

# Essential role of calcineurin in response to endoplasmic reticulum stress

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**Depletion of calcium ions (Ca<sup>2+</sup>) from the endoplasmic reticulum (ER) of yeast cells resulted in the activation of the unfolded protein response (UPR) signaling pathway involving Ire1p and Hac1p. The depleted ER also stimulated Ca<sup>2+</sup> influx at the plasma membrane through the Cch1p–Mid1p Ca<sup>2+</sup> channel and another system. Surprisingly, both Ca<sup>2+</sup> influx systems were stimulated upon accumulation of misfolded proteins in the ER even in the presence of Ca<sup>2+</sup>. The ability of misfolded ER proteins to stimulate Ca<sup>2+</sup> influx at the plasma membrane did not require Ire1p or Hac1p, and Ca<sup>2+</sup> influx and signaling factors were not required for initial UPR signaling. However, activation of the Ca<sup>2+</sup> channel, calmodulin, calcineurin and other factors was necessary for long-term survival of cells undergoing ER stress. A similar calcium cell survival (CCS) pathway operates in the pathogenic fungi and promotes resistance toazole antifungal drugs. These findings reveal an unanticipated new regulatory mechanism that couples ER stress to Ca<sup>2+</sup> influx and signaling pathways, which help to prevent cell death and promote resistance to an important class of fungistatic drugs.**

*Keywords:* *Candida albicans*/capacitative calcium entry/cell death/ER stress/*Saccharomyces cerevisiae*

## Introduction

The endoplasmic reticulum (ER) of mammalian cells accumulates high levels of Ca<sup>2+</sup> from the cytoplasm through the action of high-affinity Ca<sup>2+</sup> pumps of the sarco-endoplasmic reticulum Ca<sup>2+</sup> ATPase (SERCA) family (Moller *et al.*, 1996; MacLennan *et al.*, 1997). This pool of Ca<sup>2+</sup> is important for the translocation, folding, glycosylation, disulfide bonding and sorting of secretory proteins in the ER through its direct interactions with a variety of ER-resident molecular chaperones and enzymes (Meldolesi and Pozzan, 1998). Agents that cause sustained depletion of Ca<sup>2+</sup> from the ER, such as Ca<sup>2+</sup> ionophores and chelators, inhibitors of SERCA pumps and activators of Ca<sup>2+</sup> release channels, can all affect the efficiency of protein folding in the ER and thereby stimulate the unfolded protein response (UPR) signaling pathways (Lamarche *et al.*, 1985; Drummond *et al.*, 1987; Price *et al.*, 1992; Gill *et al.*, 1996). UPR signaling in mammalian cells involves the transmembrane protein

kinases Ire1 $\alpha$ , Ire1 $\beta$  and PERK, which dimerize and become activated in response to the accumulation of unfolded proteins in the ER (Shi *et al.*, 1998; Tirasophon *et al.*, 1998; Wang *et al.*, 1998; Harding *et al.*, 1999). The activated Ire1 $\alpha$  and Ire1 $\beta$  kinases induce the transcription of the genes encoding ER-resident molecular chaperones and inhibit the translation of mRNAs encoding secretory proteins (Harding *et al.*, 1999; Kaufman, 1999). Therefore, activation of UPR signaling pathways serves to enhance ER functioning on demand.

A second response to depletion of Ca<sup>2+</sup> from the ER is the stimulation of Ca<sup>2+</sup> influx through the plasma membrane, a process known as store-operated or capacitative Ca<sup>2+</sup> entry (CCE) (Putney, 1986, 1990). This process serves to replenish the Ca<sup>2+</sup>-depleted organelles and to activate calcium signaling pathways. The molecular mechanisms by which plasma membrane Ca<sup>2+</sup> channels become activated upon depletion of Ca<sup>2+</sup> from the ER are not yet clear.

Recent evidence indicates that yeast may employ a CCE-like mechanism that helps to replenish secretory organelles with Ca<sup>2+</sup> and to activate calcium signaling pathways (Locke *et al.*, 2000). Yeast cells lack homologs of SERCA-type Ca<sup>2+</sup> pumps, but express Pmr1p, a homolog of mammalian secretory pathway Ca<sup>2+</sup> ATPases (SPCAs) (Rudolph *et al.*, 1989; Antebi and Fink, 1992; Strayle *et al.*, 1999). Pmr1p localizes to the Golgi complex and is important for supplying the Ca<sup>2+</sup> and Mn<sup>2+</sup> required for efficient glycosylation, processing and sorting reactions in this compartment (Dürr *et al.*, 1998). Like mammalian cells treated with inhibitors of SERCA, *pmr1* null mutants exhibit high rates of Ca<sup>2+</sup> influx via a high-affinity Ca<sup>2+</sup> channel composed of Cch1p and Mid1p (Locke *et al.*, 2000). Cch1p is homologous to the catalytic subunit of voltage-gated Ca<sup>2+</sup> channels in animals and, together with a putative regulatory subunit Mid1p, forms a Ca<sup>2+</sup> influx pathway that can also be stimulated during the mating response (Iida *et al.*, 1994; Fischer *et al.*, 1997; Paidhungat and Garrett, 1997; Muller *et al.*, 2001). The elevated Ca<sup>2+</sup> influx in *pmr1* mutants activates the calcineurin-dependent transcription factor Tcn1p (Matheos *et al.*, 1997; Stathopoulos and Cyert, 1997; Mendizabal *et al.*, 1998), which induces Pmc1p, a homolog of plasma membrane Ca<sup>2+</sup> ATPases (PMCA) localized to the vacuole (Cunningham and Fink, 1994, 1996; Halachmi and Eilam, 1996; Marchi *et al.*, 1999). All these phenotypes of *pmr1* mutants can be suppressed by expression of mammalian SERCA1a (Degand *et al.*, 1999; Locke *et al.*, 2000). Together, these findings indicate that Pmr1p is the functional equivalent of mammalian SERCA and that the Cch1p–Mid1p Ca<sup>2+</sup> channel can become activated by a CCE-like mechanism.

Roles for Ca<sup>2+</sup> in the yeast ER have been difficult to elucidate. The yeast calnexin homolog appears to function

well without  $\text{Ca}^{2+}$  (Parlati *et al.*, 1995). Additionally, free  $\text{Ca}^{2+}$  in the lumen of the yeast ER is 10–100 times lower than that of mammalian ER (Strayle *et al.*, 1999). Mutants lacking Pmr1p accumulated half as much  $\text{Ca}^{2+}$  in the ER, but this decrease failed to stimulate UPR signaling (Dürr *et al.*, 1998), indicating that high luminal  $\text{Ca}^{2+}$  may not be required for protein folding in the ER of yeast. Because SERCA homologs have been identified in animals, plants, protozoa and even other fungi (Burk *et al.*, 1989), the loss of SERCA-like enzymes in the evolution of budding yeast may reflect decreased necessity for  $\text{Ca}^{2+}$  in ER folding reactions. Alternatively, other  $\text{Ca}^{2+}$  ATPases such as Pmr1p, Pmc1p or possibly the newly identified P-type ATPase Cod1p/Spf1p (Cronin *et al.*, 2000) may have compensated for the ancient loss of SERCA in yeast.

