A genome-wide survey suggests an osmoprotective role for vacuolar Ca^{2+} release in cell wall-compromised yeast

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ABSTRACT **In yeast, osmotic upshock causes a release of vacuolar Ca^{2+} through the mechanosensitive transient receptor potential channel, Yvc1. We screened the collection of 4810 yeast gene deletants twice for alterations in this response in an attempt to find elements that regulate the amount of vacuolar Ca^{2+} or the Yvc1 channel. Severe overresponders and underresponders to upshock were further scrutinized for their calcium content with ^{45}Ca and their Yvc1 electrophysiological activities under patch-clamp. The severe underresponders have lower calcium content but no change in Yvc1 activity. The strong overresponders, most of which are deleted of genes involved in cell wall metabolism, have higher calcium content. Wall mutations are known to up-regulate Ca^{2+}-calcineurin-dependent genes. It appears that stress on the cell wall induces Ca^{2+} accumulation, adaptively anticipating the need in defense or repair against future stress, including osmotic stress.—Loukin, S., Zhou, X., Kung, C., Saimi, Y. A genome-wide survey suggests an osmoprotective role for vacuolar Ca^{2+} release in cell wall-compromised yeast. *FASEB J.* 22, 2405–2415 (2008)**

Key Words: Saccharomyces cerevisiae · *Yvc1* · *TRPY1* · *transient receptor potential ion channel* · *CCH1* · *calcineurin*

Ca^{2+} IS A WELL-KNOWN SECOND messenger to relay stimuli or stresses into cellular responses in plants, animals, and unicellular eukaryotes. For all free-living cells, dehydration and overhydration are key stresses. In the laboratory, a sudden osmotic downshock simulates rain, whereas upshock simulates dehydration due to evaporation or freezing. Experiments with the budding yeast, *Saccharomyces cerevisiae*, show that downshock apparently let in Ca^{2+} from the medium (1, 2) by opening a membrane mechanosensitive channel of unknown molecular identity (3). Upshock, on the other hand, opens a different mechanosensitive channel in the vacuolar membrane to release vacuolar Ca^{2+} into the cytoplasm (4). This channel, Yvc1 (TRPY1) (5, 6), belongs to the transient receptor potential (TRP) superfamily of channels that sense chemical, thermal, or mechanical stimuli in animals (7). It has a ~320 pS unitary conductance, passes cations, and can be opened by membrane stretch or deformation as examined directly under patch-clamp (5, 6). When confronted with a hypertonic solution, water evacuates passively from the cytoplasm and then from the vacuole. The deformation of the vacuolar membrane is apparently the direct cause of opening of the Yvc1 channel and the release of vacuolar Ca^{2+} (4). Although there may be additional elements (8), this minimal model adequately explains the Ca^{2+} release. The rise of cytoplasmic Ca^{2+}, readily registered by the luminescence of transgenic aequorin (1, 4), begins within a few seconds of osmotic upshock and generally peaks between 1 and 2 min. This transient response precedes the much slower upshock-induced changes in transcriptional profile (9) and the activation of the high osmolarity glycerol pathway (10). Thus, yeast has apparently evolved several levels of defense against dehydration, played out in different time domains.

For a given upshock, the amount of Ca^{2+} release obviously depends on two elements: the source, which is the amount of Ca^{2+} in the vacuole, and the shock-to-flux pathway, which may comprise auxiliary or regulatory elements beside the Yvc1 channel. To identify these elements, if any, and to see how vacuolar Ca^{2+} may be regulated, we examine the yeast deletome for deletants showing significantly larger or smaller responses to upshock. We have indeed identified such over- and underresponders and further examined them experimentally in two ways using current technology: To examine the source, we measured the calcium content by tracing ^{45}Ca. To examine the pathway, we directly examine the channel molecular behavior under patch-clamp in excised vacuolar membrane patches. We found no obvious channel abnormalities among the severe underresponding deletants. On the other hand, we found that the Ca^{2+} pool is surprisingly dynamic and established a hitherto unknown connection between cell wall status and vacuolar Ca^{2+} content. These findings echo those showing widespread calcineurin-dependent transcriptional up-regulation in

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wall-compromised mutants (11). Together, these findings indicate that stress on the cell wall leads to an adaptive Ca^{2+}-based defense and inaugurate research into how this occurs.

MATERIALS AND METHODS

Yeast strains and media

A copy of the yeast deletion library of parental strain BY4742 (*MAT*α, *his3*Δ*1, leu2*Δ*0, lys2*Δ*0,* and *ura3*Δ*0*) was purchased from Open Biosystems (Huntsville, AL, USA). Individual deletants in the library were transformed with the aequorin-expressing plasmid pEVP11/AEQ as described in Loukin *et al.* (2). All growth was carried out in a sulfate-depleted variant of CMD-leu described in Denis and Cyert (4) and referred to as "DCD-leu" here. Double deletants with *yvc1* were generated by creating *yvc1::URA3* alleles in various overresponding deletants using the polymerase chain reaction-mediated one-step gene disruption method described in Baudin *et al.* (12).

Deletome screening

For the high-throughput screening of the deletion library, pin replicants of the 96-well plates containing frozen stocks of the pEVP11/AEQ transformed deletion library were grown overnight in DCD-leu at 30°C. Then 1 μl of the resulting saturated cultures were transferred to 50 μl of DCD-leu containing 2 μM colenterazine (Biotium, Hayward, CA, USA) and grown for 48 h at 20°C in the dark. Immediately before screening, 20 μl of these cultures was diluted into 80 μl of 200 mM sorbitol (to minimize desiccation) and placed in a Mirthras LB940 microplate luminometer (Berthold, Bad Wildbad, Germany). Upshock responses for each well were elicited by injection of 100 μl of a solution containing 4 M NaCl; 20 mM 2-(*N*-morpholino)ethane sulfonic acid (MES), pH 7.2; and 20 mM EGTA (second screen only); and the luminometric response was measured for 45 s. Luminescence data were acquired and analyzed using MikroWin2000 (Berthold). Luminometric responses were calculated relative to the median responding well from each 96-well plate (see Supplemental Table S1). Before each screen, growth in each well was visually scored with 4 being maximal, and 3, 2, and 1 being ~1/3, 1/9, and 1/30 of this density sequentially, and 0 being no obvious growth (see Supplemental Table S1). For the underresponding deletants, those that grew well and had a response less than 5% of the plate median were retested individually. If on retest, they maintained signals less than 10% of wild-type levels, they were further examined for functional aequorin expression by measuring total luminometric responses after addition of 0.5% digitonin. Deletants with less than 10% wild-type levels of aequorin were discarded. Deletants that were retested and passed both these tests are labeled "retest +" in Supplemental Table S1, and those that were retested and failed either test are labeled "retest −".

Other luminometric measurements

Luminometric measurements of the individual strains shown in Figs. 1, 3, 5, and 7 were done as above except that 200 μl of a solution containing 2 M NaCl, 10 mM MES, and 10 mM EGTA (unless otherwise stated) were added to 20 μl of cells. For the experiments shown in Fig. 1, except for those marked "standing culture," cells were grown in 2-ml roller cultures and transferred to 96-well plates for luminometric measurements as above. For the long-term cultures in Fig. 5, as well as the standing culture in Fig. 1*B*, 96-well plates were inoculated with 75 μl/well of cultures grown overnight in DCD-leu containing colenterazine diluted 1:10 into the same medium, overlaid with 75 μl of mineral oil to prevent desiccation, and placed in the plate luminometer, wherein hypertonic responses were elicited at the given intervals by injection of 150 μl of 3 M NaCl; 15 mM MES, pH 7.2; and 15 mM EGTA. For the long-term roller cultures in Fig. 1*B, C*, five 5-ml staggered cultures were used with the most concentrated being a 1:10 dilution from an overnight cultures and the subsequent cultures being serially diluted 1:3. Cultures were realigned on the basis of the measured logarithmic growth rate.

