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# Internal $Ca^{2+}$ release in yeast is triggered by hypertonic shock and mediated by a TRP channel homologue

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Calcium ions, present inside all eukaryotic cells, are important second messengers in the transduction of biological signals. In mammalian cells, the release of $Ca^{2+}$ from intracellular compartments is required for signaling and involves the regulated opening of ryanodine and inositol-1,4,5-trisphosphate ($IP_3$) receptors. However, in budding yeast, no signaling pathway has been shown to involve $Ca^{2+}$ release from internal stores, and no homologues of ryanodine or $IP_3$ receptors exist in the genome. Here we show that hyperosmotic shock provokes a transient increase in cytosolic $Ca^{2+}$ in vivo. Vacuolar $Ca^{2+}$, which is the major intracellular $Ca^{2+}$ store in yeast, is required for this response, whereas extracellular $Ca^{2+}$ is not. We aimed to identify the channel responsible for this regulated vacuolar $Ca^{2+}$ release. Here we report that Yvc1p, a vacuolar membrane protein with homology to transient receptor potential (TRP) channels, mediates the hyperosmolarity induced $Ca^{2+}$ release. After this release, low cytosolic $Ca^{2+}$ is restored and vacuolar $Ca^{2+}$ is replenished through the activity of Vcx1p, a $Ca^{2+}/H^+$ exchanger. These studies reveal a novel mechanism of internal $Ca^{2+}$ release and establish a new function for TRP channels.

## Introduction

Eukaryotic cells can sense a wide variety of environmental stresses, including changes in temperature, pH, osmolarity, and nutrient availability. They respond to these changes through a variety of signal transduction mechanisms, including activation of $Ca^{2+}$-dependent signaling pathways. In mammalian cells, various stimuli are known to induce the release of $Ca^{2+}$ from the endoplasmic or sarcoplasmic reticulum, the primary $Ca^{2+}$ stores. In yeast, the $Ca^{2+}$ concentration in the cytosol ($[Ca^{2+}]_{cyt}$)* has been shown to increase in response to the mating pheromone α factor (Iida et al., 1990), hypotonic shock (Batiza et al., 1996, Beeler et al., 1997), and addition of glucose to starving cells (Nakajima-Shimada et al., 1991). However, none of these increases in $[Ca^{2+}]_{cyt}$ has been shown to depend on internal $Ca^{2+}$ release, as opposed to influx from the external media. In the case of hypotonic shock, increases in $[Ca^{2+}]_{cyt}$ are partially independent from external $Ca^{2+}$, but there is no direct evidence of internal $Ca^{2+}$ release (Batiza et al., 1996). Therefore, although yeast possesses many of the conserved elements involved in $Ca^{2+}$ signaling (i.e., calmodulin, adenylate cyclase, and various protein kinases), signaling through internal $Ca^{2+}$ release is still speculative in yeast.

If internal $Ca^{2+}$ release exists in yeast, the vacuole is likely to be involved in this function, as it plays a major role in $Ca^{2+}$ homeostasis. Indeed, free $Ca^{2+}$ concentration in the yeast vacuole reaches 1.3 mM, compared with only 10 μM in the endoplasmic reticulum (Halachmi and Eilam, 1989; Strayle et al., 1999). Therefore, the yeast vacuole is the functional counterpart of the mammalian endoplasmic and sarcoplasmic reticulum for $Ca^{2+}$ storage. Two transporters play complementary roles in sequestering $Ca^{2+}$ into the vacuole: (a) Vcx1p, a low-affinity $Ca^{2+}/H^+$ exchanger that rapidly sequesters $Ca^{2+}$ into the vacuole; and (b) Pmc1p, a high-affinity $Ca^{2+}$ ATPase required for maintaining low $[Ca^{2+}]_{cyt}$ (Cunningham and Fink, 1994, 1996; Pozos et al., 1996; Miseta et al., 1999). It has been reported that vacuolar membrane vesicles could release $Ca^{2+}$ in the presence of IP3 (Belde et al., 1993); however, the mechanism and the physiological relevance of this effect have not been addressed. Although $Ca^{2+}$ influx into the vacuole has been well characterized, no protein has been shown to effect vacuolar $Ca^{2+}$ release.

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*Abbreviations used in this paper: $[Ca^{2+}]_{cyt}$, concentration of $Ca^{2+}$ in the cytosol; GFP, green fluorescent protein; HA, hemagglutinin; HOG, high-osmolarity glycerol; IP3, inositol-1,4,5-trisphosphate; TRP, transient receptor potential.

Key words: calcium signaling; ion channels; osmotic pressure; vacuoles; *Saccharomyces cerevisiae*


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All cells must repeatedly adapt to hypertonic shock caused by variations in water availability or solutes concentration. Yeast cells are particularly exposed to such changes, and therefore have developed multiple responses to hypertonic stress. Within minutes, cells shrink and the cytoskeleton disassembles (Morris et al., 1986; Chowdhury et al., 1992). Adaptation to these new conditions requires transcriptional induction of stress-responsive genes, as well as the accumulation of intracellular glycerol (Brown et al., 1986; Albertyn et al., 1994; Hirayama et al., 1995; Tamás et al., 1999). This transcriptional activation is mediated in part by the high-osmolarity glycerol (HOG) response pathway, which is composed of a mitogen-activated kinase cascade regulated by at least two independent osmosensors (Brewster et al., 1993; Posas et al., 1998).