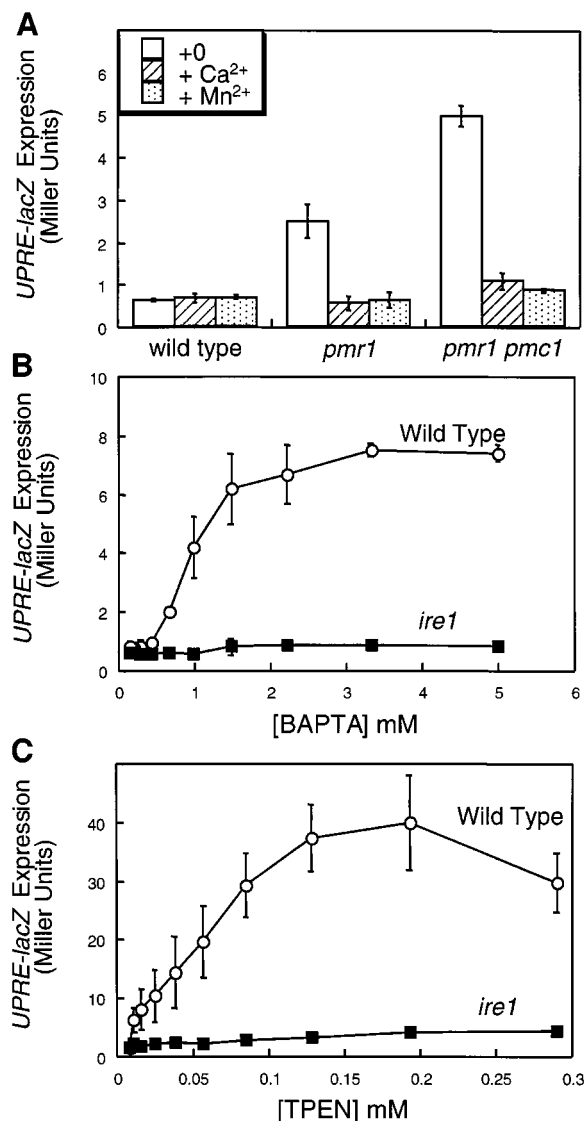
Here, we re-examined the roles of  $\text{Ca}^{2+}$  in the yeast ER using more stringent conditions and confirm that  $\text{Ca}^{2+}$  accumulation in the ER can enhance protein folding there much as in mammalian cells. Surprisingly, inhibitors of either protein folding or ergosterol biosynthesis in the ER were sufficient to stimulate  $\text{Ca}^{2+}$  influx and/or activate calcium signaling pathways that were essential for cell survival. This calcium cell survival (CCS) pathway was also active in the pathogenic yeasts *Candida albicans* and *Candida glabrata*, where it provides resistance to common antifungal drugs. Thus, existing antifungal therapies might be improved by blocking the CCS pathway with drugs that inhibit either the mechanism linking ER stress to  $\text{Ca}^{2+}$  influx or the essential targets of calcineurin.

## Results

### Activation of the UPR upon depletion of secretory $\text{Ca}^{2+}$ stores

Mutants lacking the secretory pathway  $\text{Ca}^{2+}$  ATPase Pmr1p exhibit only mild defects in the sorting and processing of secretory proteins and no significant activation of UPR signaling during growth in standard media (Dürr *et al.*, 1998). However, such conditions permit increased  $\text{Ca}^{2+}$  influx from the medium along with up-regulation and mislocalization of the vacuolar  $\text{Ca}^{2+}$  ATPase Pmc1p (Marchi *et al.*, 1999; Locke *et al.*, 2000), which might partially replenish secretory  $\text{Ca}^{2+}$  and thus compensate for the loss of Pmr1p. To address this possibility, we measured expression of *UPRE-lacZ*, a reporter of UPR signaling, in *pmr1 pmc1* double mutants and in *pmr1* mutants grown in medium deficient in  $\text{Ca}^{2+}$ . Under these conditions, *UPRE-lacZ* expression was induced in *pmr1* mutants and in *pmr1 pmc1* double mutants but not in wild-type yeast cells (Figure 1A). Induction of *UPRE-lacZ* in the mutants was suppressed by supplementing the medium with high concentrations of  $\text{CaCl}_2$  or  $\text{MnCl}_2$  (Figure 1A), by expression of rabbit SERCA1a, or by disruption of the UPR signaling factor Ire1p (data not shown). Thus, genetic depletion of  $\text{Ca}^{2+}$  and  $\text{Mn}^{2+}$  from the secretory pathway led to activation of UPR signaling.

Chelators of  $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$  and other metal ions were also able to induce *UPRE-lacZ* expression in wild-type cells. Cells treated with either the membrane-impermeant chelator *bis-(o-aminophenoxy)-ethane-N,N,N',N'*-tetracetic acid (BAPTA) (Figure 1B) or the membrane-permeant chelator *N,N,N',N'*-tetrakis-(2-pyridylmethyl)



**Fig. 1.**  $\text{Ca}^{2+}$  starvation and chelation can stimulate the UPR signaling pathway. (A) Wild-type, *pmr1* mutant and *pmr1 pmc1* double mutant cells (strains K601, K609 and K613, respectively) bearing the *UPRE-lacZ* reporter gene (plasmid pCZY1) were grown to log phase in SC medium lacking uracil,  $\text{Ca}^{2+}$  and  $\text{Mn}^{2+}$  with or without a supplement of 10 mM  $\text{CaCl}_2$  or 100  $\mu\text{M}$   $\text{MnCl}_2$ . After 4 h, the cells were harvested and assayed for  $\beta$ -galactosidase activity. Bars represent the mean of three independent transformants ( $\pm$  SD). The Pmr1p  $\text{Ca}^{2+}$  pump prevented activation of the UPR signaling pathway in medium lacking  $\text{Ca}^{2+}$ . (B and C) Wild-type and *ire1* mutant cells (strains K1257 and K1259, respectively) bearing the *UPRE-lacZ* reporter gene were grown to log phase in SC medium lacking uracil and containing 50 mM K-MES buffer pH 6.5, and then treated with the indicated concentrations of either the high-affinity membrane-impermeant  $\text{Ca}^{2+}$  chelator BAPTA or the low-affinity membrane-permeant  $\text{Ca}^{2+}$  chelator TPEN. Cells were incubated and processed for  $\beta$ -galactosidase activity as in (A).

ethylenediamine (TPEN) (Figure 1C) exhibited high levels of expression of *UPRE-lacZ* (Figure 1B). In both cases, *UPRE-lacZ* induction was partially suppressed by over-expression of Pmr1p (data not shown) and abolished in *ire1* mutants (Figure 1B and C). Taken together, these results provide evidence for a role of  $\text{Ca}^{2+}$  and the intracellular  $\text{Ca}^{2+}$  pump Pmr1p in preventing activation of

the UPR, presumably by promoting the activities of Ca<sup>2+</sup>-dependent chaperones in the ER.

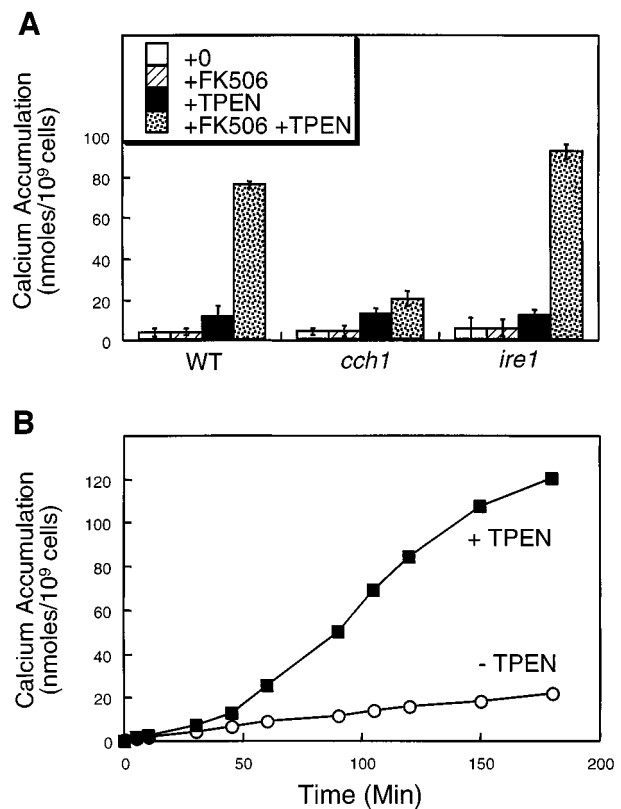
### TPEN treatment stimulates Ca<sup>2+</sup> influx and signaling