Calcium content measurements

Total cellular calcium content was measured using the method described in Cunningham and Fink (13). Briefly, parallel cultures with or without $^{45}Ca^{2+}$ (40 cpm/pmol; total Ca^{2+} added with isotope negligible) were grown from 1:10 dilutions. Luminometric responses of these cultures were measured as described above. Calcium content was measured by transferring 100 μl of cells from the $^{45}Ca^{2+}$ cultures to 1 ml of ice-cold buffer A (5 mM HEPES, pH 6.5, and 10 mM $CaCl_2$). After 1 min, cells were filtered under vacuum onto Millipore 0.45-μm HA filters (Millipore, Bedford, MA, USA) and rinsed 3× with 5 ml of ice-cold buffer A with a 10-s rinse time each. All measurements were done in triplicate and are presented as cpm relative to wild-type cultures assayed in parallel. Cultures were all at similar densities of OD 4.0.

Electrophysiological measurements

See Palmer *et al.* (5) for the methods of cell culture, enzyme digestion, spheroplast formation, osmotic adjustment, vacuolar presentation, pipette micromanipulation, gigaseal formation, membrane-patch excision, electrophysiological recording techniques, and data handling. Data were filtered at 1 kHz at the point of acquisition using an 8-pole Bessel filter and analyzed using pCLAMP 9 software (Axon Industries, Foster City, CA, USA).

Whole deletome screen

The peak responses as well as other relevant data from the whole deletome screen are presented in Excel format in Supplemental Table S1.

RESULTS

Growth-phase dependence of the response to osmotic upshock

A transient elevation in cytoplasmic Ca^{2+} measured by transgenic aequorin luminescence can be elicited by the addition of osmoticum in the wild type but not in *yvc1*Δ (**Fig. 1*A***) (4). We examined this response in more detail before basing the screen of thousands of strains on it. Culture state can account for a large variation in the response to osmotic upshock. We examined the behavior of cultures over 100 h after inoculation, through their logarithmic phase, diauxic lag, and post-diauxic growth phase. Surprisingly, this response is completely lacking in logarithmically growing cells (Fig. 1*B, C*). The absence of response is not due to a

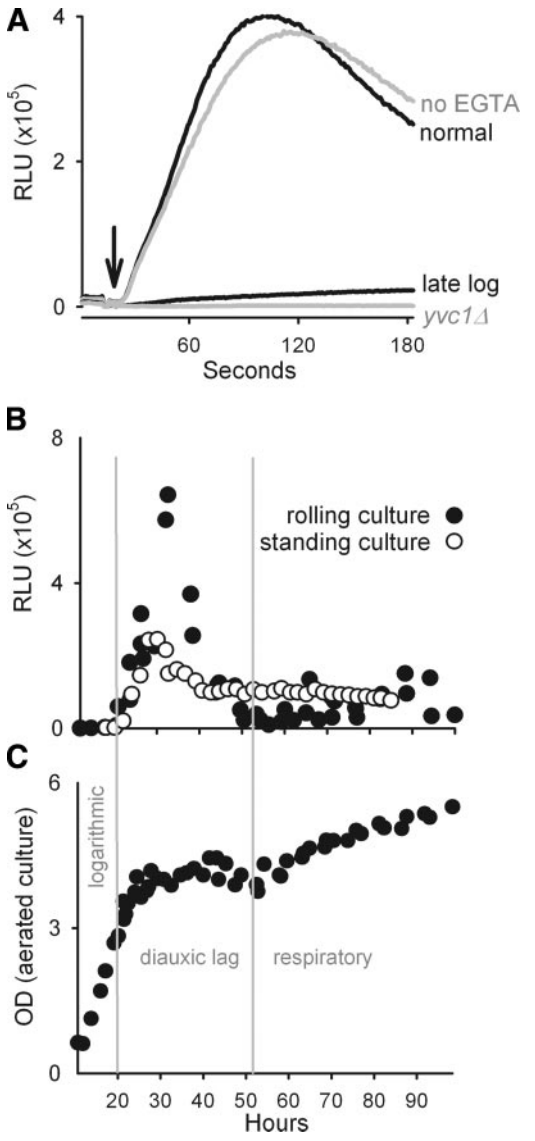

Figure 1. The wild-type response to osmotic upshock and its variation through culture phases. *A*) Responses in relative luminometric units per second (RLU) to the addition of the osmoticum (arrow) over time. Wild-type postlogarithmic culture at OD 3.1 (black curve) shows a response peaking at ~100 s. This response is due to an internal Ca^{2+} release, as EGTA is included in the shock osmoticum. Withholding the chelator (gray) generally has little effect, demonstrating that the signal usually has only a minor component from external Ca^{2+}. The Ca^{2+} is released through the Yvc1 channel, since deleting *YVC1* (*yvc1*△) removes the response completely (4). Wild-type culture in late logarithmic phase (OD 2.4) has little or no signal (late log). *B*) The peak of the upshock response is plotted against culture age ≤100 h after inoculation, showing the strongest responses during diauxic lag. Aerated rolling cultures (●) and standing cultures (○) are compared. The former shows responses with a more pronounced rise and fall. *C*) Optical density of the rolling culture in *B*. The approximate demarcations of three growth phases are marked.

lack of vacuolar Ca^{2+} as measured by ^{45}Ca (see below) or to a lack of aequorin in the cells examined here. Digitonin permeabilization showed that log-phase cells actually have more available aequorin than postlogarithmic cells (data not shown). The upshock response peaks in the diauxic lag (Fig. 1*B*, *C*), during which the cells shift from fermentation to respiration. However, respiration cannot be the determinant of the response, because the response subsides almost completely in the respiring postdiauxic cells (Fig. 1*B*, *C*). We also tested nonrespiring *petite* cells and found a rise and fall of the upshock response similar to that of the wild type (data not shown). Compared with more well-aerated roller cultures, the rise and fall of the response in the standing cultures is less pronounced. More important, the standing culture gives a constant response between 40 and 70 h after inoculation (Fig. 1*B*, ○). For the purpose here, we did not investigate further why the standing cultures give this constant response but used such cultures and this time window in the deletome screen below. Despite our attempt to control the culture conditions, variability among the thousands of cultures cannot be completely avoided in this or any similar high-throughput screens.

Screen for deletants with altered upshock responses

We screened the 4810 members of the yeast deletome individually for those with increased or decreased response. This collection comprises nearly all the viable strains, each deleted of a nonessential open reading frame (ORF) in the yeast genome of ~5800 ORFs. The entire deletant library was transformed individually with a plasmid bearing an aequorin gene. For each round of screening, we made standing cultures of these strains on more than 50 96-well plates. Each strain in a well was tested in the same optimal time window determined above, *i.e.*, 44–52 h after inoculation, carried out by adding 1 vol of culture into 10 vol of fresh medium. A variety of osmotica can elicit the upshock response; 2 M NaCl (1:1 dilution of cells in medium with 4 M NaCl) gave large and consistent results and was used here. The upshock test was carried out directly on the 96-well culture plates using an automated injector to deliver the NaCl serially. The light signal from Ca^{2+}-aequorin was registered with an automated microplate luminometer.

In the first round of the deletome screening, we encountered a small, inconsistent, and *YVC1*-indpendent component in the luminometric signal in some cases. Although it was minor compared to the *YVC1*-dependent component, it might undermine our search for underresponders. This component originates from external Ca^{2+} and can be eliminated by chelation. Accordingly, we added EGTA to the NaCl shock solutions in the second round of deletome screening. The peak responses and ranking of all deletants from both screens are presented in Supplemental Table S1.

Identification of deletants with increased upshock responses

As expected of this type of high-throughput screens, the two rounds of screening did not give identical

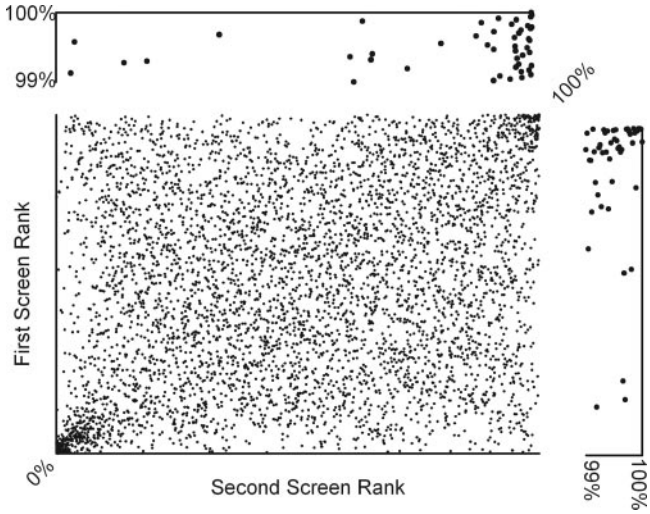

Figure 2. Comparison of the two screens of the deletome. Comparison of rankings between the two screens performed on the 4810 individual deletants, with 100% being the strongest response. The main panel shows the entire range, with two clusters apparent: those that scored high in both screens (top right) and those that scored low in both (bottom left). The majority of the latter result from cultures that did not grow well (Supplemental Table S1) and therefore were not in the postlogarithmic phase, the point at which responses were expected. The top and right panels show just the top 1% of the first and second screen, respectively.

results (**Fig. 2**). Nonetheless, clear consensual overresponders can be identified. Of the top 1% of responders (13 of 48), 27% were common to both screens. Of the top 1% responders in the first screen, 66% were in the top 10% of the second screen (Fig. 2, top panel). Of the top 1% responders in the second screen, 70% were in the top 10% of the first screen (Fig. 2, right panel).

