostasis seems to be the primary reason for salt-induced cell death of yeast cells. Due to the defective targeting and subsequent degradation of the Ena1p sodium transporter in *sro7Δ* mutants, these cells experience increased intracellular $Na^+/K^+$ ratios when exposed to NaCl stress (Larsson *et al.*, 1998; Wadskog, 2003). The *ENA1* gene encodes a P-type ATPase that uses ATP to drive sodium ions out of the cell, and this pump has a primary role in maintaining cation homeostasis in NaCl-stressed yeast (Haro *et al.*, 1991; Wieland *et al.*, 1995). Interestingly, expression of the *ENA1* gene is subject to a highly complex regulation, being controlled by a number of distinct regulatory pathways (Serrano *et al.*, 1999; Wadskog and Adler, 2003). It is tempting to speculate that this elaborate control of *ENA1* expression reflects a strict need to fine-tune ion homeostasis to prevent creating an intracellular environment that favors the activation of a cell death program. An additional indication for a role of *ENA1* in this context stems from the observation that the human anti-

apoptotic protein Bcl-2 promoted transcriptional activation of *ENA1* when expressed in a salt-sensitive *cnb1Δ* mutant (Huh *et al.*, 2002).

### The Yca1p Metacaspase Promotes Salt-induced Cell Death

The *YCA1* gene in *S. cerevisiae* encodes a metacaspase that is activated by $H_2O_2$ and aging and is required for apoptotic demise of yeast (Madeo *et al.*, 2002). Yca1p undergoes proteolytic processing similar to mammalian caspases and cleaves petidyl-caspase substrates, giving direct support for the existence of a specific effector of apoptosis in yeast. To investigate whether the apoptotic traits of NaCl-exposed cells were dependent on Yca1p, we compared wild-type and *sro7Δ* mutants with the corresponding strains lacking *YCA1*. For wild-type cells, deletion of *YCA1* significantly improved survival rate at high salinity (Figure 4B), which suggests a general role for Yca1p in salt-induced apoptosis. The marked increase in caspase activity of cells exposed to harsh NaCl stress (Figure 3) gives further support to this view. Two additional observations provide evidence that Yca1p serves as an effector of the salt-induced cell death in yeast.

Yca1p was more strongly activated in the salt sensitive *sro7*Δ mutants than in the more tolerant wild-type cells (Figure 5E), and deletion of *YCA1* markedly affected both survival rate and DNA cleavage of the NaCl stressed strains (Figures 4, B–D, and 5, B–D). These results suggest that Yca1p mediates an apoptosis-like cell death, which is rapid and efficient. However, only a fraction of the cells seems to be susceptible to the Yca1p-mediated cell death. The first dramatic decrease of cell viability of *sro7*Δ cells was followed by a more moderate rate of killing that seemed independent of Yca1p. This decrease in viability may result from a more general effect of Na$^+$ toxicity on the *sro7*Δ mutant and explain why the *sro7*Δ*yca1*Δ mutant displays a salt-sensitive phenotype on plate assays.