In cultured mammalian cells, TPEN treatment rapidly diminishes ER free Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>er</sub>) and activates the CCE pathway, which leads to elevation of cytosolic free Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>c</sub>) within 1 min of application (Hofer *et al.*, 1998). We have previously reported evidence of a CCE-like pathway in yeast (Locke *et al.*, 2000), but the use of genetic methods prevented a kinetic analysis of the CCE-like response. Therefore, we tested whether TPEN addition could stimulate Ca<sup>2+</sup> influx and [Ca<sup>2+</sup>]<sub>c</sub> elevation in yeast on a time scale similar to that observed in mammalian cells. TPEN treatment stimulated <sup>45</sup>Ca<sup>2+</sup> accumulation in wild-type cells ~10-fold in the presence of the calcineurin inhibitor FK506 and ~2.5-fold in the absence of FK506 (Figure 2A). FK506 was used because it prevents calcineurin-dependent feedback inhibition of the Cch1p–Mid1p Ca<sup>2+</sup> channel (Locke *et al.*, 2000; Muller *et al.*, 2001). Mutants lacking Cch1p exhibited a markedly diminished response to TPEN in the presence of FK506 (Figure 2A), indicating that TPEN may activate this Ca<sup>2+</sup> channel in much the same way as a *pmr1* mutation. Mutants lacking Ire1p or Hac1p exhibited a wild-type response to TPEN (Figure 2A and data not shown). Thus, TPEN appears to phenocopy *pmr1* mutants and to trigger the CCE-like pathway in yeast, which is independent of UPR signaling.

In time course experiments, the effect of TPEN treatment on <sup>45</sup>Ca<sup>2+</sup> accumulation became apparent after a lag of at least 30 min and reached a maximum after ~150 min (Figure 2B). Similar kinetics were observed using cytoplasmic aequorin as a probe for [Ca<sup>2+</sup>]<sub>c</sub> (data not shown). Therefore, the Ca<sup>2+</sup> influx observed in response to TPEN appears to be much slower in yeast relative to mammalian cells.

### Misfolded secretory proteins stimulate Ca<sup>2+</sup> influx and signaling

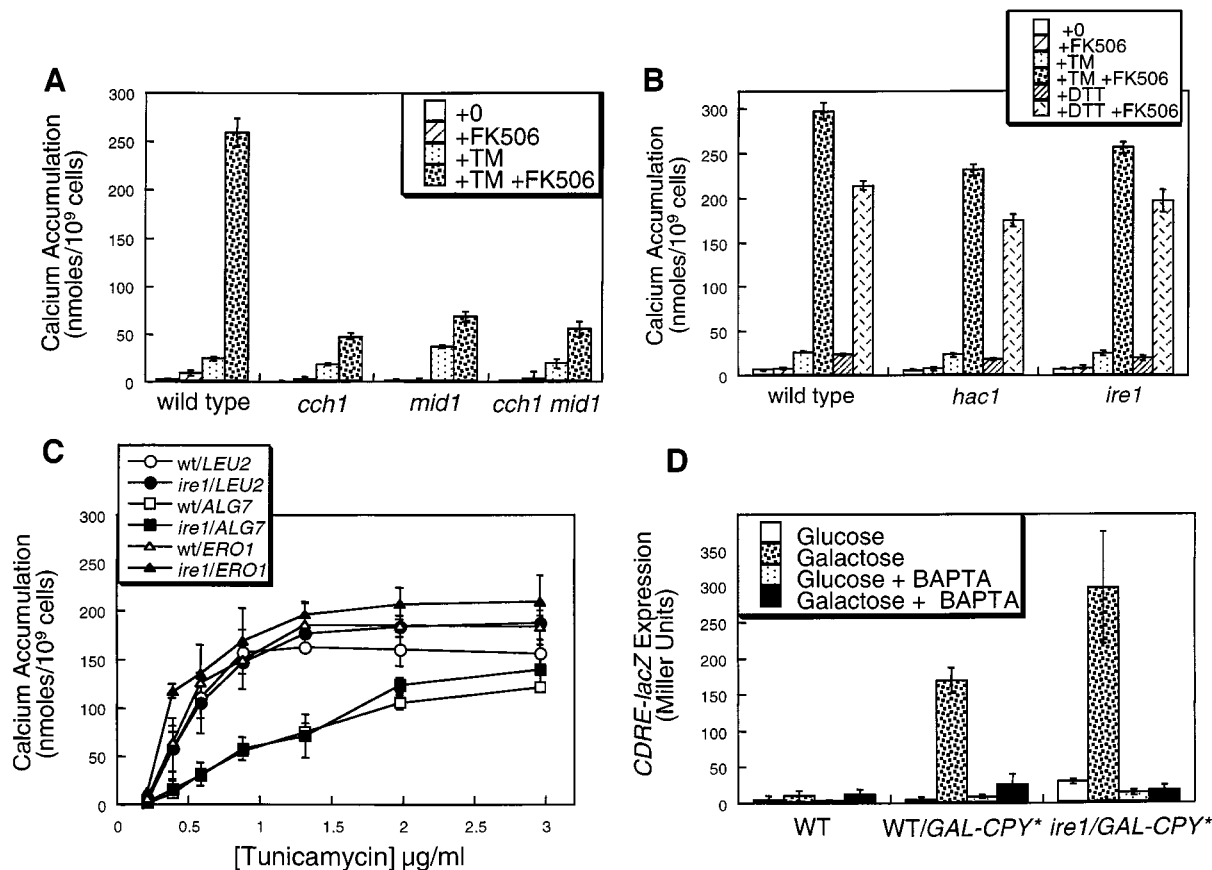
The relatively slow response of yeast cells to TPEN indicates that these cells may utilize a distinct mechanism for coupling Ca<sup>2+</sup> influx to depletion of [Ca<sup>2+</sup>]<sub>er</sub>. To test whether this coupling involves the accumulation of misfolded proteins, we measured <sup>45</sup>Ca<sup>2+</sup> accumulation in cells treated with tunicamycin (TM), an inhibitor of N-glycosylation in the ER (Tkacz and Lampen, 1975; Lehle and Tanner, 1976) that causes accumulation of misfolded proteins and stimulation of UPR signaling. TM treatment significantly stimulated <sup>45</sup>Ca<sup>2+</sup> accumulation in wild-type cells and in *cch1* mutants, *mid1* mutants and *cch1 mid1* double mutants (Figure 3A), suggesting the existence of a Ca<sup>2+</sup> influx pathway that is independent of Cch1p–Mid1p. In the presence of FK506, TM stimulated even greater levels of <sup>45</sup>Ca<sup>2+</sup> accumulation, which was abolished in *cch1* and *mid1* mutants (Figure 3A). These effects of TM were completely independent of Ire1p and Hac1p (Figure 3B) but were clearly diminished in cells overexpressing Alg7p, the enzyme in the ER that is the direct target of TM (Figure 3C). The time course of Ca<sup>2+</sup> accumulation in response to TM was similar to that of TPEN (data not shown). These findings indicate that



**Fig. 2.** TPEN stimulates Ca<sup>2+</sup> influx via the Cch1p–Mid1p Ca<sup>2+</sup> channel independently of Ire1p. (A) <sup>45</sup>Ca<sup>2+</sup> accumulation in wild-type cells, *cch1* mutants and *ire1* mutants (strains K1257, RG05040 and K1259 respectively) was measured after incubation for 4 h in SC medium supplemented with trace amounts of <sup>45</sup>CaCl<sub>2</sub> plus 2.0 μg/ml FK506 and 129 μM TPEN as indicated. Bars represent the mean of three independent experiments (± SD). (B) <sup>45</sup>Ca<sup>2+</sup> accumulation in wild-type cells (strain K601) was measured at various time-points after treatment with either FK506 (open symbols) or TPEN plus FK506 (filled symbols) as in (A).

specific inhibition of Alg7p and N-glycosylation in the ER can activate two Ca<sup>2+</sup> influx systems through a mechanism that is independent of Ire1p and Hac1p. Identical results were obtained using dithiothreitol (DTT) as an inhibitor of disulfide bond formation in the ER and its target Ero1p (Figure 3B and data not shown). Thus, multiple inhibitors of protein folding or processing in the ER (e.g. TPEN, TM and DTT) all stimulated Ca<sup>2+</sup> accumulation independently of a functional UPR signaling pathway.