We further scrutinized the top 1% responders from each of the two rounds of the high-throughput screening in repeated individual retests. They were first grown on 96-well plates, which also contained wild-type cells in every row for local comparison. They were then tested at 48 and at 72 h after inoculation (1:100 dilution to fresh medium). We further screened several of the top responders by growing them for 2 days after a 1:10 dilution and found that they had a growth-phase variation of their upshock responses very similar to that of the wild type (Fig. 1*C*), although the responses were uniformly stronger than those of the wild type (data not shown). **Table 1** lists the top 26 highest ranking upshock overresponders showing 4-fold or greater response than wild type in individual retests.

The responses to osmotic upshock of the overresponders are not only stronger, but also faster than those of the wild type (**Fig. 3**). In general, the larger the response is, the earlier it peaks, presumably reflecting the positive feedback of the Ca^{2+}-dependent Ca^{2+} release, an innate property of the Yvc1 channel (6), as well as the ensuing activation of the Ca^{2+} resequestration mechanisms. To test whether the increased signal from these deletants is through novel pathways (leaks?) or through the usual conduit (the Yvc1 channel), we constructed and tested six double mutants, adding *yvc1::URA3* to each of the named deletions. As shown in Fig. 3, although single mutants (deletants) *gas1*Δ, *bst1*Δ, *sur4*Δ, *pmr1*Δ, and *alg6*Δ and the deletant of YEL059W clearly overresponded to upshock, the six corresponding double mutants (*gas1*Δ *yvc1*Δ, *bst1*Δ *yvc1*Δ, *sur4*Δ *yvc1*Δ, *pmr1*Δ *yvc1*Δ, *alg6*Δ *yvc1*Δ, and YEL059WΔ *yvc1*Δ) gave no response. Thus, the strong responses of these representative deletants with wall defects were not due to leakage but are exaggerated output through the wild-type Yvc1 channels.

Overresponders tend to have cell wall defects

Given the nature of high-throughput screening and probable growth variation among the deletants, true overresponding deletants will have been missed in our screens (see Discussion). Still, a clear pattern emerges from the consensus of the two screens: unusually large osmotic upshock response correlates with deletants, predicted to be compromised in their cell wall structure. Striking among the top responders is the preponderance of genes annotated by the Gene Ontology (GO) process (http://www.geneontology.org/) as related to "protein amino acid glycosylation," a key process in wall synthesis (GO:0006486) (**Fig. 4**, top middle panel). Nine of the 52 deletants in the deletome so annotated are among the top 26 strongest overresponders (Table 1), a random probability value of $P = 10^{-5}$. Also prominent among the top responders are those annotated as being calcofluor-sensitive, a symptom of wall weakening (Fig. 4, top right). Seven of the 66 deletants in the deletome annotated being as calcofluor-sensitive (14) were among the strongest responders (Table 1). Representative annotated phenotypes, not related directly to the cell wall, such as amino acid metabolism, cytoskeleton, or salt sensitivity, are not associated with upshock overresponse (Fig. 4).

Glycosylated proteins are major component of the cell wall, and calcofluor sensitivity is indicative of weakened wall structure. Twenty-two of the 26 top overresponders in Table 1 can be associated with cell wall metabolism. *GAS1*, *KRE1, SMI1, KRE6*, and *ROT2* (rank numbers 1, 10, 15, 16, and 23 among the 4810 deletants) (Table 1) are all annotated as being involved in the process of cell wall metabolism and biosynthesis (GO:0007047). The number-2 overresponder, YEL059WΔ, is probably not a true ORF. This sequence *in trans* does not restore wild-type responses in YEL059Δ (data not shown). Its 5′ end lies within 30 bp of the 5′ end of *SOM1*, encoding a mitochondrial peptidase, but *som1*Δ did not overrespond in the initial screen (Supplemental Table S1) or on recheck (data not shown). Its 3′ end lies <300 bp 5′ to *PCM1*, an essential *N*-acetylglucosamine-phosphate mutase involved in the synthesis of chitin (15). Thus, the most likely causative effect of YEL059W deletion is to downregulate the essential *PCM1*. *HUR1* (number 13) overlaps *PMR1*, and we found that plasmid-borne *HUR1* does not diminish the upshock overresponse in *hur1*Δ

TABLE 1. *Top responding deletants*

Gene/ORF	Function	Peak RLU[a]	Second rank[b]	First rank[b]	CS[c,d]	GLYCO[d,e]	CW[d,f]
GAS1	β-1,3-Glucanosyltransferase	15.80	1	197	+		+
YEL059W	Hypothetical protein (upstream of *PCM1,* a chitin synthase)	14.39	2	11			~
GDA1	Guanosine diphosphatase, involved in the transport of GDP-mannose into Golgi	7.98	50	25		+	
SUR4	Elongase, involved in fatty acid and sphingolipid biosynthesis	7.86	14	12			
BST1	GPI inositol deacylase of ER	7.61	207	9			~
ALG5	Glucosyltransferase, involved in *N*-linked glycosylation in ER	7.30	42	10		+	
ALG6	Glucosyltransferase, involved in transfer of core oligosaccharides to proteins	7.17	95	30		+	
LAS21	Integral plasma membrane protein involved in the synthesis GPI	7.11	11	85	+		
GUP1	Plasma membrane protein with a possible role in proton symport of glycerol	6.41	13	4	+		
KRE1	Cell wall glycoprotein involved in β-glucan assembly; serves as a K1 killer	6.39	22	182			+
YBL083C	Hypothetical protein, overlaps *ALG3*	6.20	32	68		~	
SNU66	Component of the U4/U6.U5 snRNP complex involved in pre-mRNA splicing	5.99	21	293			
HUR1	Protein required for hydroxyurea resistance; overlaps PMR1	5.86	3	3		~	
OST3	Subunit of oligosaccharyltransferase complex of ER lumen	5.70	44	3636		+	
SMI1	Protein involved in the regulation of cell wall synthesis	5.59	132	15	+		+
KRE6	Protein required for β-1,6 glucan biosynthesis	5.55	159	6			+
VAN1	Component of the mannan polymerase I	5.39	33	19	+	+	
OCH1	Mannosyltransferase of *cis*-Golgi apparatus	5.18	8	1		+	
ALG8	Glucosyl transferase, involved in *N*-linked glycosylation in ER	5.13	9	215		+	
MAK10	Noncatalytic subunit of N-terminal acetyltransferase of the NatC type	4.91	388	26			
PMR1	Ca^{2+}/Mn^{2+} ATPase required for Ca^{2+} and Mn^{2+} transport into Golgi	4.87	4	2		+	
TNA1	High-affinity nicotinic acid plasma membrane permease	4.74	7	63			
ROT2	Glucosidase II catalytic subunit required for normal cell wall synthesis	4.48	28	452			+
SHE4	Endocytosis, polarization of actin and asymmetric mRNA localization	4.47	24	158	+		
CSF1	Protein required for fermentation at low temperature	4.24	39	4112	+		
DIE2	Dolichyl-phosphoglucose-dependent glucosyltransferase of ER	4.04	6	874		+	

[a]Peak RLU is presented as the fraction of wild type assayed in parallel. [b]Second and first ranks are the rank of the peak response of the deletant in the second and first deletome screen, respectively. [c]CS, stains annotated as being calcofluor sensitive. [d]Direct annotations are marked +, indirect associations are marked ~. Direct annotations and functions are from the *Saccharomyces* Genome Database. [e]GLYCO, stains annotated as being involved in protein glycosylation. [f]CW, stains involved in cell wall metabolism and synthesis. ER, endoplasmic reticulum.