### Role of SRO7 and SRO77 in Apoptosis-like Cell Death

Because interactions between Sro77p and Yca1p are suggested by two-hybrid screens (Uetz *et al.*, 2000; Drees *et al.*, 2001), we wanted to investigate the role of *SRO77* in salt-induced apoptosis-like cell death. Interestingly, deletion of *YCA1* in *sro7*Δ*sro77*Δ or *sro77*Δ backgrounds did not improve viability on NaCl treatment (Figure 5, A and B), indicating that the function of Yca1p is dependent on *SRO77*. Indeed, our measurement of salt-induced Yca1p activation shows that the caspase activity does not increase in mutants lacking *SRO77*, but remains similar to that of cells having a deleted *YCA1* gene. The *SRO*-encoded proteins have been implicated in a late step in exocytosis by their ability to physically interact with Sec9p, a t-soluble *N*-ethyl-maleimide-sensitive factor attachment (t-SNARE) protein receptor involved in the fusion of post-Golgi vesicles with the plasma membrane (Lehman *et al.*, 1999). Given the proposed role in vesicle targeting, the opposing consequences of loss of function of *SRO7* and *SRO77* on the susceptibility of yeast to NaCl-induced cell death, suggest that the exocytosis machinery might sensitively attune the responsiveness of the cells to the exogenous apoptotic stimuli. The observed hyperactivation of the caspase in *sro7*Δ strain (Figure 5E) and the fact that these mutants show signs of DNA strand breakage (Figure 2C), even before exposure to NaCl stress, indicates that loss of *SRO7* promotes a generally increased disposition for apoptosis-like cell death. The precise mechanistic relationship between Yca1p and the Sro proteins remains, however, to be determined. Because both *sro77*Δ*yca1*Δ (Figure 5, C and D) and *sro7*Δ*sro77*Δ*yca1*Δ (our unpublished data) cells exhibit diagnostic markers of apoptosis when exposed to sufficiently high NaCl concentration, our findings also indicate the existence of a Yca1p-independent pathway(s) for cellular suicide in *S. cerevisiae*. This pathway(s) may be under negative control that is dependent on Sro77p.

### Are There Links to the Function of the Authentic Tumor Suppressor?

Whether the observed sensitivity of *sro7*Δ and *sro7*Δ*sro77*Δ mutants to NaCl-induced cell death bears any relevance to tumor development in *Drosophila* larvae lacking the authentic tumor suppressor *l(2)gl* is as yet speculative. Homozygous *l(2)gl* mutants show phenotypes involving loss of epithelial cell polarity and neoplastic overgrowth of tissues (Wodarz, 2000). Recent studies have indicated that loss of epithelial cell polarity may confer increased susceptibility to apoptosis (Weaver *et al.*, 2002). This sensitivity has been suggested to counterbalance the proliferative advantage that is associated with nonpolarized cells, thereby reducing the possibility for malignant transformation (Humbert *et al.*, 2003). Interestingly, De Lorenzo *et al.* (1999) have reported

that a mutant form of *l(2)gl* induces apoptosis of the germ line during oogenesis, indicating that a defective tumor suppressor may promote apoptosis. In most epithelial cells the Na$^+$, K$^+$ ATPase is selectively sorted to the basolateral membrane (Dunbar and Caplan, 2001). Whether *l(2)gl* mutants exhibit defective epithelial distribution of this sodium transporter and suffer from a consequent disruption of inorganic ion homeostasis remain to be determined. Additional studies of the relationship between ion homeostasis and epithelial tumor development/apoptosis may further our understanding of the signals controlling growth, survival, and death of cells.

## REFERENCES

Albertyn, J., Hohmann, S., Thevelein, J.M., and Prior, B.A. (1994). *GPD1*, which encodes glycerol-3-phosphate dehydrogenase is essential for growth under osmotic stress in *Saccharomyces cerevisiae* and its expression is regulated by the high-osmolarity glycerol response pathway. Mol. Cell. Biol. *14*, 4135–4144.

Ansell, R., Granath, K., Hohmann, S., Thevelein, J., and Adler, L. (1997). The two isoenzymes for yeast NAD-dependent glycerol 3-phosphate dehydrogenase encoded by *GPD1* and *GPD2*, have distinct roles in osmoadaption and redox regulation. EMBO J. *16*, 2179–2187.

Beers, E.P., and McDowell, J.M. (2001). Regulation and execution of programmed cell death in response to pathogens, stress and developmental cues. Curr. Opin. Plant Biol. *4*, 561–567.

Bilder, D., Li, M., and Perrimon, N. (2000). Cooperative regulation of cell polarity and growth by *Drosophila* tumor suppressors. Science *289*, 113–116.

De Lorenzo, C., Strand, D., and Mechler, B.M. (1999). Requirement of *Drosophila l(2)gl* function for survival of the germline cells and organization of the follicle cells in a columnar epithelium during oogenesis. Int. J. Dev. Biol. *43*, 207–217.

de Nadal, E., Calero, F., Ramos, J., and Arino, J. (1999). Biochemical and genetic analyses of the role of yeast casein kinase 2 in salt tolerance. J. Bacteriol. *181*, 6456–6462.