To test whether accumulation of a misfolded protein in the ER is sufficient to stimulate Ca<sup>2+</sup> responses, the effects of overexpressing a folding-incompetent mutant of carboxypeptidase Y termed CPY\* (Hiller *et al.*, 1996) were measured. Overexpression of CPY\* in wild-type or *ire1* mutants stimulated <sup>45</sup>Ca<sup>2+</sup> accumulation (data not shown) and [Ca<sup>2+</sup>]<sub>c</sub> elevation as assessed by monitoring expression of a calcineurin-dependent reporter gene *CDRE-lacZ* (Figure 3C). Thus, accumulation of a single misfolded protein species in the ER appeared to be sufficient to stimulate Ca<sup>2+</sup> influx and to activate calcineurin signaling. These findings indicate the existence of a novel UPR-independent regulatory pathway emanating from the ER that can sense the accumulation of



**Fig. 3.** Accumulation of misfolded proteins in the ER stimulates  $\text{Ca}^{2+}$  influx and signaling independently of Ire1p. (A)  $^{45}\text{Ca}^{2+}$  accumulation in wild type, *cch1* and *mid1* mutants was measured as in Figure 2A except that TM (2.5  $\mu\text{g}/\text{ml}$ ) was used instead of TPEN. (B)  $^{45}\text{Ca}^{2+}$  accumulation in wild type, *ire1* and *hac1* mutants (strains K1257, K1259 and RG05650) was measured as in (A) except that DTT (2.5 mM) was included as indicated. (C)  $^{45}\text{Ca}^{2+}$  accumulation in wild-type (open symbols) and *ire1* mutant cells (closed symbols) that were overexpressing Alg7p, Ero1p or no additional proteins in the ER (plasmids pMK13XRV, pKT001 or vector control) was measured after treatment with varying amounts of TM in the presence of 2  $\mu\text{g}/\text{ml}$  FK506. Complementary results were obtained using DTT instead of TM (data not shown). (D) Expression of the *CDRE-lacZ* reporter gene (plasmid pAMS366) was measured in wild-type cells and *ire1* mutants expressing a folding-incompetent mutant of carboxypeptidase Y (denoted CPY\*) under control of the galactose promoter (strains DNY1047 and DNY1048) after growth for 4 h in SC medium lacking uracil but supplemented with either 2% glucose or 2% galactose as indicated. Induction of CPY\* in galactose medium stimulated *CDRE-lacZ* expression through a process that was sensitive to both 4 mM BAPTA and FK506 (data not shown). Wild-type cells lacking CPY\* (strain K601) were included as a control. Bars represent the mean of three independent transformants ( $\pm$  SD).

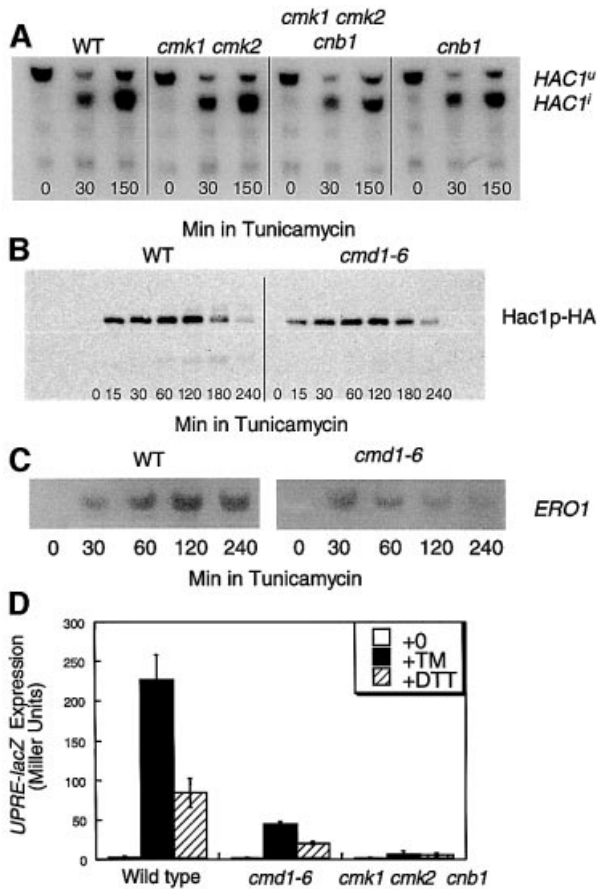
misfolded proteins and stimulate  $\text{Ca}^{2+}$  influx at the plasma membrane as well as calcineurin signaling.

### Roles for $\text{Ca}^{2+}$ influx and signaling during the response to unfolded protein accumulation

What are the roles of  $\text{Ca}^{2+}$  during the response to ER stress? One possibility is that UPR signaling itself may depend on  $\text{Ca}^{2+}$  influx or signaling. Consistent with this possibility, *UPRE-lacZ* induction was 5-fold lower in response to BAPTA relative to TPEN, the former of which prevents  $[\text{Ca}^{2+}]_c$  elevation (Figures 1 and 2). To determine whether UPR signaling might involve calcium signaling factors, several assays of UPR signaling were performed on a panel of yeast strains lacking either  $\text{Ca}^{2+}$ /calmodulin (*cmd1-6* mutants),  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinases (*cmk1 cmk2* double mutants), calcineurin (*cnb1* mutants), calcineurin-dependent transcription factor (*tcn1* mutants) or combinations thereof.

Early events in the UPR signaling pathway include the Ire1p-dependent splicing of the immature *HAC1<sup>u</sup>* pre-mRNA to the mature *HAC1<sup>i</sup>* mRNA, which is

efficiently translated to promote accumulation of the Hac1p transcription factor (Sidrauski and Walter, 1997). Northern blot analysis of *HAC1* transcripts in wild type, *cnb1* mutants, *cmk1 cmk2* and *cmk1 cmk2 cnb1* triple mutants revealed no significant difference in either the kinetics or extent of TM-induced *HAC1* mRNA splicing (Figure 4A and data not shown), although the basal levels of *HAC1* transcription were slightly decreased. Western blot analysis of a functional epitope-tagged Hac1p-HA also revealed no significant differences between *cmd1-6* mutants, *cmk1 cmk2 cnb1* triple mutants and wild-type cells after treatment with TM (Figure 4B and data not shown). Finally, northern blot analysis of *ERO1* transcripts, a target of Hac1p that is highly induced by UPR signaling (Frand and Kaiser, 1998), was performed in wild type, *cmd1-6* mutants and *cmk1 cmk2 cnb1* triple mutants at various times after treatment with TM. At early time-points, *ERO1* expression was strongly induced in all these strains but *ERO1* expression was not fully maintained in *cmd1-6* mutants or *cmk1 cmk2 cnb1* triple mutants at later time-points (Figure 4C and data not shown). The induction