(data not shown), suggesting that the overresponse originates from the *pmr1* defect. The deletant of *PMR1* proper is an overresponder (number 21). *pmr1*Δ lacks a Golgi Ca^{2+}/Mn^{2+} ATPase (16), so whether its upshock overresponse is related to its role in Ca^{2+} metabolism or to problems in protein glycosylation due to Mn^{2+} transport is unclear, although the latter effect follows the trend connecting overresponse to wall defect. YBL083C (number 11) overlaps *ALG3*, encoding a manosyltransferase, which is responsible for core *N*-glycosylation along with *ALG 5* and *ALG 6*. (*alg3*Δ itself did not grow well in either deletome screen.) *BST1* (number 5) encodes a glycosylphosphatidylinositol (GPI) -anchor deacylase, responsible for the sequestration of misfolded GPI proteins (17), many of which, including the top responder *gas1*Δ, are metabolic or structural components of the cell wall (18). *LAS21* and *PER1* (numbers 8 and 31) are both involved in GPI metabolism (19, 20). Besides the deletants named above, 11 of the strong overresponders are annotated to be involved in glycosylation, sphingolipid synthesis, or other endoplasmic reticulum or Golgi functions, disruptions of which are expected to compromise the cell wall. In sum, 22 of the 26 top overresponders to osmotic upshock are compromised in the syntheses of cell wall components (Table 1). *A priori*, one could imagine mutations affecting cytoskeletons, lipids (2), vacuolar biogenesis, and so on to perturb the upshock response. No such groupings correlate with upshock overresponse.

Calcineurin but not Pkc1 pathway deletants overrespond

Given the striking prevalence among the overresponders of deletants associated with cell wall metabo-

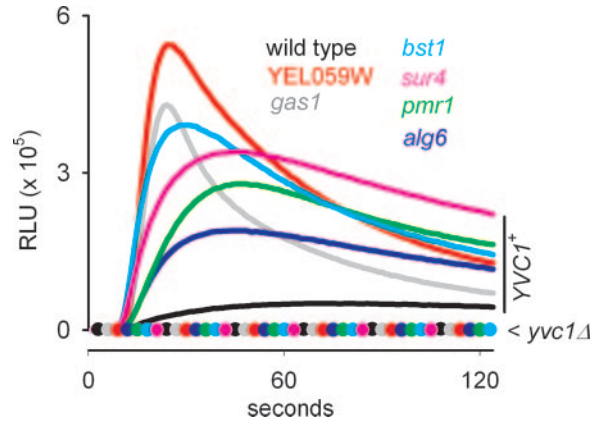

Figure 3. The overresponse of selected deletants requires the Yvc1 channel. Luminometric responses of selected overresponding deletants and (with wild-type *YVC1*$^+$, colored lines) their corresponding double mutants (with *yvc1*Δ alleles, symbols) are shown.

lism, it was curious that no deletants in the *PKC1* "cell wall-integrity" pathway similarly overresponded. As stated above, the stringent growth-phase dependence of the hypertonic Ca^{2+} release resulted in variability, and it was possible that the overresponse in these deletants was simply missed. Viable nonredundant deletants of the *PKC1* pathway, including deletants of the putative osmosensors *SLG1* and *MID2*, the kinases *BCK1* and *SLT2*, and the transcriptional regulator *RIM1*, were therefore tested at regular intervals as they crossed the diauxic shift. Indeed it was the case that none of them significantly overresponded (data not shown). The lack of the role of the *Pkc1* pathway in the hypertonic response is considered in the Discussion below.

The deletant of the calcineurin regulatory subunit *cnb1*Δ ranked 87th strongest responder among the 4810 deletants in the first screen. As with the Pkc1 pathway deletants, we carefully retested *cnb1*Δ and found it to have a peak response 2.9× that of wild type (data included in Fig. 7 below). This increased response is unlikely to be due to transcriptional regulation, as *crz1*Δ, the deletant of the calcineurin-responsive transcriptional factor (21), did not overrespond when similarly tested (data not shown).

Deletants with decreased responses

For the identification of deletants with reduced responses to osmotic upshock, we scrutinized the data from the EGTA-containing shock series. An inherent difficulty here is that we must winnow out the underresponders owing to poor growth from the true underresponders. Nearly 200 deletant strains having re-

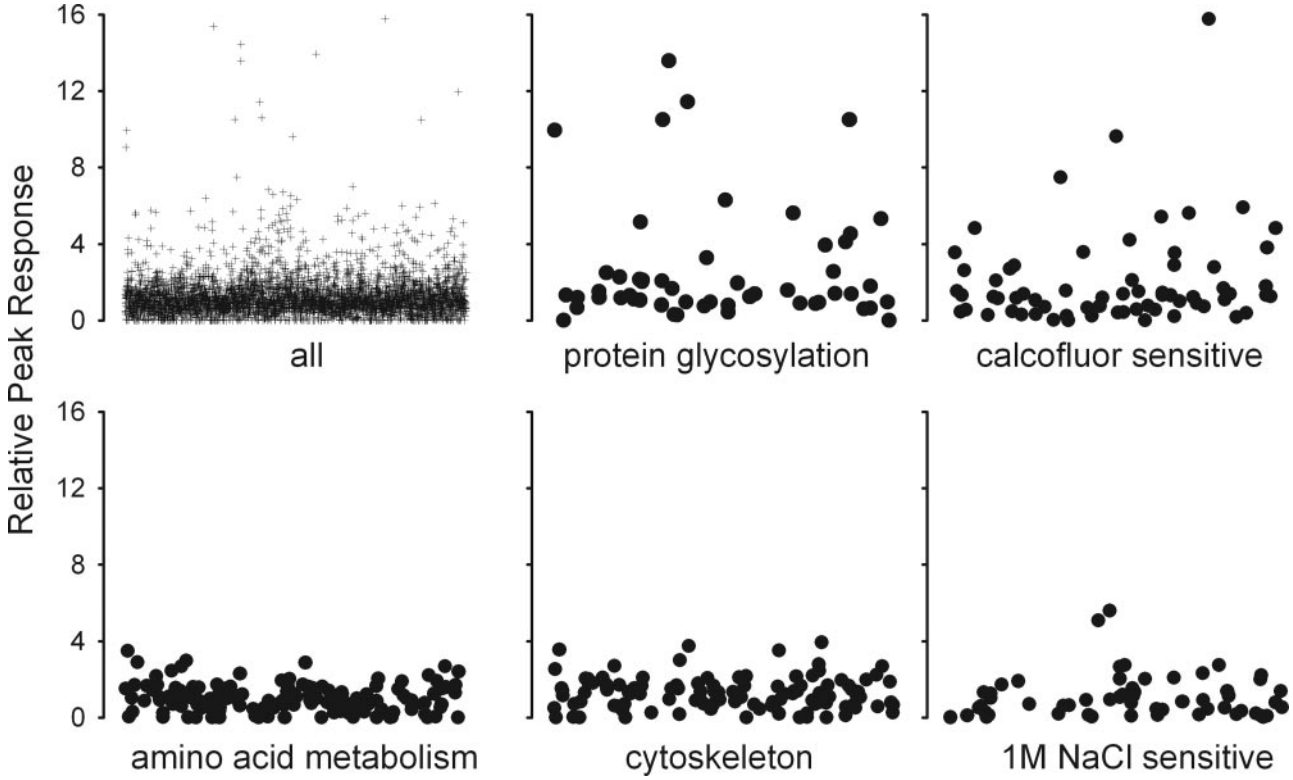

Figure 4. Responses of deletants annotated with specific processes or phenotypes. The relative peak responses from the entire second deletome screen are shown at top left. The top row shows those annotated with the GO process of protein amino acid glycosylation or the phenotype of calcofluor sensitivity (14). The association of these two phenotypes with an unusually strong upshock response is evident. The bottom row shows the peak responses of the subset of deletants within control annotation groups of three phenotypes for comparison. There is no association between these phenotypes with upshock overresponse. All annotations were retrieved from the *Saccharomyces* Genome Database (http://www.yeastgenome.org/).

sponses less than 5% of the median of the 96-well plate (Supplemental Table S1) were reexamined. Assessed in the initial screen, 63% of these were cultures with obvious growth defects and were not tested further. The rest were retested individually, and those having *bona fide* low signals were further examined for the presence of active aequorin by digitonin permeabilization (see Materials and Methods). Excluding those with little aequorin, surprisingly few deletants had sharply reduced signals.