Although the response to hypertonic shock has been intensively studied, whether it involves $Ca^{2+}$ signaling is unknown. In addressing this question, we show that: (a) hypertonic shock induces a transient increase in cytosolic $Ca^{2+}$ concentrations; (b) the $Ca^{2+}$ flux comes from the vacuole; and (c) Yvc1p, a homologue of transient receptor potential (TRP) channels, is required for this release. Yvc1p has recently been cloned by Palmer et al. (2001), and has been shown to be a cation-selective channel that can conduct $Ca^{2+}$, $K^+$, or $Na^+$. This conductance had been previously characterized by electrophysiological methods (Wada et al., 1987; Bertl and Slayman, 1990, 1992; Bertl et al., 1992). The electrophysiological properties of Yvc1p and its presence in the vacuolar fraction suggested that Yvc1p could be a vacuolar $Ca^{2+}$ channel. In this study we verify this hypothesis using living cells; we demonstrate in vivo, for the first time, that Yvc1p is a vacuolar channel that mediates $Ca^{2+}$ release in response to hyperosmotic stress.

## Results and discussion

To analyze the effect of hypertonic shock on $[Ca^{2+}]_{cyt}$, we added media containing high NaCl, KCl, or sorbitol to cells expressing the cytosolic, luminescent $Ca^{2+}$ reporter aequorin (Nakajima-Shimada et al., 1991; Batiza et al., 1996), and monitored luminescence (Fig. 1 a). All of these treatments induced an increase in $[Ca^{2+}]_{cyt}$, which peaked ~1 min after the hypertonic shock. $[Ca^{2+}]_{cyt}$ rapidly decreased and returned to its basal level by 5 min. In comparison, addition of $CaCl_2$ to the extracellular medium induced a sudden increase in $[Ca^{2+}]_{cyt}$ that peaked within the first second and then decreased rapidly (Fig. 1 a) (Miseta et al., 1999). As shown previously, this decrease is due to $Ca^{2+}$ sequestration into the vacuole (Miseta et al., 1999). These experiments show that hyperosmotic shock induces a transient increase in cytosolic $Ca^{2+}$, and that the timing of this response is slower than that induced by simple addition of external $Ca^{2+}$.

To further investigate this novel $Ca^{2+}$ response, we examined whether the hyperosmolarity induced $Ca^{2+}$ flux comes from an external or an internal source. We repeated these experiments using media containing the $Ca^{2+}$ chelators EGTA or 1,2-bis(2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid (BAPTA), as well as using low $Ca^{2+}$ SD medium (see Materials and methods), and observed no differences under these conditions (unpublished data). This strongly suggests that external $Ca^{2+}$ is not required for the observed cytosolic $Ca^{2+}$ peak. Next, we asked if the $Ca^{2+}$ flux is released from internal stores. Because the vacuole plays an important role in $Ca^{2+}$ storage and homeostasis, we investigated the hyperosmolarity induced $Ca^{2+}$ flux in mutants with defects in vacuolar $Ca^{2+}$ storage. In a *pmc1Δvcx1Δ* strain lacking both transporters for vacuolar $Ca^{2+}$ storage, $Ca^{2+}$ is not sequestered in the vacuole, and consequently, vacuolar $[Ca^{2+}]$ is dramatically reduced (Cunningham and Fink, 1996; Pozos et al., 1996). Therefore, if the hyperosmolarity induced $Ca^{2+}$ flux comes from the vacuole, we expect it to be reduced in this strain. Wild-type, *pmc1Δ*, *vcx1Δ*, and *pmc1Δvcx1Δ* strains were subjected to high osmolarity shock (0.8 M NaCl). Strikingly, the $Ca^{2+}$ increase was completely absent in the *pmc1Δvcx1Δ* strain (Fig. 1 b). In contrast, the single mutant *pmc1Δ* had a $Ca^{2+}$ peak comparable to wild-type strain, and the $Ca^{2+}$ response was increased in the *vcx1Δ* strain (Fig. 1 b). This last observation confirms that