**Fig. 4.** Calcium signaling factors are not required for efficient UPR signaling. (A) Alternative splicing of *HAC1* transcripts was stimulated by tunicamycin independently of calcium signaling factors. *HAC1* splicing was monitored by northern blot analysis of total RNA isolated from wild-type (K601), *cmk1 cmk2* (K481), *cmk1 cmk2 cnb1* (K488) and *cnb1* (K603) strains after 0, 30 or 150 min in 2.5  $\mu$ g/ml TM. (B) Accumulation of Hac1p was stimulated by tunicamycin treatment independently of calcium signaling factors. Hac1p accumulation was monitored by western blotting of total cell protein extracted from wild-type (K601) cells and *cmd1-6* mutants (JGY148) carrying epitope-tagged Hac1p-HA (plasmid pMB004) that had been treated with 2.5  $\mu$ g/ml TM for the indicated periods of time. (C) Northern blot analysis of *ERO1* gene expression was performed as in (B) on wild-type and *cmd1-6* mutant cells at various times after treatment with TM. Mutants defective in calmodulin signaling reproducibly induced *ERO1* transcripts much more transiently than wild-type cells. (D) *UPRE-lacZ* expression was measured in wild type (strain K601), *cmd1-6* mutants (strain JGY148) and *cmk1 cmk2 cnb1* triple mutants (strain K488) after 4 h growth in SC medium supplemented with either 2.5 mg/ml TM or 2.5 mM DTT as indicated.

of *UPRE-lacZ* was markedly decreased in *cmd1-6* mutants and *cmk1 cmk2 cnb1* triple mutants as measured after 4 h of TM treatment (Figure 4D). These results indicate that calcium signaling factors are not required for the initial stages of UPR signaling. However, calcium signaling factors may be important to sustain UPR signaling during long periods of TM treatment.

#### Calcineurin signaling prevents cell death in TM

Wild-type yeast cells treated with TM remain viable for extended periods of time but fail to proliferate, apparently due to reversible arrest in either the  $G_1$  or  $G_2$  phase of the cell division cycle (Klebl *et al.*, 1984). Mutants deficient

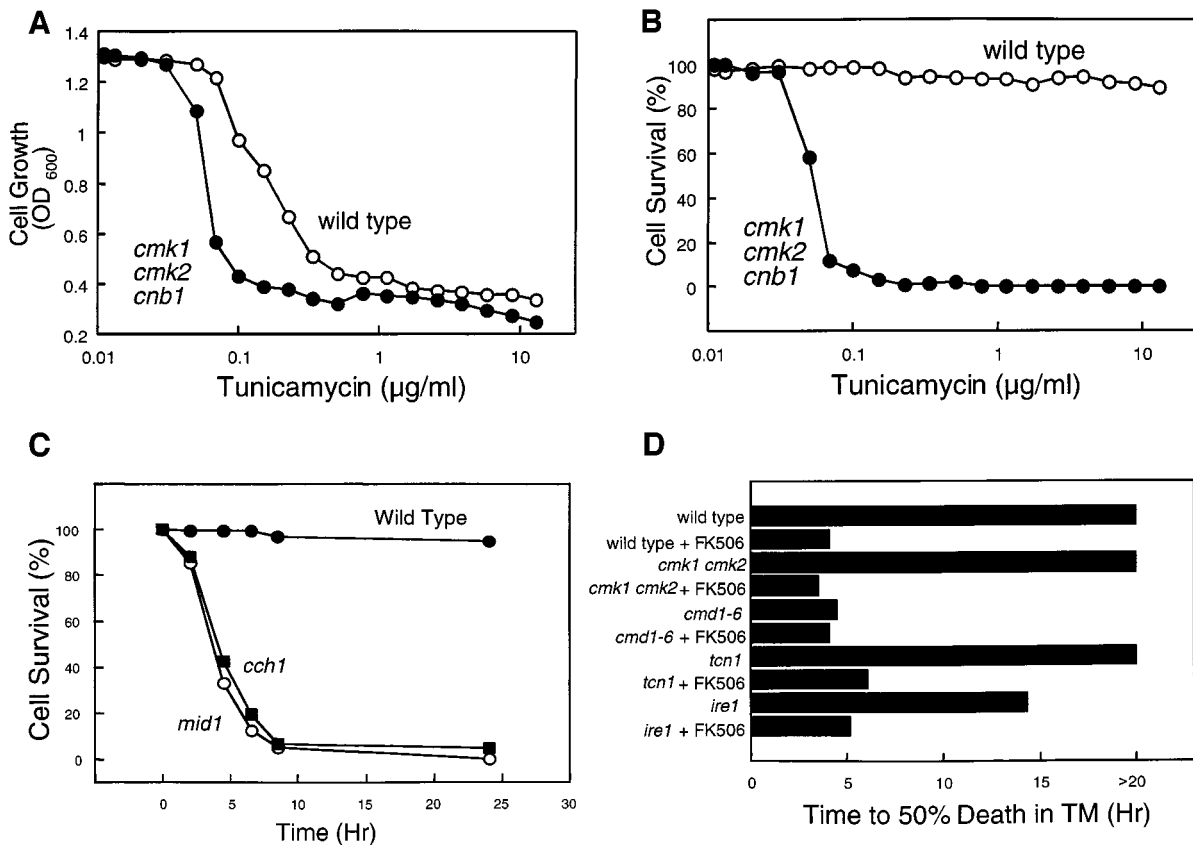
in Alg7p behaved similarly (Lennon *et al.*, 1997). We therefore explored the possible role of calcium signaling factors in cell growth and cell death after TM treatment. In growth assays (measurement of culture turbidity by optical density at 600 nm), *cmk1 cmk2 cnb1* triple mutants were approximately four times more sensitive to TM than the wild-type strain (Figure 5A). However, in cell viability assays (exclusion of the vital dye methylene blue) wild-type cells remained almost completely viable after 6 h of treatment with TM whereas the *cmk1 cmk2 cnb1* triple mutant cells exhibited striking levels of cell death (Figure 5B). In time course experiments, *cch1* and *mid1* mutants died rapidly in response to TM (Figure 5C). Therefore,  $Ca^{2+}$  influx and signaling factors were required for survival of arrested cells during prolonged treatment with TM.

The relative contributions of individual calcium signaling factors were estimated by determining the time after TM treatment that resulted in death in 50% of various mutants. Wild-type cells and *cmk1 cmk2* double mutants remained arrested but highly viable for at least 20 h of TM treatment but died rapidly if FK506 was also included (Figure 5D). In contrast, *cmd1-6* mutants died rapidly with or without FK506. These findings indicate that cell survival requires activation of calcineurin by  $Ca^{2+}$ /calmodulin. Mutants lacking the calcineurin-dependent transcription factor Tcn1p behaved like wild-type cells in survival assays (Figure 5D), indicating that calcineurin promotes cell survival independently of Tcn1p. Interestingly, Ire1p was not required for this role of calcineurin because *ire1* mutants survived much longer without FK506 than with FK506 (Figure 5D). These findings demonstrate that calcium signaling factors such as Cch1p, Mid1p, calmodulin and calcineurin promote long-term survival of cells treated with TM and that this role is not shared with UPR signaling factors. This CCS pathway has not been described previously.