Interestingly, the deletants with greatly reduced signals are all associated directly with Ca^{2+} transport. They include both deletions of *YVC1* in the deletome (YOR087W and YOR088W, as annotated in the original genome sequence.). They also include the gene deletion of the Ca^{2+} channel homolog *CCH1* (22), the *CCH1*-associated *MID1* (23) as well as the vacuolar Ca^{2+} ATPase *PMC1* (13). It has been reported that deletion of *PMC1* does not alter the hypertonic response (4). In that report, deletion of the other vacuolar Ca^{2+} transporter gene, *VCX1*, did abolish the response in conjunction with *PMC1* deletion and enhanced the response when deleted alone, presumably because Vcx1 is responsible for rapid sequestration of Ca^{2+} (24). In our strains *VCX1* deletion did not enhance the response (data not shown), indicating that it is less active here and hence *pmc1* deletion here would mimic the double deletion in the previous study.

To guard against the possibility that we might have missed the peak response windows of *yvc1*Δ, *cch1*Δ, *mid1*Δ, or *pmc1*Δ, we monitored their upshock response for several hours in postlogarithmic cultures (**Fig. 5**). Peak responses, although sharply reduced, could indeed be seen in all these deletants excepting *yvc1*Δ, which lacked the response completely (Fig. 5, inset).

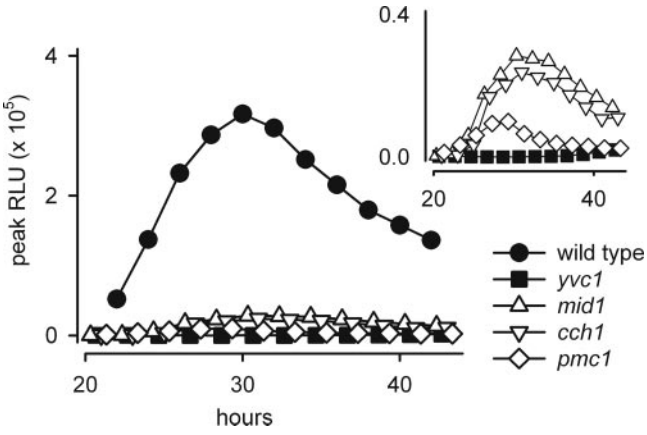

Figure 5. Peak luminometric signals of underresponding deletants cultured through the diauxic lag. Peak luminometric signals to the osmotic upshock were assessed every 2 h. *mid1*Δ, *cch1*Δ, and *pmc*Δ give little signal compared with the wild type. Inset: as cells enter postlogarithmic growth, small signals are evident in these deletants; *yvc1*Δ is the only deletant among the four that completely lacks a signal.

Yvc1 activity not altered by these deletions

One of the goals of this survey was to uncover gene products that might alter the activity of the Yvc1 channel. Such gene products could be regulatory subunits or enzymes that covalently or noncovalently modify the channel protein or its environment. Even when a gene product is annotated for one function, it may still play other roles in directly or indirectly affecting Yvc1 activities. These activities can be scrutinized by examining the behavior of individual channel molecules. Although technically demanding, it is possible to generate yeast spheroplasts, expose the vacuoles within, form gigaseals on selected vacuolar membrane, excise membrane patches, and examine the molecular activities of the Yvc1 channel directly (5, 6). Some of the overresponders and the key underresponders from our screen were examined in this manner. **Figure 6** shows results from such examinations of three overresponding *gas1*Δ, YEL059WΔ, and *pmr1*Δ, (numbers 1, 2, and 21 in Table 1) as well the underresponding *cch1*Δ, *mid1*Δ, and *pmc1*Δ.

To the extent of sampling by excised patches, channel density in the vacuolar membrane as evidenced by the number of unitary conductances was not significantly altered in these deletants. Like other TRP superfamily members, the Yvc1 channel is equipped with a filter that passes cations (7). This filter and other permeability features in the conducting path determine the unitary conductance. None of the deletions tested affected this parameter (320 pS in symmetric 180 mM KCl) (6). Subtler changes by channel protein modifications could result in changes in open probabilities or in channel kinetics (the transition rates between different open or closed conformations). Neither the open probability nor the channel kinetics appeared significantly altered in these deletants (Fig. 6). Yvc1 is an inward rectifier: cytoplasmic negativity increases its open probability. This property is not affected in these deletants. Cytoplasmic Ca^{2+} activates Yvc1 by changing its kinetics and increasing the open probability, forming the physiologically important Ca^{2+}-dependent Ca^{2+} release (CICR) feedback loop. The response to added Ca^{2+} was also unaltered in these deletants (data not shown). In short, deletions of *GAS1, YEL059W, PMR1, CCH1, MID1,* or *PMC1* do not seem to have influence in Yvc1 channel molecular behavior. Thus, the alterations causing the increase or decrease in the upshock-induced Ca^{2+} release from the vacuole in these deletants apparently lie upstream of the Yvc1 channel and not in the channel activity itself.

Cellular calcium content correlates with upshock response

An obvious potential change, which could lead to changes in the upshock response, is simply a change in vacuolar Ca^{2+} content. Total cellular calcium as assessed with ^{45}Ca is largely a measure of vacuolar calcium (25). We discovered that the upshock overre-

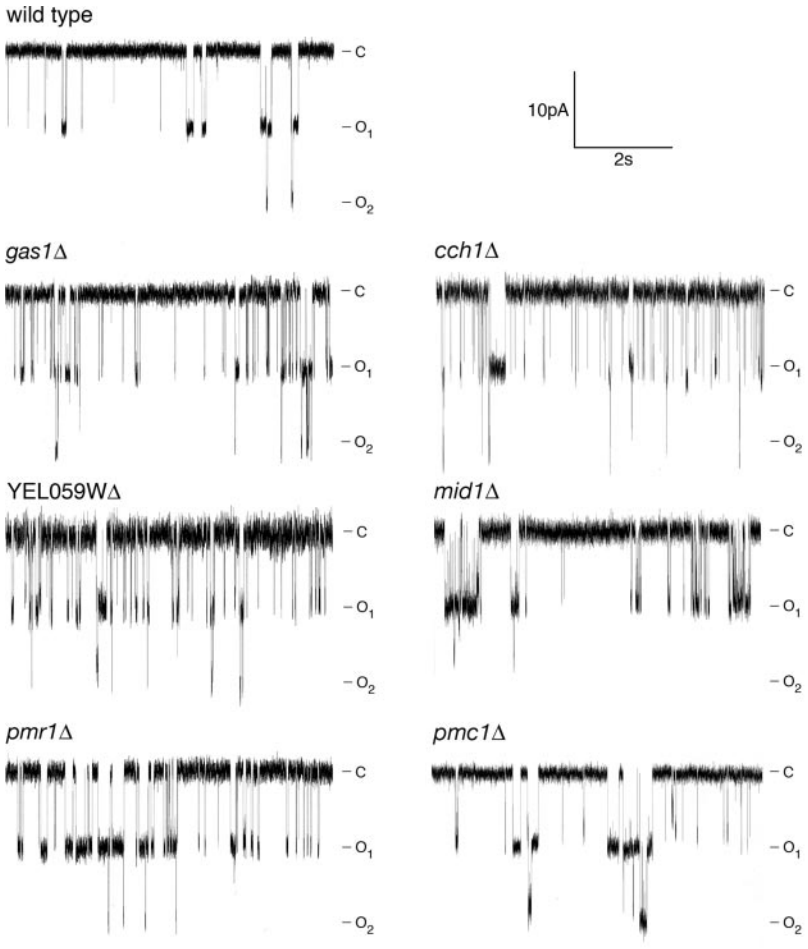

Figure 6. Yvc1 channel activity is not altered in over- or underresponding deletants. Sample traces of Yvc1 activity recorded from plasma-side-out patches from vacuoles isolated from the stated strains. Activities of two conducting units are apparent in each excised patch. Currents flowing into the cytoplasmic side are shown downward by convention. The current levels of all closed (C), one open (O_1), and two open (O_2) channels are marked. No significant difference in the unitary conductance or channel kinetics can be discerned between the wild type (top left) and the selected overresponders (remainder left) *gas1*Δ, YEL059WΔ, and *pmr1*Δ or the underresponders (right) *cch1*Δ, *mid1*Δ, and *pmc1*Δ. Pipette solutions were 180 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 5 mM HEPES, 1 mM 1,2-bis(2-aminophenoxy)-ethane-*N,N,N′,N′*-tetraacetic acid) and calculated free Ca^{2+} was 10 μM. Bath solution was the same plus 100 mM sorbitol. Test voltages were −30 mV (cytoplasmic side-negative).