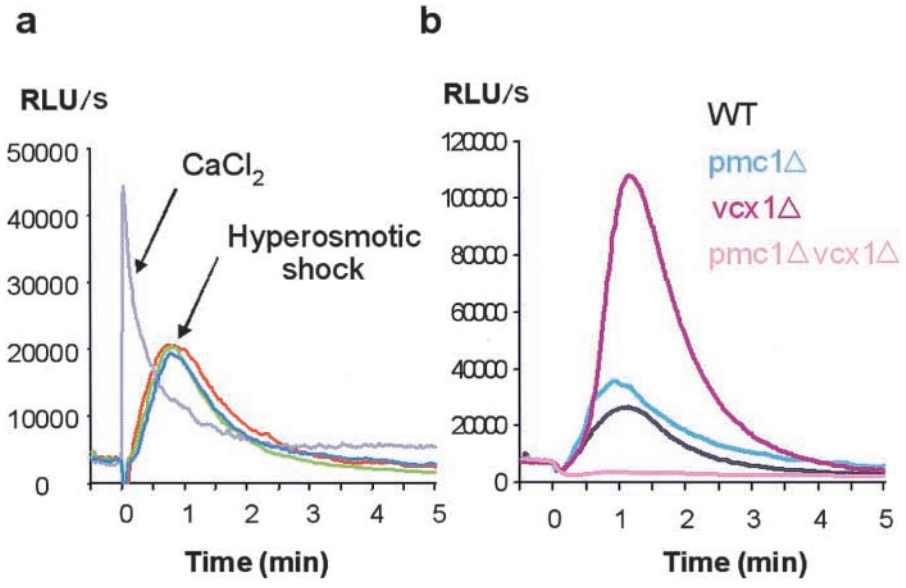


Figure 1. **Changes in $[Ca^{2+}]_{cyt}$ in response to hypertonic shock.** (a) Luminescence response in a wild-type strain (YPH499) after addition (Time = 0) of 0.1 M $CaCl_2$ (gray), or after hypertonic shock treatments: 0.8 M NaCl (red); 0.9 M KCl (yellow); and 0.7 M sorbitol (blue). As measured with an osmometer, the osmolality of media with 0.8 M NaCl is the same as with 0.9 M KCl, and is ~1.2-fold higher than with 0.7 M sorbitol. (b) Luminescence response after treatment with 0.8 M NaCl in wild-type (YPH499, black), *pmc1Δ* (TPYp, blue), *vcx1Δ* (KKY127, purple), and *pmc1Δvcx1Δ* (KKY124, pink) strains. All strains were transformed with PEVP11/AEQ.

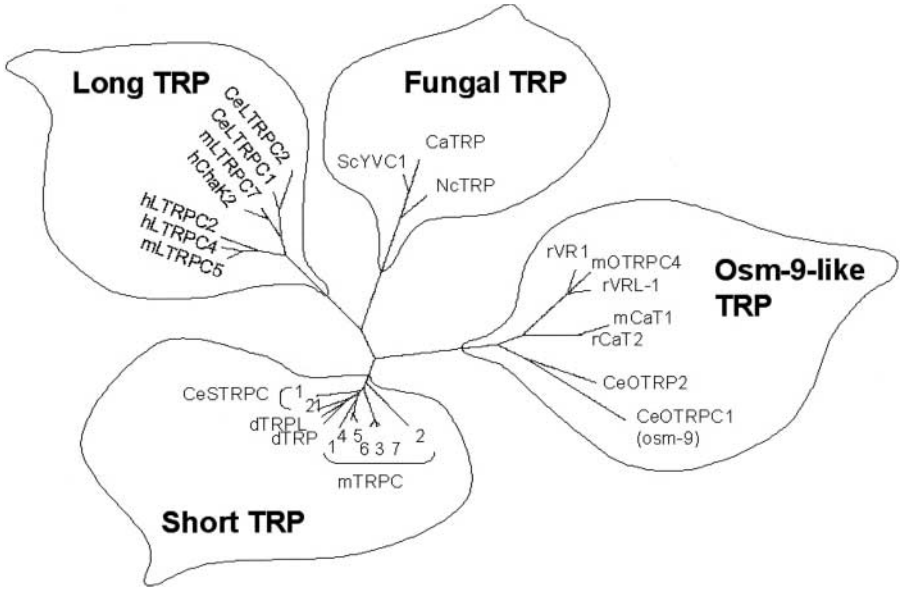


Figure 2. **Phylogenetic tree of TRP family of ion channels displaying the new fungal subfamily.** Ca, *C. albicans*; Ce, *C. elegans*; d, *D. melanogaster*; h, human; m, mouse; Nc, *N. crassa*; r, rat; Sc, *S. cerevisiae*; and TRP homologues cluster into four subfamilies.

Vcx1p plays a critical role in rapidly sequestering a sudden pulse of cytosolic $Ca^{2+}$ into the vacuole. Indeed, *vcx1Δ* cells also display a delay in restoring low $[Ca^{2+}]_{cyt}$ after addition of extracellular $Ca^{2+}$ (Miseta et al., 1999). Together, these results strongly suggest that the hyperosmolarity induced $Ca^{2+}$ flux is generated by release of $Ca^{2+}$ from the vacuole. This is the first time that $Ca^{2+}$ release from the vacuole has been shown in vivo in yeast in response to a specific signal.

As a next step, we aimed to identify the channel responsible for this $Ca^{2+}$ release. We examined the yeast genome for putative $Ca^{2+}$ channels and found a candidate ORF, recently characterized as *YVC1*, that shows significant homology to the TRP family of ion channels (Palmer et al., 2001). The first TRP channel was discovered in *Drosophila melanogaster* and is required for phototransduction (Montell and Rubin, 1989). Multiple homologues have since been identified in mammals, *Xenopus*, squid, and worms, and are involved in such diverse sensory functions as pain, heat, olfaction, and osmolarity signaling; they may also be involved in replenishing intracellular $Ca^{2+}$ stores (Putney and McKay, 1999; Harteneck et al., 2000; Clapham et al., 2001). TRP channels have been the subject of intense investigation recently, yet their gating mechanisms and biological role are not fully understood (Harteneck et al., 2000; Clapham et al., 2001; Montell, 2001). The discovery of a TRP homologue in *Saccharomyces cerevisiae* prompted us to search other fungal genomes for YVC1 homologues. We found a single homologue in *Candida albicans*, *Neurospora crassa*, and in 5 of the 14 hemiascomycetous yeast genomes that have been partially sequenced (Souciet et al., 2000). Next, we analyzed the phylogenetic relationship between these new fungal TRP channels and animal TRPs from worm and mammals (Fig. 2). The resulting tree shows that the newly defined cluster of fungal TRPs forms a distinct subfamily (Fig. 2), in addition to the previously described Short, Osm-like, and Long subfamilies (Harteneck et al., 2000; Clapham et al., 2001), also defined, respectively, as TRPC, TRPV, and TRPM subfamilies (Montell, 2001).