Drees, B.L., *et al.* (2001). A. protein interaction map for cell polarity development. J. Cell Biol. *154*, 549–571.

Dunbar, L.A., and Caplan, M.J. (2001). Ion pumps in polarized cells: sorting and regulation of the Na$^+$, K$^+$- and H$^+$, K$^+$-ATPases. J. Biol. Chem. *276*, 29617–19620.

Engelberg-Kulka, H., and Glaser, G. (1999). Addiction modules and programmed cell death and antideath in bacterial cultures. Annu. Rev. Microbiol. *53*, 43–70.

Fröhlich, K.U., and Madeo, F. (2000). Apoptosis in yeast - a monocellular organism exhibits altruistic behaviour. FEBS Lett. *473*, 6–9.

Fröhlich, K.U., and Madeo, F. (2001). Apoptosis in yeast: a new model for aging research. Exp. Gerontol. *37*, 27–31.

Gateff, E. (1978). Malignant neoplasms of genetic origin in *Drosophila* melanogaster. Science *200*, 1448–1459.

Haro, R., Garciadeblas, B., and Rodriguéz-Navarro, A. (1991). A novel P-type ATPase from yeast involved in sodium transport. FEBS Lett. *291*, 189–191.

Huh, G.H., Damsz, B., Matsumoto, T.K., Reddy, M.P., Rus, A.M., Ibeas, J.I., Narasimhan, M.L., Bressan, R.A., and Hasegawa, P.M. (2002). Salt causes ion disequilibrium-induced programmed cell death in yeast and plants. Plant J. *29*, 649–659.

Humbert, P., Russell, S., and Richardson, H. (2003). Dlg, Scribble and Lgl in cell polarity, cell proliferation and cancer. Bioessays *25*, 542–553.

Joza, N., Kroemer, G., and Penninger, J.M. (2002). Genetic analysis of the mammalian cell death machinery. Trends Genet. *18*, 142–149.

Katsuhara, M. (1997). Apoptosis like cell death in barley roots under salt stress. Plant Cell Physiol. *38*, 1091–1093.

Kerr, J.F. (2002). History of the events leading to the formulation of the apoptosis concept. Toxicology *181–182*, 471–474.

Larsson, K., Böhl, F., Sjöström, I., Akhtar, N., Strand, D., Mechler, B., Grabowski, R., and Adler, L. (1998). The *Saccharomyces cerevisiae SOP1* and *SOP2* genes, which act in cation homeostasis, can be functionally substituted by the *Drosophila lethal(2)giant larvae* tumor suppressor gene. J. Biol. Chem. *273*, 33610–33618.

Larsson, K., Eriksson, P., Ansell, R., and Adler, L. (1993). A gene encoding *sn*-glycerol 3-phosphate dehydrogenase (NAD$^+$) complements an osmosensitive mutant of *Saccharomyces cerevisiae*. Mol. Microbiol. *10*, 1101–1111.

Latterich, M., Fröhlich, K.U., and Schekman, R. (1995). Membrane fusion and the cell cycle: Cdc48p participates in the fusion of ER membranes. Cell *82*, 885–893.

Laun, P., Pichova, A., Madeo, F., Fuchs, J., Ellinger, A., Kohlwein, S., Dawes, I., Fröhlich, K.U., and Breitenbach, M. (2001). Aged mother cells of *Saccharomyces cerevisiae* show markers of oxidative stress and apoptosis. Mol. Microbiol. *39*, 1166–1173.

Lehman, K., Rossi, G., Adamo, J.E., and Brennwald, P. (1999). Yeast homologues of tomosyn and lethal giant larvae function in exocytosis and are associated with the plasma membrane SNARE, Sec9. J. Cell Biol. *146*, 125–140.