#### The CCS pathway is required for resistance to azole antifungal drugs and operates in pathogenic fungi

To appreciate the range of conditions that stimulate the CCS pathway, we tested several additional inhibitors and mutations that affect ER function. Like TM, addition of DTT caused much more rapid cell death in mutants lacking the calcium signaling factors in comparison with wild type (data not shown). To inhibit protein folding without the use of inhibitors such as TM or DTT, we examined the survival of temperature-sensitive *kar2* mutants in which the ER-localized Hsp70-family chaperone homologous to mammalian BiP could be conditionally inactivated. After a shift from 23°C to the semi-permissive temperature of 30°C, two different *kar2-ts* mutants rapidly arrested growth relative to wild type, but remained viable for at least 24 h (Figure 6A). Viability of the mutants was rapidly lost at the semi-permissive temperature if FK506 was included (Figure 6A). Thus, genetic deficiencies in the ER protein folding machinery appear to require calcium signaling pathways for cell survival.

To test whether the CCS pathway might operate in other fungi, cell survival assays were performed on pathogenic strains of *C.albicans* and *C.glabrata* treated with TM and FK506. In both of these species, treatment with TM plus



**Fig. 5.** Tunicamycin treatment arrests growth of wild-type cells and causes death of cells deficient in  $\text{Ca}^{2+}$  influx and signaling factors. After treatment of log-phase yeast cultures with TM, dead cells were stained with methylene blue and counted. Cell survival reflects the percentage live cells of total cells in each culture. Wild type and *cmk1 cmk2 cnb1* triple mutants (strains K601 and K488) were treated with varying amounts of TM for 6 h and then assayed for cell growth (A) or cell survival (B) as described in Materials and methods. (C) Survival of wild type, *cch1* and *mid1* mutants (strains K601, ELY117, ELY138) was measured at various times after addition of TM (2.5 µg/ml). (D) The time required for various yeast strains to undergo 50% cell death was determined in time course experiments similar to those in (C).

FK506 resulted in rapid cell death whereas treatment with TM alone arrested growth without causing cell death (Figure 6B). Treatment with FK506 alone had no effect on growth or death of these cells. Thus, the CCS pathway appears to operate in diverse fungi.

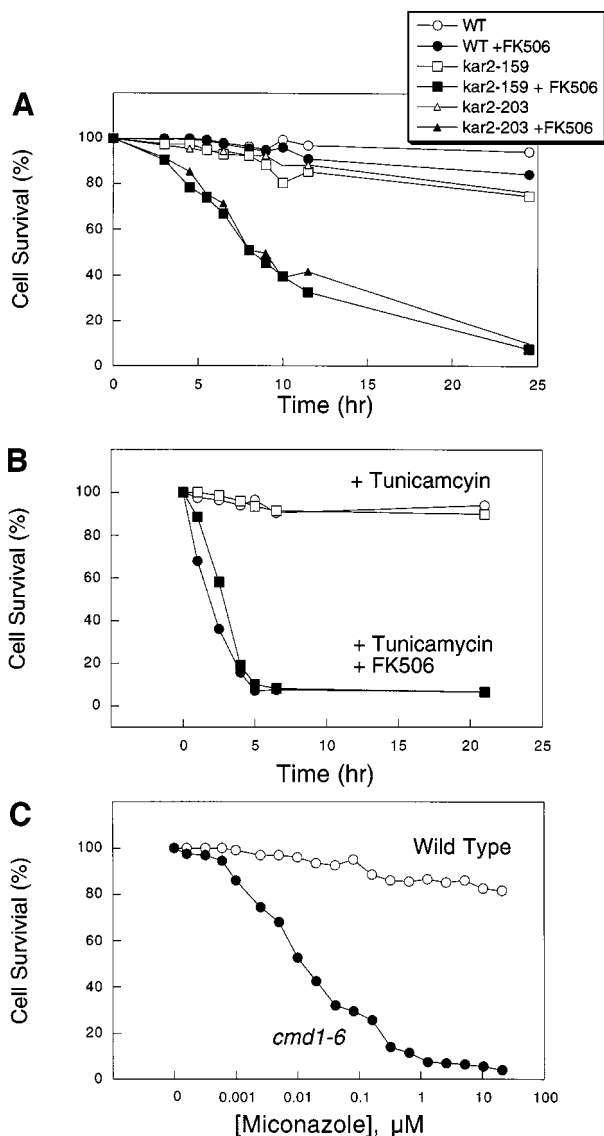
The cytotoxic effects of FK506 during TM treatment resemble the cytotoxic effects of FK506 observed in pathogenic strains of *C. albicans* that had been treated with azole-class antifungal drugs that inhibit ergosterol biosynthesis in the fungal ER (Marchetti *et al.*, 2000). To determine whether the CCS pathway can provide tolerance to azole-class antifungal drugs in *Saccharomyces*, we measured the survival of wild-type and *cmd1-6* mutant cells after a 6 h treatment with various concentrations of miconazole (Figure 6C). Wild-type yeast cells arrested but remained highly viable at concentrations of miconazole up to 20 µM whereas *cmd1-6* mutant cells arrested and died at concentrations >0.2 µM. Miconazole treatment stimulated  $^{45}\text{Ca}^{2+}$  accumulation in wild-type cells, but did not stimulate expression of *UPRE-lacZ* (data not shown). These results indicate that the CCS pathway becomes activated in response to miconazole and promotes cell survival. The CCS pathway may therefore be an important determinant of cellular resistance to azole-class antifungal drugs.

## Discussion

This report provides evidence that  $\text{Ca}^{2+}$  accumulation in the ER is important for folding or processing of secretory proteins in yeast, much as it is in other eukaryotes. Accumulation of unfolded proteins or inhibition of ergosterol synthesis in the ER of yeast was sufficient to stimulate  $\text{Ca}^{2+}$  influx via the Cch1p–Mid1p channel and other pathways, which promoted cell survival through the activation of calcineurin. The existence of this CCS pathway in fungi may provide new opportunities for treatment of fungal infections as well as a model system for studies of similar pathways in human cells.

### Roles of $\text{Ca}^{2+}$ in the ER

Until now, little evidence was available to support the notion that the ER of yeast employs  $\text{Ca}^{2+}$  for the folding or processing of secretory proteins. Previously it was shown that mutants lacking Pmr1p failed to retain a heterologous secretory protein in the ER (Rudolph *et al.*, 1989) and failed to degrade misfolded proteins in the ER, although UPR signaling was not elevated (Dürer *et al.*, 1998). Our findings indicate that high extracellular  $\text{Ca}^{2+}$  and consequent up-regulation of Pmc1p suppresses the loss of Pmr1p and thus obscures a role for  $\text{Ca}^{2+}$  in the modifica-



**Fig. 6.** Calcineurin signaling is required for survival of *kar2-ts* mutants and for survival of *Saccharomyces* and *Candida* species in response to azoles or other ER stress agents. (A) FK506 prevents survival of *kar2-159* (strain MS137) and *kar2-203* (strain MS1028) at a semi-permissive temperature (30°C). Cell survival assays were performed as described in Figure 5. (B) FK506 (filled symbols) prevents survival of *C. albicans* (circles) and *C. glabrata* (squares) in medium containing TM. (C) Calmodulin signaling was required for survival in medium containing miconazole. Survival of wild type and *cmd1-6* mutants was measured after treatment with varying amounts of miconazole for 6 h in SC medium.

tion or folding of secretory proteins. Inactivation of Pmc1p and/or removal of excess  $\text{Ca}^{2+}$  from the medium led to high UPR signaling in *pmr1* mutants (Figure 1). Treatment with the extracellular chelator BAPTA or intracellular chelator TPEN caused similar effects in wild-type cells. Though these chelators are not specific for  $\text{Ca}^{2+}$ , their effects were at least partially reversed by overexpression of Pmr1p or rabbit SERCA1a (not shown), indicating they deplete cells of a Pmr1p substrate.  $\text{Mn}^{2+}$  was effective at suppressing secretory defects of *pmr1* mutants (Figure 1A), confirming an earlier observation that  $\text{Mn}^{2+}$  often substitutes for  $\text{Ca}^{2+}$  in the secretory pathway of yeast (Loukin and Kung, 1995).