As a first step toward characterizing yeast Yvc1p, we determined its localization in vivo using a COOH-terminal green fluorescent protein (GFP) fusion. Interestingly, Yvc1–GFP was specifically localized to the vacuolar membrane (Fig. 3 a). This localization of the yeast TRP homologue is in contrast to other TRP channels studied thus far, which localize to the plasma membrane (Pollock et al., 1995; McKay et al., 2000; Xu and Beech, 2001). Next, we characterized the effect of Yvc1p levels on yeast cell growth. Although *yvc1Δ* had no apparent growth defects, cells expressing high levels of Yvc1p were extremely sensitive to the presence of $CaCl_2$ in the medium (Fig. 3 b). Furthermore, this sensitivity was $Ca^{2+}$-specific, as $MgCl_2$ at the same concentration did not affect growth (Fig. 3 b), and cells overexpressing *YVC1* did not show increased sensitivity to NaCl or KCl (0.6 to 1.2 M) (unpublished data). This $Ca^{2+}$ sensitivity strongly suggests that Yvc1p, like some other TRP channels, participates in $Ca^{2+}$ homeostasis and acts to increase cytosolic $[Ca^{2+}]$. Based on this finding, as well as its localization to the vacuolar membrane, Yvc1p is a good candidate for a $Ca^{2+}$ channel that mediates vacuolar $Ca^{2+}$ release. This hypothesis is also consistent with the electrophysiological properties of YVC1, which has been shown to be permeable to $Ca^{2+}$, among other cations (Bertl and Slayman, 1990, 1992; Bertl et al., 1992; Palmer et al., 2001).

We tested whether *YVC1* was involved in the hyperosmolarity induced $Ca^{2+}$ increase by examining $[Ca^{2+}]_{cyt}$ in cells lacking or overexpressing *YVC1*. The *yvc1Δ* strain displayed no significant increase in $[Ca^{2+}]_{cyt}$ after hypertonic treatment (Fig. 3 c). In contrast, *YVC1* overexpression greatly enhanced the magnitude of the $Ca^{2+}$ peak induced by high osmolarity (Fig. 3 c). These results indicate that Yvc1p mediates increased $[Ca^{2+}]_{cyt}$ in response to hypertonic shock. To confirm that this *YVC1*-mediated $Ca^{2+}$ release is dependent on vacuolar $Ca^{2+}$, we examined $[Ca^{2+}]_{cyt}$ in *pmc1Δ*, *vcx1Δ and pmc1Δvcx1Δ* strains carrying a *yvc1Δ* allele or overexpressing *YVC1*. The *pmc1Δvcx1Δ* strain showed no vacuolar $Ca^{2+}$ release even when *YVC1* was overexpressed (Fig. 3 d). This is likely due to low vacuolar $[Ca^{2+}]$, and shows that