sponders clearly have more calcium than the wild type. *gas1*Δ and *yel059w*Δ, the two strongest overresponders, have 2–3× the amount of the wild type. Other representative overresponders tested, *sur4*Δ, *bst5*Δ, *alg6*Δ, and *pmr1*Δ (rank numbers 4, 5, 7, and 21), as well as *cnb1*Δ also have significantly higher calcium content in repeated measurements (**Fig. 7**, left). One possible reason why *cnb1*Δ cells have higher Ca^{2+} content is that calcineurin is known to negative-regulate one of the two vacuolar Ca^{2+} pumps, Vcx1, and that this regulation is thought to be post-translational (26), consistent with the lack of increased response in the *crz1*Δ deletant. We also found the upshock underresponders to have clearly less calcium than the wild type. *cch1*Δ and *mid1*Δ have half and *pmc1*Δ has about one-quarter of the wild-type calcium content. The one exception to this trend again is *yvc1*Δ, wherein the cells retain a wild-type complement of calcium yet have no luminometric response. This result shows that the deletion of the Ca^{2+} releasing channel has no influence on the uptake or maintenance of vacuolar Ca^{2+}.

We are struck by the systematic variations of Ca^{2+} content in the deletants. Because the upshock response of the wild type varies with growth stage (Fig. 1), we wondered whether cell Ca^{2+} content varies accordingly. We examined the nonresponding wild-type cells in logarithmic growth and found that they in fact have higher calcium content per cell than the responding postlogarithmic cells (data not shown). Thus, the lack of response in the log-phase cells is not because of a

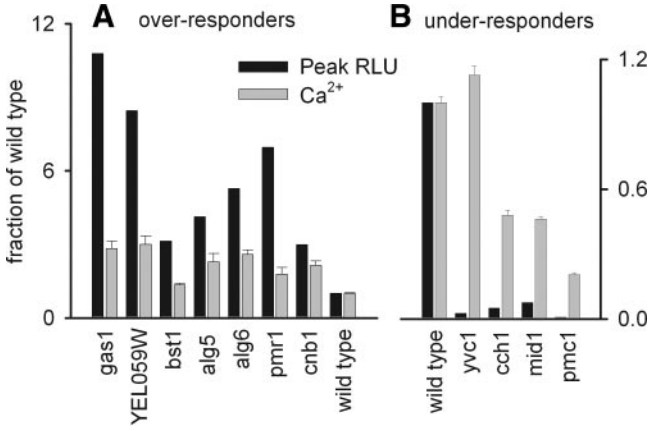

Figure 7. Ca^{2+} Content of the over- and underresponding deletants. Shown are the cellular calcium content, measured with ^{45}Ca, in the overresponding (*A*) and underresponding (*B*) deletants. Calcium contents are presented as average ± SD (n=4). Peak luminometric responses were measured from parallel nonradioactive cultures. Peak responses correlate with calcium content, with the notable exception of *yvc1*Δ.

lack of vacuolar Ca^{2+}. On the other hand, cell-calcium level clearly dynamically responds to stresses induced by various gene deletions (see Discussion).

DISCUSSION

The purpose of the two rounds of deletome screens and the extensive follow-up experiments was to find genetic elements that might regulate the source (vacuolar Ca^{2+} content) and the pathway (Yvc1 channel) of the osmotic upshock response. The rank orders of the responses from the 4810 deletant strains in the two screens are listed in Supplemental Table S1. The follow-up ^{45}Ca accumulation measurement and patch-clamp analyses are tests appropriate only for limited individual strains. Patch-clamping, especially, is a delicate and laborious procedure for detailed analyses of individual molecular behavior. It is not practical as a screening device for thousands of strains, not even for a second-level rescreen for several tens of strains. Because of these limitations, only the very strong overresponders (top 1% in either of the two screens, giving more than 4× the wild-type response) and very clear underresponders (with less than 5% of the median response) are more thoroughly analyzed here.

We did not uncover any direct regulators of Yvc1 activity. Representative overresponders and all of the severe underresponders from the screens have normal Yvc1 channel activities (Fig. 6). If the Yvc1 channel requires additional subunits or modifying enzymes, then these gene products will have to be essential or redundant or their deletions led to slow growth. A major caveat is that we could only scrutinize the severe underresponders. Those responding better than 5% of the median were not reexamined. Among those responding less than 5%, only a few robustly growing deletants came through our stringent rescreens as underresponders to upshock. These included *yvc1*Δ as well as *cch1*Δ, *mid1*Δ, and *pmc1*Δ (Fig. 5). All except *yvc1*Δ had reduced calcium content (Fig. 7) and normal Yvc1 channel activities (Fig. 6). It has previously been reported that *MID1* or *CCH1* deletion lowers the upshock response (27), but signal reductions we observed were much more pronounced. The most likely explanation is that much of the Ca^{2+} pulse in the previous work was from an external source, as evidenced by the susceptibility of the signal to EGTA there. *PMC1* encodes the vacuolar Ca^{2+} ATPase, the pump that accumulates Ca^{2+} in the vacuole ATPase. That *pmc1*Δ has a low vacuolar Ca^{2+} content (Fig. 7) is consistent with its low upshock response (Fig. 5).

In our search for underresponders, a lack of response due to growth-phase variation would result in a false-positive result, winnowed in the individual retests. In the search for overresponders, however, a lower response due to growth-phase variation would result in a false-negative result, which, together with the thousands of negatives, would not be individually retested. We therefore expect more overresponders than those listed in Table 1 and Supplemental Table S1. As with the underresponders, overresponders correlate well with their calcium content (Fig. 7, left). The simplest interpretation is that increased vacuolar Ca^{2+} is what causes the increased luminometric response.

The preponderance of deletants associated with cell wall metabolism is striking. Twenty-two of the top 26 responders (Table 1) that had luminometric signals greater than 4× that of the wild type in the retest can be directly or indirectly associated with cell wall metabolism. One may argue that the preponderance of wall-related mutants is simply a pathological curiosity: a compromised cell wall somehow leads to Ca^{2+} leakage into the cytosol, which is subsequently sequestered in the vacuole. First of all, this view glosses over the fact that the barrier for Ca^{2+} entry is not the cell wall but the cell membrane. Second, molecular changes in the wall after a mutation do not necessarily result in physical weakening of the wall, especially because of the compensatory metabolic adjustments to the mutation. Nonetheless, it is possible that wall defects can lead to subtler changes in the permeability of the Cch1/Mid1 channel (28) or the mechanosensitive conductance on the plasma membrane (3). Interestingly, TRP channel activity has been associated with cell wall regulation in *Schizosaccharomyces pombe* as well, wherein the polycystic kidney disease-related ion channel homolog (*pkd2*) expression level correlates with cell wall damage and the channel itself interacts with a Rho-GTPase involved in cell wall synthesis and shape determination (29).