### Communication between the ER and plasma membrane

Most mammalian cell types monitor  $\text{Ca}^{2+}$  levels in the ER and rapidly activate the CCE mechanism in response to even transient  $\text{Ca}^{2+}$  depletion (Putney, 1986). For example, TPEN treatment depletes  $[\text{Ca}^{2+}]_{\text{er}}$  very rapidly, and within seconds the depleted organelle triggers CCE at the plasma membrane and elevation of  $[\text{Ca}^{2+}]_{\text{c}}$  (Hofer *et al.*, 1998). In yeast, TPEN stimulated  $\text{Ca}^{2+}$  entry and elevated  $[\text{Ca}^{2+}]_{\text{c}}$  (Figure 2A), but these effects required at least 1 h of treatment (Figure 2B). The different kinetics might reflect a lower permeability of yeast cells to TPEN, or possibly a distinct mechanism in yeast cells for regulating  $\text{Ca}^{2+}$  entry such as ER stress. Several other agents and conditions that stimulate UPR signaling in yeast, including TM, DTT, CPY\*, *kar2-ts* mutation and *pmr1* mutation, also stimulated  $\text{Ca}^{2+}$  accumulation. Their effects on  $\text{Ca}^{2+}$  influx and signaling were completely independent of Ire1p and Hac1p, two crucial components of the UPR signaling pathway. Furthermore, an inhibitor of ergosterol biosynthesis in the ER also stimulated calcium signaling but did not activate UPR signaling (data not shown). These findings suggest that more general forms of ER stress can regulate  $\text{Ca}^{2+}$  entry in yeast. Though the signaling pathways employed to couple ER stress to  $\text{Ca}^{2+}$  influx remain to be identified, our results exclude the UPR signaling pathway.

All of the ER stress agents and conditions reported here appeared to stimulate two separate  $\text{Ca}^{2+}$  influx systems, one of which required Mid1p and Cch1p and the other remaining unidentified. Mid1p homologs are conserved in other fungi and are required for resistance to echinocandin-class drugs in *Schizosaccharomyces pombe* (Carnero *et al.*, 2000; Tasaka *et al.*, 2000). Cch1p is homologous to the catalytic subunit of voltage-gated  $\text{Ca}^{2+}$  channels (VGCCs) conserved in fungi and animals, but is most closely related to an uncharacterized family of four-repeat channels that is conserved in the genomes of nematodes, fruit flies and mammals (Lee *et al.*, 1999). The possibility that ER stress activates these channels in mammalian cells has not been ruled out. In fact, ER stresses such as TM treatment or even Alzheimer's disease mutations in presenilin-1 can affect CCE (Pahl and Baeuerle, 1996; Yoo *et al.*, 2000) and can also affect the activity of Cch1p homologs that contribute to neuronal cell death and neurodegenerative disease (Paschen and Frandsen, 2001). Defining the signal transduction pathway that couples ER stress to Cch1p–Mid1p activation in yeast may therefore help to elucidate similar mechanisms in human cells.

### Roles for $\text{Ca}^{2+}$ influx in response to ER stress

Yeast mutants lacking  $\text{Ca}^{2+}$ /calmodulin (*cmd1-6*) or its effectors (*cmk1 cmk2 cnb1* triple mutants) appeared indistinguishable from wild-type cells in their ability to execute the early stages of UPR signaling (Figure 4). However, these mutants did not maintain long-term expression of target genes and largely failed to accumulate  $\beta$ -galactosidase activity expressed from the *UPRE-lacZ* reporter gene. These defects might reflect a role for  $\text{Ca}^{2+}$  and calcium signaling factors at a late step in the UPR signaling pathway. Alternatively, the inability to maintain UPR signaling may be a consequence of the dramatically

**Table I.** Fungal strains used in this study

Strain	Genotype	Source or reference
W303-1A	<i>MATa ade2-1 can1-100 his3-11,14 leu2-3 112 trp1-1 ura3-1</i>	Wallis <i>et al.</i> (1989)
K609	<i>pmr1::HIS3</i>	Cunningham and Fink (1994)
K613	<i>pmr1::HIS3 pmc1::TRP1</i>	Cunningham and Fink (1994)
JGY148	<i>cmd1-6</i>	Geiser <i>et al.</i> (1991)
K481	<i>cmk1::HIS3 cmk2::TRP1</i>	This study
K488	<i>cmk1::HIS3 cmk2::TRP1 cnb1::LEU2</i>	This study
K603	<i>cnb1::LEU2</i>	Cunningham and Fink (1994)
DMY14	<i>tcn1::G418</i>	Matheos <i>et al.</i> (1997)
ELY117	<i>cch1::TRP1</i>	Locke <i>et al.</i> (2000)
ELY138	<i>mid1::LEU2</i>	Locke <i>et al.</i> (2000)
ELY151	<i>cch1::TRP1 mid1::LEU2</i>	Locke <i>et al.</i> (2000)
DNY1047	<i>LEU2::GAL-CPY*</i>	Gift of Davis Ng
DNY1048	<i>LEU2::GAL-CPY* ire1::TRP1</i>	Gift of Davis Ng
BY4741	<i>MATa his3-1 leu2-0 ura3-0</i>	Brachmann <i>et al.</i> (1998)
RG05650	BY4741 <i>hacl::G418</i>	Research Genetics
RG05040	BY4741 <i>cnb1::G418</i>	Research Genetics
K1257	BY4741	This study
K1259	BY4741 <i>ire1::G418</i>	This study
MS10	<i>MATa ura3-52 leu2-3 leu2-112 ade2-101</i>	Vogel <i>et al.</i> (1990)
MS137	MS10 <i>kar2-159</i>	Sanders <i>et al.</i> (1992)
MS1028	MS10 <i>kar2-203</i>	Scidmore <i>et al.</i> (1993)
BG2	<i>C.glabrata</i> wild type	Cormack and Falkow (1999)
Ca5314	<i>C.albicans</i> wild type	Fonzi and Irwin (1993)

accelerated cell death observed in the mutants during ER stress.

Yeast strains lacking Cch1p, Mid1p, calmodulin or calcineurin functions died rapidly in response to ER stress agents whereas mutants lacking Tcn1p, the only known calcineurin-dependent transcription factor, remained fully viable for long periods of time, like wild-type cells (Figure 6). This Tcn1p-independent cell survival pathway appears to be distinct from the calcineurin-dependent growth of cells with cell wall defects (Mazur *et al.*, 1995; Carnero *et al.*, 2000), which requires Tcn1p (Matheos *et al.*, 1997). Survival of yeast undergoing ER stress was also independent of Vcx1p and Hsl1p (M.Bonilla and K.W.Cunningham, unpublished results), two other suspected targets of calcineurin (Cunningham and Fink, 1996; Mizunuma *et al.*, 2001). Because a similar pathway appears to operate in pathogenic fungi and promote resistance to the azole class of antifungal drugs (Figure 6; Del Poeta *et al.*, 2000; Marchetti *et al.*, 2000; Cruz *et al.*, 2002), identification of the targets of calcineurin responsible for cell survival under these conditions may be useful in the development of new antifungal therapies.