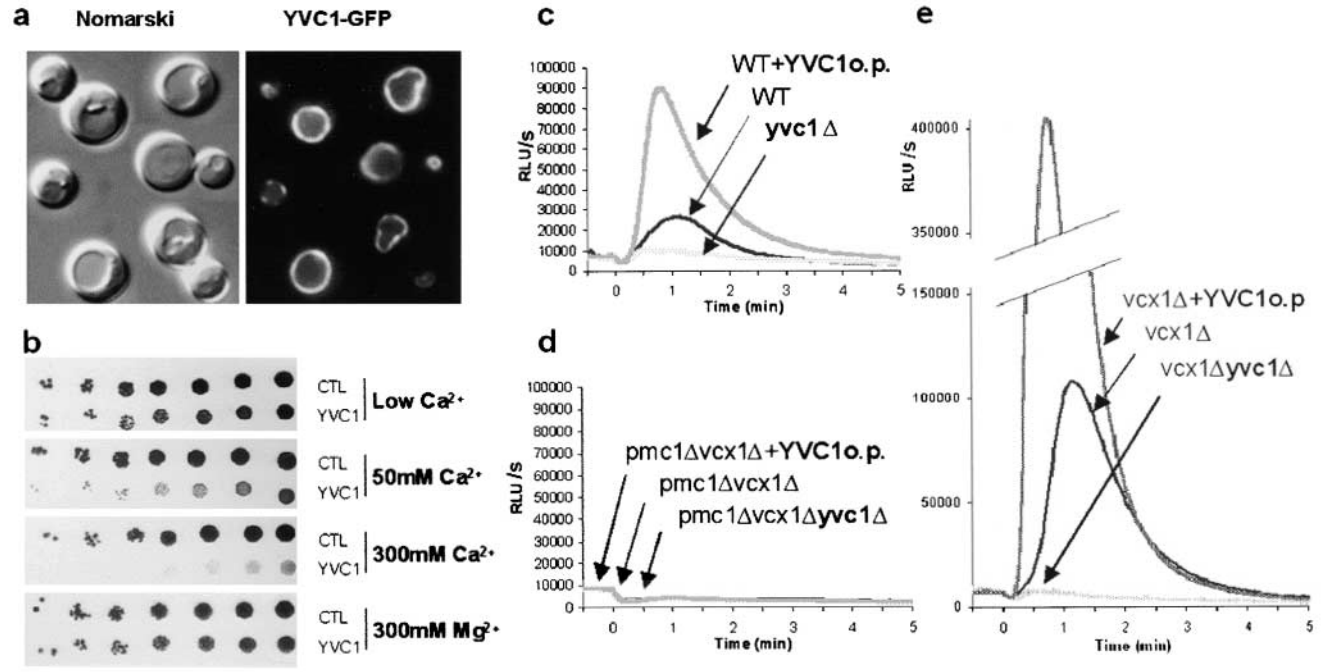


Figure 3. **Functional characterisation of Yvc1p.** (a) Yvc1–GFP localization to the vacuolar membrane, as visualized by fluorescence microscopy using an FITC filter. (b) *YVC1* overexpression causes $Ca^{2+}$ sensitivity, as shown by serial fivefold dilutions of wild-type (YPH499) strain transformed with a control (CTL) or the pYVC1-L-HA plasmid allowing high expression levels. Because *YVC1*-expressing cells grew slightly more slowly than control cells on regular media (unpublished data), low $Ca^{2+}$ SD medium (see Materials and methods) was used to perform this experiment in order to have identical growth on the control plate. (c) *YVC1* is required for vacuolar $Ca^{2+}$ release in response to hypertonic shock. Luminescence response of the wild-type strain (YPH499), carrying pYVC1-U for *YVC1* overexpression (WT+YVC1o.p.) or a control plasmid (WT), and of the *yvc1Δ* strain (VDY23) carrying a control plasmid (yvc1Δ). (d) Same experiment as c in *pmc1Δvcx1Δ* (KKY124) and *pmc1Δvcx1Δyvc1Δ* (VDY40) strains. (e) Same experiment as c in *vcx1Δ* (KKY127) and *vcx1Δyvc1Δ* (VDY31) strains.

*YVC1*-dependent $Ca^{2+}$ release comes from the vacuole. As expected, changes in $[Ca^{2+}]_{cyt}$ observed in the *Δpmc1* background lacking or overexpressing *YVC1* were equivalent to those seen in the wild-type strain (unpublished data). In a *vcx1Δ* background, deletion of *YVC1* completely eliminated the $Ca^{2+}$ increase induced by hypertonic shock (Fig. 3 e). In contrast, overexpression of *YVC1* in the *vcx1Δ* strain caused a dramatic increase in this $Ca^{2+}$ response (Fig. 3 e). Thus, overexpression of *YVC1* and mutational inactivation of *VCX1* both increase the amplitude of the hyperosmolarity induced $Ca^{2+}$ peak, and these two effects are additive. These observations underscore the importance of Vcx1p in antagonizing and potentially modulating *YVC1*-dependent $Ca^{2+}$ release. Together, these results show that following hypertonic shock, Yvc1p effects $Ca^{2+}$ release from the vacuole into the cytosol, and that this release is followed by rapid $Ca^{2+}$ sequestration into the vacuole by Vcx1p (Fig. 4).

Yeast actively sequester $Ca^{2+}$ in their vacuole. In these studies we establish that, as in other eukaryotic cells, this $Ca^{2+}$ can be released into the cytosol in response to external stimuli. We also show that this release is followed by refilling of the internal store. However, two key questions remain: (a) What leads to Yvc1p channel opening?; and (b) What are the physiological consequences of $Ca^{2+}$ release? We investigated whether other environmental changes besides hypertonic shock induced $Ca^{2+}$ release by Yvc1p. First, we found that the $Ca^{2+}$ peak induced by injection of extracellular $Ca^{2+}$ (Fig. 1 a) was not affected by *YVC1* deletion or overexpression (unpublished data). Thus, in vivo, a brief increase in $[Ca^{2+}]_{cyt}$ is apparently not sufficient to trigger Yvc1p opening, although the YVC1 cation conductance observed in isolated vacuoles is activated by $Ca^{2+}$ (Wada et al., 1987;

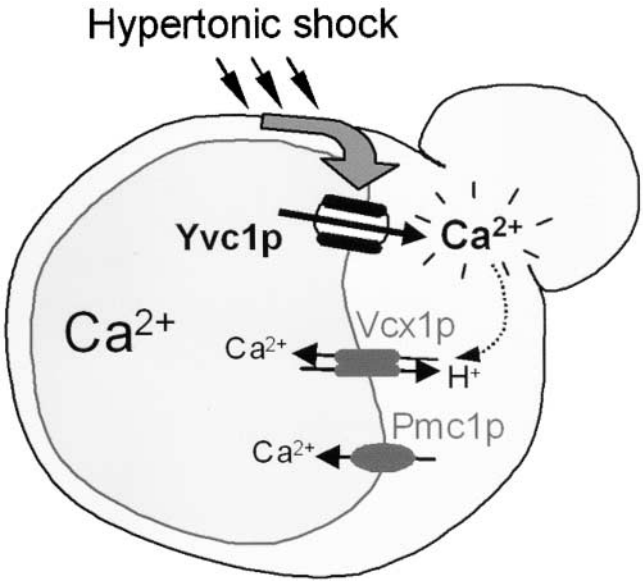


Figure 4. **Model for vacuolar $Ca^{2+}$ release and sequestration.** Hypertonic shock induces vacuolar $Ca^{2+}$ release by YVC1, which requires the presence of at least one of the $Ca^{2+}$ transporters Pmc1p or Vcx1p. After $Ca^{2+}$ release, Vcx1p rapidly sequesters $Ca^{2+}$ into the vacuole and decreases cytosolic $[Ca^{2+}]$.