It appears that cell Ca^{2+} is dynamic, and yeast has evolved to store Ca^{2+} for defense and repair when its wall is under stress. This view is in agreement with current knowledge on cell wall regulation. Lagorce *et al.* (11) examined the effects on the yeast transcriptome by five mutations, (*gas1, knr4, kre6, fks1,* and *mnn9*) chosen for their effects on the cell wall. They found that 40% of the genes in the yeast genome that are transcriptionally up-regulated in response to these mutations contain the calcineurin-dependent response element (CDRE) motif and concluded that Ca^{2+}-calcineruin-dependent pathways are a crucial part of the cell wall compensatory mechanism. Interestingly, four of the five mutants chosen for the transcriptome analysis by Lagorce *et al.* (11) scored very high in our upshock response: *gas1*Δ ranks as number 1, *knr4*Δ (=*smi1*Δ) ranks as number *15,* and *kre6*Δ ranks as number 16 among the 4810 deletants (Table 1). *fks1*Δ also ranked in the upper 4th percentile in each of our two screens (Supplemental Table S1). In *fks1*Δ, the cell is expected to rely on the alternative 1,3-β-glucan synthase encoded by *FKS2* (*GSC2*), which is known to be positively regulated by Ca^{2+} through calcineurin. An increase in calcium content in *fks1*Δ will therefore adaptively dovetail the need for this positive regulation.

It may seem curious that mutants in the Pkc1 cell wall integrity pathway do not overrespond as they may be predicted to have similarly compromised cell walls. One possibility is that while the Pkc1 pathway functions during episodic events such as bud emergence (30) or

in response to a sudden hypotonic challenge (31), the wall defects in the hyperresponders resulting from loss of protein manosylation (*e.g.*, *gda1*Δ and *alg5*Δ), glucan synthesis (*e.g.*, *gas1*Δ and *kre6*Δ), or chitin synthesis (*e.g.*, YEL059Δ) would be anticipated to have more chronically unstable walls. There are at least two other key differences. First, disruption of the Pkc1 pathway leads to sensitivity to hypotonic, not hypertonic, stress examined here and the latter, in fact, inhibits the Pkc1 pathway (31). Second, Pkc1 functions in budding, but the hypertonic response is most prominent when cells are in fact not budding during the diauxic shift (Fig. 1). It should be noted that deletion of one gene associated with the Pkc1 pathway (32), *SMI1* (*KNR4*) (15th strongest responder) (Table 1), does indeed overrespond. This probably results from the fact that *smi1*Δ deletants have decreased glucan levels (33).

In a search for mutations synthetically lethal with calcineurin deletion, Garrett-Engele *et al.* (34) isolated mutations in *FKS1* (a moderately strong responder here, see above) and in members of the Pkc1 pathway. This led to the conclusion that calcineurin functions in an independent parallel pathway to Pkc1 to regulate cell wall synthesis (34) and, in fact, *FKS1* has been shown to be dually regulated by both the Pkc1 pathway and calcineurin (35). It seems possible that hypertonic Ca^{2+} release through Yvc1 functions in this or another parallel pathway involved in maintaining cellular integrity. That cnb1 deletion here resulted in an increased response, and vacuolar Ca^{2+} (Fig. 7) could reflect chronically weakened walls, because calcineurin-responsive transcriptional elements (21) are found above many genes involved with cell wall construction (11). Alternatively, the overresponse of *cnb1*Δ could reflect an increase in Vcx1 activity pumping Ca^{2+} into the vacuole, which is negatively regulated posttranslationally by calcineurin (26). The fact that deletion of the gene of the calcineurin-dependent transcriptional regulator, *CRZ1* (21), did not increase the response favors the latter interpretation.

As measured with ^{45}Ca, *gas1*Δ and *kre6*Δ as well as several wall mutants clearly have higher calcium contents (Fig. 7, left). By extension, one would also expect that *knr4*Δ, *fks1*Δ, and many of the upshock overresponders listed in Table 1 and Supplemental Table S1 have raised calcium content. To keep it manageable, Lagorce *et al.* (11) limited their transcriptome analysis to five mutations, and the five were chosen because they affect cell wall composition differently. Presumably, many other mutations affecting the cell wall also upregulate the calcineurin-dependent pathways. The concomitant increase in calcium content and the transcription of CDRE-regulated genes in many different cell wall mutants strongly suggest that these are coordinated as adaptive responses to general cell wall stress.

It is striking that neither the study of Lagorce *et al.* (11) nor the present study is hypothesis-driven. Both are molecular surveys on how yeast cells respond to mutation-induced stress. Yet, devoid of preconceived notions, both uncovered aspects of calcium-related mechanism: accumulation of Ca^{2+} and up-regulation of Ca^{2+}-calcineruin-controlled genes. Together, it seems plausible that yeast has evolved a defensive mechanism that stockpiles Ca^{2+} when it suffers a cell wall stress. This Ca^{2+} is then ready to be released as a second messenger to up-regulate CDRE-containing genes as well as other defense systems against additional stress, such as an osmotic upshock, to which wall-compromised cells may be especially vulnerable (**Fig. 8**). In the wild, yeast cells face a barrage of enzymatic attacks against their cell walls (36), leading to stresses simulated with mutations here and by Lagorce *et al.* (11). How the cell wall stress leads to the entry of Ca^{2+} remains to be investigated in the future. Normally, the Cch1/Mid1 channel accounts for this entry, as evidenced by the lack of calcium in *cch1*Δ and *mid1*Δ (Fig. 7, right). Whether Ca^{2+} enters through this route when the wall is stressed can be tested with double mutants that combine the overresponding with the underresponding mutations uncovered in this study, such as *gas1*Δ *cch1*Δ, YEL059WΔ *cch1*Δ, and others. FJ

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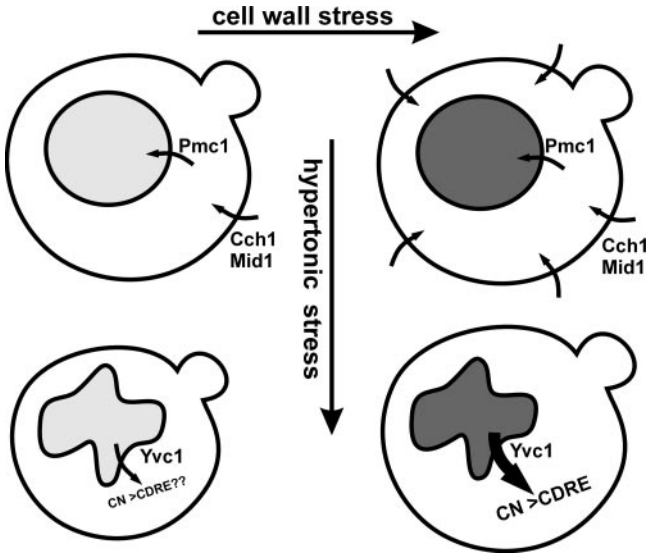

Figure 8. A model in which vacuolar Ca^{2+} increase functions as an adaptive safeguard against hypertonic stress in wall-compromised yeast cells. This model is based on our results together with the finding that many genes up-regulated in cell wall mutants contain CDREs (11). Normally, Ca^{2+} enters through the Cch1/Mid1, which is then sequestered into the vacuole by Pmc1 (top left). It appears that stress on the cell wall (from attacking enzymes common in the wild or by gene deletion in the laboratory) induces additional Ca^{2+} entry either through Cch1/Mid1 or another route (top right). This stockpiling of Ca^{2+} appears adaptive, probably an evolved mechanism, as it better readies the cells for existing stress and additional insults. Compounded osmotic stress, for example, will then elicit a stronger release of Ca^{2+}, a second messenger for downstream defense or repair mechanism, including the up-regulation of calcineurin (CN) -dependent genes (bottom row).