Madeo, F., Fröhlich, E., and Fröhlich, K.U. (1997). A yeast mutant showing diagnostic markers of early and late apoptosis. J. Cell Biol. *139*, 729–734.

Madeo, F., Fröhlich, E., Ligr, M., Grey, M., Sigrist, S.J., Wolf, D.H., and Fröhlich, K.U. (1999). Oxygen stress: a regulator of apoptosis in yeast. J. Cell Biol. *145*, 757–767.

Madeo, F., *et al*. (2002). A caspase-related protease regulates apoptosis in yeast. Mol. Cell *9*, 911–917.

Matsuyama, S., Nouraini, S., and Reed, J.C. (1999). Yeast as a tool for apoptosis research. Curr. Opin. Microbiol. *2*, 618–623.

Ohshiro, T., Yagami, T., Zhang, C., and Matsuzaki, F. (2000). Role of cortical tumour-suppressor proteins in asymmetric division of *Drosophila* neuroblast. Nature *408*, 593–596.

Peng, C.Y., Manning, L., Albertson, R., and Doe, C.Q. (2000). The tumour-suppressor genes lgl and dlg regulate basal protein targeting in *Drosophila* neuroblasts. Nature *408*, 596–600.

Qi, H., Li, T.-K., Kuo, D., Nur-El-Kamal, A., and Li, L.F. (2003). Inactivation of Cdc13p triggers *MEC1*-dependent apoptotic signals in yeast. J. Biol. Chem. *278*, 15136–15141.

Serrano, R., *et al*. (1999). A glimpse of the mechanisms of ion homeostasis during salt stress. J. Exp. Bot. *50*, 1023–1036.

Severin, F.F., and Hyman, A.A. (2002). Pheromone induces programmed cell death in *S. cerevisiae*. Curr. Biol. *12*, R233–R235.

Uetz, P., *et al*. (2000). A comprehensive analysis of protein-protein interactions in *Saccharomyces cerevisiae*. Nature *403*, 623–627.

Uren, A.G., and Vaux, D.L. (1996). Molecular and clinical aspects of apoptosis. Pharmacol. Ther. *72*, 37–50.

Wadskog, I., and Adler, L. (2003). Ion homeostasis in *Saccharomyces cerevisiae* under NaCl stress. In: S. Hohmann and P. Mager, eds. Topics in current genetics: yeast stress responses (vol. 1). Berlin, Springer-Verlag, 201–239.

Wadskog, I. (2003). Functional studies of the Sro yeast homologues of the *Drosophila* lethal(2) giant larvae tumor suppressor. [Ph.D. thesis]. Göteborg, Sweden: Götenborg University.

Vaux, D.L., and Korsmeyer, S.J. (1999). Cell death in development. Cell *96*, 245–254.

Weaver, V.M., Lelievre, S., Lakins, J.N., Chrenek, M.A., Jones, J.C., Giancotti, F., Werb, Z., and Bissell, M.J. (2002). beta4 Integrin-dependent formation of polarized three-dimensional architecture confers resistance to apoptosis in normal and malignant mammary epithelium. Cancer Cell *2*, 205–216.

Webb, S., Harrison, D.J., and Wyllie, A.H. (1997). Apoptosis: an overview of the process and its relevance in disease. Adv. Pharmacol. *41*, 1–34.

Wieland, J., Nietsche, A.M., Strayle, J., Steiner, H., and Rudolph, H.K. (1995). The *PMR2* gene cluster encodes functionally distinct isoforms of a putative Na$^+$ pump in the yeast plasma membrane. EMBO J. *14*, 3870–3882.

Wodarz, A. (2000). Tumor suppressors: linking cell polarity and growth control. Curr. Biol. *10*, R624–R626.

Wyllie, A.H., *et al*. (1999). Apoptosis and carcinogenesis. Br. J. Cancer *80*, 34–37.

Wyllie, A.H., Kerr, J.F., and Currie, A.R. (1980). Cell death: the significance of apoptosis. Int. Rev. Cytol. *68*, 251–306.