Bertl and Slayman, 1990, 1992; Bertl et al., 1992; Palmer et al., 2001). Other conditions, such as hypotonic shock or the addition of 0.03% SDS or 7% ethanol, also induced a transient increase in cytosolic $Ca^{2+}$ (Batiza et al., 1996; unpublished data); however, *YVC1* was similarly not required for these $Ca^{2+}$ peaks (unpublished data). Therefore, the response of Yvc1p to hypertonic shock appears to be specific. We are currently investigating the role of *YVC1*-mediated $Ca^{2+}$ release in hypertonic stress signaling. The signaling pathway activated by hypertonic shock has been well characterized in yeast, and is composed of the HOG mitogen-activated kinase cascade (Posas et al., 1998). Further studies will examine the relationship between components of the HOG pathway and the $Ca^{2+}$ increase mediated by *YVC1*.

In conclusion, we show that internal $Ca^{2+}$ release in yeast is mediated by a novel class of $Ca^{2+}$ release channel, which is unrelated to $IP_3$ or ryanodine receptors. Instead, this release requires a homologue of the TRP family of ion channels, Yvc1p. Like TRP channels in multicellular organisms, YVC1 acts in sensory transduction. However, YVC1 is the first TRP channel homologue shown to mediate $Ca^{2+}$ release from an intracellular store.

## Materials and methods

### Yeast strains and media

Strains were isogenic to YPH499 (*MAT*a *ura3–52 lys2–801 ade2–101 trp1-Δ63 his3-Δ200 leu2-Δ1*) (Sikorski and Hieter, 1989). TPYp is *Mat*α *pmc1Δ::TRP1*; KKY127 is *Mat*a *vcx1Δ*; KKY124 is *Mat*α *pmc1Δ::TRP1 vcx1Δ*; VDY23 is *MAT*a *yvc1Δ::Kan*$^R$; VDY25 is *Mat*α *pmc1Δ::TRP1 yvc1Δ::Kan*$^R$; VDY31 is *Mat***a** *vcx1Δ yvc1Δ::Kan*$^R$; and VDY40 is *Mat*α *pmc1Δ::TRP1 vcx1Δ yvc1Δ::Kan*$^R$. SD medium (Sherman et al., 1986) contained twice the recommended levels of supplements with 3.5 g of ammonium chloride per liter substituted for ammonium sulfate. Low $Ca^{2+}$ SD medium ($[Ca^{2+}] \sim 0.24$ μM) was the SD medium in which $CaCl_2$ was omitted and calcium pantothenate was replaced by sodium pantothenate as described previously (Iida et al., 1994).

### Vector construction and gene deletion

YVC1–GFP plasmid (VDp88) was constructed by cloning a PCR fragment containing *YVC1* and 737 pb upstream sequence into the SacI/NheI sites of pGRU2, provided by Bertrand Daignan-Fornier (Institut de Biochemie et Génétique Cellulaires, Bordeaux, France). Hemagglutinin (HA)-tagged *YVC1* overexpression plasmids were constructed by a two-step PCR: PCR-amplified 3× (HA) was used as a downstream primer to amplify *YVC1* (YOR087/088W). This fragment was cut by Xho/BglII and cloned into Xho/Bam sites in pVT100L or pVT100U (Vernet et al., 1987), leading to pYVC1-HA-L for high expression of *YVC1*, and pYVC1-HA-U, for moderate expression, respectively. We verified that full-length Yvc1-HA protein (~78 kD) was expressed in yeast by Western blotting. pYVC1-U, used for aequorin experiments, is a nontagged version of pYVC1-HA-U: the HA tag was removed by a NotI digest and self-religation. To delete *YVC1*, a fragment comprising ORF *YOR087/088* and 736 bp of upstream sequence was cloned between the XhoI and BamHI sites of pcDNA3.1 (Invitrogen). This plasmid was then cut by EcoRI, which removed the sequence from position −41 to +1780 from the ATG, and a cassette containing the *kan*$^r$ gene was inserted into this site. The resulting plasmid was used to amplify a deletion cassette that was used to transform yeast. Kanamycin-resistant colonies were selected and *Δyvc1* knockout was checked by PCR as described previously (Güldener et al., 1996).

### Aequorin experiments

Yeast carrying the PEVP11/AEQ plasmid, provided by Patrick H. Masson (University of Wisconsin-Madison, Madison, WI) (Batiza et al., 1996) were inoculated from a saturated overnight culture to $OD_{600} = 0.5$ in SD media with 2 μM coelenterazine, and were grown overnight at room temperature to reconstitute aequorin from apoaequorin. For each experiment, an aliquot of 250 μl ($OD_{600} = 2–3$) was harvested. Cells were resuspended in 100 μl SD media and transferred to luminometer tubes. The baseline luminescence was recorded every second for 30 s (1-s integration) using a Berthold LB9507 luminometer, and was reported in relative luminescence units/s. Hypertonic shock was performed by adding 100 μl SD containing twice the desired final concentration of sorbitol, KCl, or NaCl. To ensure that total reconstituted aequorin was not limiting in our assays, we measured the maximal luminescence after addition of 0.1% digitonin. The maximal luminescence was 4,000,000 relative luminescence units or more, which is 10× higher than the highest signal observed in our assays. Because light units cannot be accurately converted into intracellular $Ca^{2+}$ concentrations, our results are presented as relative quantities.