REFERENCES

1. Batiza, A. F., Schulz, T., and Masson, P. H. (1996) Yeast respond to hypotonic shock with a calcium pulse. *J. Biol. Chem.* **271,** 23357–23362
2. Loukin, S. H., Kung, C., and Saimi, Y. (2007) Lipid perturbations sensitize osmotic down-shock activated Ca^{2+} influx, a yeast "deletome" analysis. *FASEB J.* **21,** 1813–1820
3. Gustin, M. C., Zhou, X. L., Martinac, B., and Kung, C. (1988) A mechanosensitive ion channel in the yeast plasma membrane. *Science* **242,** 762–765
4. Denis, V., and Cyert, M. S. (2002) Internal Ca^{2+} release in yeast is triggered by hypertonic shock and mediated by a TRP channel homologue. *J. Cell Biol.* **156,** 29–34
5. Palmer, C. P., Zhou, X.-L., Lin, J., Loukin, S. H., Kung, C., and Saimi, Y. (2001) A TRP homolog in *Saccharomyces cerevisiae* forms an intracellular Ca^{2+}-permeable channel in the yeast vacuolar membrane. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 7801–7805
6. Zhou, X. L., Batiza, A. F., Loukin, S. H., Palmer, C. P., Kung, C., and Saimi, Y. (2003) The transient receptor potential channel on the yeast vacuole is mechanosensitive. *Proc. Natl. Acad. Sci. U. S. A.* **100,** 7105–7110
7. Clapham, D. E. (2003) TRP channels as cellular sensors. *Nature* **426,** 517–524
8. Nass, R., and Rao, R. (1999) The yeast endosomal Na^+/H^+ exchanger, Nhx1, confers osmotolerance following acute hypertonic shock. *Microbiology* **145**(Pt. 11), 3221–3228
9. Gasch, A. P., Spellman, P. T., Kao, C. M., Carmel-Harel, O., Eisen, M. B., Storz, G., Botstein, D., and Brown, P. O. (2000) Genomic expression programs in the response of yeast cells to environmental changes. *Mol. Biol. Cell* **11,** 4241–4257
10. Brewster, J. L., de Valoir, T., Dwyer, N. D., Winter, E., and Gustin, M. C. (1993) An osmosensing signal transduction pathway in yeast. *Science* **259,** 1760–1763
11. Lagorce, A., Hauser, N. C., Labourdette, D., Rodriguez, C., Martin-Yken, H., Arroyo, J., Hoheisel, J. D., and Francois, J. (2003) Genome-wide analysis of the response to cell wall mutations in the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem.* **278,** 20345–20357
12. Baudin, A., Ozier-Kalogeropoulos, O., Denouel, A., Lacroute, F., and Cullin, C. (1993) A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **21,** 3329–3330
13. Cunningham, K. W., and Fink, G. R. (1994) Calcineurin-dependent growth control in *Saccharomyces cerevisiae* mutants lacking PMC1, a homolog of plasma membrane Ca^{2+} ATPases. *J. Cell Biol.* **124,** 351–363
14. Lesage, G., Shapiro, J., Specht, C. A., Sdicu, A. M., Menard, P., Hussein, S., Tong, A. H., Boone, C., and Bussey, H. (2005) An interactional network of genes involved in chitin synthesis in *Saccharomyces cerevisiae*. *BMC Genet.* **6,** 8
15. Hofmann, M., Boles, E., and Zimmermann, F. K. (1994) Characterization of the essential yeast gene encoding N-acetylglucosamine-phosphate mutase. *Eur. J. Biochem.* **221,** 741–747
16. Durr, G., Strayle, J., Plemper, R., Elbs, S., Klee, S. K., Catty, P., Wolf, D. H., and Rudolph, H. K. (1998) The medial-Golgi ion pump Pmr1 supplies the yeast secretory pathway with Ca^{2+} and Mn^{2+} required for glycosylation, sorting, and endoplasmic reticulum-associated protein degradation. *Mol. Biol. Cell* **9,** 1149–1162
17. Fujita, M., Yoko, O. T., and Jigami, Y. (2006) Inositol deacylation by Bst1p is required for the quality control of glycosylphosphatidylinositol-anchored proteins. *Mol. Biol. Cell* **17,** 834–850
18. Hamada, K., Fukuchi, S., Arisawa, M., Baba, M., and Kitada, K. (1998) Screening for glycosylphosphatidylinositol (GPI)-dependent cell wall proteins in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **258,** 53–59
19. Benachour, A., Sipos, G., Flury, I., Reggiori, F., Canivenc-Gansel, E., Vionnet, C., Conzelmann, A., and Benghezal, M. (1999) Deletion of GPI7, a yeast gene required for addition of a side chain to the glycosylphosphatidylinositol (GPI) core structure, affects GPI protein transport, remodeling, and cell wall integrity. *J. Biol. Chem.* **274,** 15251–15261
20. Fujita, M., Umemura, M., Yoko-o, T., and Jigami, Y. (2006) PER1 is required for GPI-phospholipase A_2 activity and involved in lipid remodeling of GPI-anchored proteins. *Mol. Biol. Cell* **17,** 5253–5264
21. Stathopoulos, A. M., and Cyert, M. S. (1997) Calcineurin acts through the CRZ1/TCN1-encoded transcription factor to regulate gene expression in yeast. *Genes Dev.* **11,** 3432–3444
22. Fischer, M., Schnell, N., Chattaway, J., Davies, P., Dixon, G., and Sanders, D. (1997) The *Saccharomyces cerevisiae* CCH1 gene is involved in calcium influx and mating. *FEBS Lett.* **419,** 259–262
23. Locke, E. G., Bonilla, M., Liang, L., Takita, Y., and Cunningham, K. W. (2000) A homolog of voltage-gated Ca^{2+} channels stimulated by depletion of secretory Ca^{2+} in yeast. *Mol. Cell. Biol.* **20,** 6686–6694
24. Miseta, A., Kellermayer, R., Aiello, D. P., Fu, L., and Bedwell, D. M. (1999) The vacuolar Ca^{2+}/H^+ exchanger Vcx1p/Hum1p tightly controls cytosolic Ca^{2+} levels in *S. cerevisiae*. *FEBS Lett.* **451,** 132–136
25. Eilam, Y., Lavi, H., and Grossowicz, N. (1985) Cytoplasmic Ca^{2+} homeostasis maintained by a vacuolar Ca^{2+} transport system in the yeast *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **131,** 623–629
26. Cunningham, K. W., and Fink, G. R. (1996) Calcineurin inhibits VCX1-dependent H^+/Ca^{2+} exchange and induces Ca^{2+} ATPases in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **16,** 2226–2237
27. Matsumoto, T. K., Ellsmore, A. J., Cessna, S. G., Low, P. S., Pardo, J. M., Bressan, R. A., and Hasegawa, P. M. (2002) An osmotically induced cytosolic Ca^{2+} transient activates calcineurin signaling to mediate ion homeostasis and salt tolerance of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **277,** 33075–33080
28. Kanzaki, M., Nagasawa, M., Kojima, I., Sato, C., Naruse, K., Sokabe, M., and Iida, H. (1999) Molecular identification of a eukaryotic, stretch-activated nonselective cation channel. *Science* **285,** 882–886
29. Palmer, C. P., Aydar, E., and Djamgoz, M. B. (2005) A microbial TRP-like polycystic-kidney-disease-related ion channel gene. *Biochem. J.* **387,** 211–219
30. Levin, D. E., and Bartlett-Heubusch, E. (1992) Mutants in the *S. cerevisiae* PKC1 gene display a cell cycle-specific osmotic stability defect. *J. Cell Biol.* **116,** 1221–1229
31. Davenport, K. R., Sohaskey, M., Kamada, Y., Levin, D. E., and Gustin, M. C. (1995) A second osmosensing signal transduction pathway in yeast: hypotonic shock activates the PKC1 protein kinase-regulated cell integrity pathway. *J. Biol. Chem.* **270,** 30157–30161
32. Martin-Yken, H., Dagkessamanskaia, A., Basmaji, F., Lagorce, A., and Francois, J. (2003) The interaction of Slt2 MAP kinase with Knr4 is necessary for signalling through the cell wall integrity pathway in *Saccharomyces cerevisiae*. *Mol. Microbiol.* **49,** 23–35
33. Hong, Z., Mann, P., Brown, N. H., Tran, L. E., Shaw, K. J., Hare, R. S., and DiDomenico, B. (1994) Cloning and characterization of KNR4, a yeast gene involved in (1,3)-β-glucan synthesis. *Mol. Cell. Biol.* **14,** 1017–1025
34. Garrett-Engele, P., Moilanen, B., and Cyert, M. S. (1995) Calcineurin, the Ca^{2+}/calmodulin-dependent protein phosphatase, is essential in yeast mutants with cell integrity defects and in mutants that lack a functional vacuolar H^+-ATPase. *Mol. Cell. Biol.* **15,** 4103–4114
35. Zhao, C., Jung, U. S., Garrett-Engele, P., Roe, T., Cyert, M. S., and Levin, D. E. (1998) Temperature-induced expression of yeast FKS2 is under the dual control of protein kinase C and calcineurin. *Mol. Cell. Biol.* **18,** 1013–1022
36. Klis, F. M., Mol, P., Hellingwerf, K., and Brul, S. (2002) Dynamics of cell wall structure in *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.* **26,** 239–256

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