## Materials and methods

### Yeast strains, plasmids and growth conditions

All yeast strains used in this study (Table I) were obtained from original sources or derived from the parental strain W303-1A (Wallis *et al.*, 1989) or BY4741 (Brachmann *et al.*, 1998) using standard molecular and genetic methods (Sambrook *et al.*, 1989). The *CMK1* and *CMK2* genes were disrupted in wild-type strain K601 by transformation with fragments of plasmids pBKScmk1Δ1 and pBKScmk2Δ2 (Pausch *et al.*, 1991) to yield strain K481 and a *MATa* derivative K523. The *CNB1* gene was disrupted in strain K523 as described (Cyert and Thorner, 1992) to yield strain K488. Strains K1257 and K1259 (*ire1::G418*) were recovered after sporulation of strain RG21907 (Research Genetics, Inc.).

Overexpression of Ero1p and Alg7p in yeast was achieved by transformation with high-dosage plasmids pKT001 (2μ *URA3 ERO1*) (Pollard *et al.*, 1998) and pMK13×RV (2μ *LEU2 ALG7*) (gift of Maria Kukuruzinska), respectively. Activation of the UPR signaling pathway

was monitored in yeast transformed with plasmid pCZY1 (2μ *URA3 UPRE-lacZ*) (Mori *et al.*, 1996). Activation of the calcium signaling pathway was monitored in yeast transformed with pAMS366 (2μ *URA3 4×CDRE-lacZ*) (Stathopoulos and Cyert, 1997). Plasmid pMB004, which expresses epitope-tagged Hac1p-HA, was constructed by inserting two repeats of the 96 bp triple-tandem HA1 sequence (Field *et al.*, 1988) into the *SpeI* site (at position +25 in the *HAC1* open reading frame) of plasmid YCp-HAC1WT (Mori *et al.*, 1996).

Yeast strains were grown in standard yeast peptone dextrose (YPD) medium, synthetic complete (SC) medium containing 2% glucose, galactose or raffinose, as described previously (Sherman *et al.*, 1986), using reagents from Difco (Detroit, MI, USA) and Sigma Chemical Co. (St Louis, MO, USA). Calcium-free SD medium was made by replacing YNB with divalent cation-free yeast nitrogen base (Q-Biogene Inc.) and supplementing with the chloride salts of Cu<sup>2+</sup>, Fe<sup>2+</sup>, Mg<sup>2+</sup> and Zn<sup>2+</sup> (but not Mn<sup>2+</sup> or Ca<sup>2+</sup>) to their standard concentrations. The Ca<sup>2+</sup> chelators BAPTA and TPEN were obtained from Sigma, dissolved in water and dimethylsulfoxide (DMSO), respectively, and diluted into SC culture medium pH 6.5, supplemented with 50 mM MES-KOH, to final concentrations of 3 mM and 130 μM, respectively. TM (Sigma) was dissolved in methanol and diluted into medium to a final concentration of 2.5 μg/ml. DTT (Gibco/Life Technologies, Inc.) was dissolved in water and diluted into YPD medium to a final concentration of 2.5 mM. These concentrations were chosen based on their maximal stimulation of *UPRE-lacZ* expression in strain W303-1A.

### β-galactosidase assays

Yeast strains transformed with plasmids carrying either the *UPRE-lacZ* or *CDRE-lacZ* reporter gene were grown to log phase in SC medium (shaking overnight at 30°C), harvested by centrifugation, then cultured in SC medium containing the indicated concentrations of chelators or inhibitors. After incubation at 30°C for 4 h, cells were harvested by centrifugation, permeabilized with chloroform and assayed for β-galactosidase activity using the colorimetric substrate ONPG as described previously (Guarente, 1983). The effects of CPY\* overexpression in wild-type (DNY1047) or *ire1* mutant (DNY1048) cells were monitored after cells were grown to log phase in SC medium supplemented with 2% raffinose, washed twice, then shifted to SC medium supplemented with 2% galactose or 2% glucose for 4 h.

### <sup>45</sup>Ca<sup>2+</sup> accumulation measurements

Total cellular accumulation of Ca<sup>2+</sup> was measured as described previously (Cunningham and Fink, 1996). Briefly, yeast cells were grown to log phase in YPD medium, harvested and resuspended in fresh YPD medium supplemented with tracer quantities (~20 μCi/ml) of



$^{45}\text{CaCl}_2$  (Amersham). After 4 h incubation at 30°C, cells were harvested rapidly by filtration onto GF/F filters (Whatman), washed with ice-cold buffer W (10 mM  $\text{CaCl}_2$ , 5 mM HEPES-NaOH pH 6.5), dried at 90°C for 1 h, placed in scintillation vials and processed for liquid scintillation counting on a Packard 1600 TR liquid scintillation counter using Opti-Fluor (Packard) scintillation cocktail.

### Northern blot analysis

Yeast cells growing in YPD medium were treated with chelators or inhibitors for 20 min at 30°C, chilled on ice and harvested by centrifugation at 700 g at 4°C, for 5 min. Total RNA was extracted and electrophoresed through agarose gels essentially as described (Zhao *et al.*, 1998). For northern blot analysis, 20 µg of total RNA was loaded and separated on a 1% agarose-formaldehyde gel [1% agarose, 20 mM morpholinepropanesulfonic acid (MOPS), 1 mM EDTA, 5 mM sodium acetate, 2.2 M formaldehyde]. RNA was blotted onto Hybond-N<sup>+</sup> membrane by capillary transfer and cross-linked by UV radiation. Membranes were blocked, hybridized overnight at 42°C with ~0.5 × 10<sup>6</sup> c.p.m./ml of [ $\alpha$ -<sup>32</sup>P]dCTP-labelled DNA probe, washed and subsequently imaged by autoradiography using X-Omat film. Probes for detecting *HAC1* and *ERO1* mRNAs were synthesized by PCR amplification of wild-type yeast genomic DNA using the primer pairs AATTCGCAATCGAAGCTTGGC plus TATATCGTCGCAGAGTGGT and AGATTAAGAACC GCCATTGC plus ATAGGCTCTCGTGTCTCTCA, respectively, purified and labelled using a random primer kit (Gibco/Life).

### Western blot analysis

Whole-cell extracts were prepared by glass bead lysis from ~2 OD<sub>600</sub> units of log-phase yeast cells carrying plasmid pMB004 (*CEN LEU2 HAC1-HA*). Cells were collected by centrifugation and resuspended in 40 µl of sorbitol breaking buffer (0.3 M sorbitol, 0.1 M NaCl, 5 mM  $\text{MgCl}_2$ , 10 mM Tris pH 7.5, protease inhibitors) (Katzmann *et al.*, 1999). Glass beads were added to the meniscus, and samples were vortexed at 4°C for 5 min in the presence of protease inhibitors [ $\text{N}\alpha$ -*p*-tosyl-L-lysine chloromethyl ketone (TLCK),  $\text{N}\alpha$ -*p*-tosyl-L-arginine methyl ester (TAME),  $\text{N}\alpha$ -benzoyl-L-arginine methyl ester (BAME), *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 4-(2-aminoethyl) benzene-sulfonyl fluoride (AEBSF), leupeptin and pepstatin all from Sigma]. Sample buffer was added to whole-cell lysates and the lysates were loaded onto SDS-PAGE gels. After running gels, proteins were transferred to PVDF and membranes were probed with 12CA5 anti-myc monoclonal antibody (Santa Cruz). A secondary horseradish peroxidase-conjugated goat anti-mouse antibody (Jackson ImmunoResearch) was used in conjunction with an ECL kit to visualize Hac1p-HA bands on the membranes.

### Methylene blue viability assays

Log-phase cultures of yeast growing in SC medium were treated with tunicamycin and/or FK506 and incubated at 30°C. At intervals, aliquots were removed and stained with 100 µg/ml methylene blue (Sigma). Live cells (unstained) and dead cells (blue stained) were immediately counted under bright-field microscopy. At least 200 live and dead cells were counted at each time-point and the data were plotted as the percentage of live cells.

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