### Phylogenetic tree

Evolutionary distances between peptide sequences aligned with ClustalW were calculated with the PHILIP protdist software (Felsenstein, 1993), and the tree was subsequently plotted by the neighbor-joining method (Saitou and Nei, 1987). The GenBank accession numbers for the proteins used are the following (available at GenBank/EMBL/DDBJ accession no.): ScYVC1 (*S. cerevisiae,* YOR087/088w) (Palmer et al., 2001); CaTRP (sequence 11894–9852 from *Candida albicans* genome contig 1.802); NcTRP (sequence 4727–6751 from *N. crassa* genome contig 6–2259); rVR1 (T09054); mOTRPC4 (AAG17543); rVRL-1 (NP_035836); mCaT1 (BAA99538); rCaT2 (BAA99541); CeOTRPC2 (CAA96644); CeOTRPC1 (T37241); CeLTRPC2 (CAA92726); CeLTRPC1 (CAB02303); mLTRPC7 (AAK57433); hChaK2 (AAK31202); hLTRPC2 (NP_003298); hLTRPC4 (NP_060106); mLTRPC5 (AAF98120);CeSTRPC1 (AAA28168); CeSTRPC2 (AAK21447); dTRPL (P48994); dTRP (P19334); mTRPC1 (AAB50622); mTRPC4 (AAC05179); mTRPC5 (AAC13550); mTRPC6 (AAC06146); mTRPC3 (NP_062383); mTRPC7 (AAD42069); and mTRPC2 (AAG29950).

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## References

Albertyn, J., S. Hohmann, J.M. Thevelein, and B.A. Prior. 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.

Batiza, A.F., T. Schulz, and P.H. Masson. 1996. Yeast respond to hypotonic shock with a calcium pulse. *J. Biol. Chem.* 271:23357–23362.

Beeler, T., K. Gable, and T. Dunn. 1997. Activation of divalent cation influx into *S. cerevisiae* cells by hypotonic downshift. *J. Membr. Biol.* 160:77–83.

Belde, P.J.M., J.H. Vossen, G.W.F.H. Borst-Pauwels, and A.P.R. Theuvenet. 1993. Inositol 1,4,5-trisphosphate releases $Ca^{2+}$ from vacuolar membrane vesicles of *Saccharomyces cerevisiae*. *FEBS Lett.* 323:113–118.

Bertl, A., and C.L. Slayman. 1990. Cation-selective channels in the vacuolar membrane of *Saccharomyces*: dependence on calcium, redox state, and voltage. *Proc. Natl. Acad. Sci. USA.* 87:7824–7828.

Bertl, A., and C.L. Slayman. 1992. Complex modulation of cation channels in the tonoplast and plasma membrane of *Saccharomyces cerevisiae*: single-channel studies. *J. Exp. Biol.* 172:271–287.

Bertl, A., D. Gradmann, and C.L. Slayman. 1992. Calcium- and voltage-dependent ion channels in *Saccharomyces cerevisiae*. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 338:63–72.

Brewster, J.L., T. de Valoir, N.D. Dwyer, E. Winter, and M. Gustino. 1993. An osmosensin signal transduction pathway in yeast. *Science*. 259:1760–1763.

Brown, A.D., K.F. Mackenzie, and K.K. Singh. 1986. Selected aspects of microbial osmoregulation. *FEMS Microbiol. Rev.* 39:31–36.

Chowdhury, S., K.W. Smith, and M.C. Gustin. 1992. Osmotic stress and the yeast cytoskeleton: phenotype-specific suppression of an actin mutation. *J. Cell Biol.* 118:561–571.

Clapham, D.E., L.W. Runnels, and C. Strübing. 2001. The TRP ion channel family. *Nat. Rev. Neurosci.* 6:387–396.

Cunningham, K.W., and G.R. Fink. 1994. Calcineurin-dependent growth control in *Saccharomyces cerevisiae* mutants lacking *PMC1*, a homologue of plasma

membrane $Ca^{2+}$ ATPases. *J. Cell Biol.* 124:351–363.

Cunningham, K.W., and G.R. Fink. 1996. Calcineurin inhibits *VCX1*-dependent $H^+/Ca^{2+}$ exchange and induces $Ca^{2+}$ ATPases in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 16:2226–2237.

Felsenstein, J. 1993. PHYLIP (Phylogeny Inference Package) version 3.5c. Department of Genetics, University of Washington, Seattle.

Güldener, U., S. Heck, T. Fiedler, J. Beinhauer, and J.H. Hegemann. 1996. A new efficient gene disruption cassette for repeated use in budding yeast. *Nucleic Acids Res.* 24:2519–2524.

Halachmi, D., and Y. Eilam. 1989. Cytosolic and vacuolar $Ca^{2+}$ concentrations in yeast cells measured with the $Ca^{2+}$-sensitive fluorescence dye indo-1. *FEBS Lett.* 256**:**55-61.

Harteneck, C., T.D. Plant, and G. Schultz. 2000. From worm to man: three subfamilies of TRP channels. *Trends Neurosci.* 23:159–166.

Hirayama, T., T. Maeda, H. Saito, and K. Shinozaki. 1995. Cloning and characterization of seven cDNAs for hyper-osmolarity-responsive (HOR) genes of *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* 249:127–138.

Iida, H., Y. Yagawa, and Y. Anraku. 1990. Cell cycle control by $Ca^{2+}$ in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 265:13391–13399.

Iida, H., H. Nakamura, T. Ono, M.S. Okumura, and Y. Anraku. 1994. *MID1*, a novel *Saccharomyces cerevisiae* gene encoding a plasma membrane protein, is required for $Ca^{2+}$ influx and mating. *Mol. Cell Biol.* 14:8259–8271.

McKay, R.R., C.L. Szymeczek-Seay, J.P. Lievremont, G.S. Bird, C. Zitt, E. Jungling, A. Luckhoff, and J.W. Putney, Jr. 2000. Cloning and expression of the human transient receptor potential 4 (TRP4) gene: localization and functional expression of human TRP4 and TRP3. *Biochem. J.* 351:735–746.

Miseta, A., R. Kellermayer, D.P. Aiello, L. Fu, and D.M. Bedwell. 1999. The vacuolar $Ca^{2+}/H^+$ exchanger Vcx1p/Hum1p tightly controls cytosolic $Ca^{2+}$ levels in *S. cerevisiae*. *FEBS Lett.* 451:132–136.

Montell, C. 2001. Physiology, phylogeny, and functions of the TRP superfamily of cation channels. *Sciences's STKE* (http://stke.sciencemag.org/cgi/content/full/OC_sigtrans;2001/90/re1).

Montell, C., and G.M. Rubin. 1989. Molecular characterization of the *Drosophila* trp locus: a putative integral membrane protein required for phototransduction. *Neuron.* 2:1313–1323.

Morris, G.J., L. Winters, G.E. Coulson, and K.J. Clarke. 1986. Effect of osmotic stress on the ultrastructure and viability of the yeast *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* 2023–2034.

Nakajima-Shimada, J., H. Iida, F.I. Tsuji, and Y. Anraku. 1991. Monitoring of intracellular calcium in *Saccharomyces cerevisiae* with an apoaequorin cDNA expression system. *Proc. Natl. Acad. Sci. USA.* 88:6878–6882.

Palmer, C.P., X.L. Zhou, J. Lin, S.H. Loukin, C. Kung, and Y. Saimi. 2001. A TRP homolog in *Saccharomyces cerevisiae* forms an intracellular Ca(2+)-permeable channel in the yeast vacuolar membrane. *Proc. Natl. Acad. Sci. USA.* 98:7801–7805.

Pollock, J.A., A. Assaf, A. Peretz, C.D. Nichols, M.H. Mojet, R.C. Hardie, and B. Minke. 1995. TRP, a protein essential for inositide-mediated $Ca^{2+}$ influx is localized adjacent to the calcium stores in *Drosophila* photoreceptors. *J. Neurosci.* 15:3747–3760.

Posas, F., M. Takekawa, and H. Saito. 1998. Signal transduction by MAP kinase cascades in budding yeast. *Curr. Opin. Microbiol.* 1:175–182.

Pozos, T.C., I. Sekler, and M.S. Cyert. 1996. The product of *HUM1*, a novel yeast gene, is required for vacuolar $Ca^{2+}/H^+$ exchange and is related to mammalian $Na^+/Ca^{2+}$ exchangers. *Mol. Cell. Biol.* 16:3730–3741.

Putney, J.W., and R.R. McKay. 1999. Capacitative calcium entry channels. *Bioessays.* 21:38–46.

Saitou, N., and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4:406–425.

Sherman, F., G.R. Fink, and J.B. Hicks. 1986. Methods in Yeast Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 186 pp.

Sikorski, R.S., and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *S. cerevisiae*. *Genetics.* 122:19–27.

Souciet, J., M. Aigle, F. Artguenare, G. Blandin, M. Bolton-Fukuhara, E. Bon, P. Brottier, S. Casararegola, J. de Montigny, B. Dujon, et al. 2000. Genomic exploration of the hemiascomycetous yeasts: a set of species for molecular evolution studies. *FEBS Lett*. 487:3–12.

Strayle, J., T. Pozzan, and H.K. Rudolph. 1999. Steady-state free Ca(2+) in the yeast endoplasmic reticulum reaches only 10 microM and is mainly controlled by the secretory pathway pump pmr1. *EMBO J.* 18:4733–4743.

Tamás, M.J., K. Luyten, F.C. Sutherland, A. Hernandez, J. Albertyn, H. Valadi, H. Li, B.A. Prior, S.G. Kilian, J. Ramos, et al. 1999. Fps1p controls the accumulation and release of the compatible solute glycerol in yeast osmoregulation. *Mol. Microbiol.* 31:1087–1104.

Vernet, T., D. Dignard, and D.Y. Thomas. 1987. A family of yeast expression vectors containing the phage f1 intergenic region. *Gene.* 52:225–233.

Wada, Y., Y. Ohsumi, M. Tanifuji, M. Kasai, and Y. Anraku. 1987. Vacuolar ion channel of the yeast *S. cerevisiae*. *J. Biol. Chem.* 262:17260–17263.

Xu, S.Z., and D.J. Beech. 2001. TrpC1 is a membrane-spanning subunit of store-operated Ca(2+) channels in native vascular smooth muscle cells. *Circ. Res.* 88:84–87.