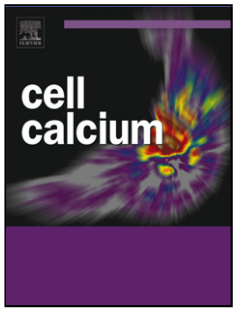

Title: HYPOTONIC STRESS-INDUCED CALCIUM SIGNALING IN *S. CEREVISIAE* INVOLVES TRP-LIKE TRANSPORTERS ON THE ENDOPLASMIC RETICULUM MEMBRANE

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HYPOTONIC STRESS-INDUCED CALCIUM SIGNALING IN *S. CEREVISIAE* INVOLVES TRP-LIKE TRANSPORTERS ON THE ENDOPLASMIC RETICULUM MEMBRANE

Running title: stress-responsive calcium signaling in yeast

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Summary

S. cerevisiae cells respond to hypotonic stress (HTS) by a cytosolic calcium rise, either generated by an influx of calcium from extracellular medium, when calcium is available, or by a release from intracellular stores in scarcity of extracellular calcium. Calcium release from intracellular compartments is peculiarly inhibited by external calcium in a calcineurin-independent and Cch1-, but not Mid1-, driven manner. HTS-induced calcium release is also negatively regulated by the ER protein Cls2 and involves a poorly characterized protein, *FLC2*/*YAL053W* gene product, previously proposed to be required for FAD transport in the ER, albeit, due to its molecular features, it was also previously classified as an ion transporter. A computational analysis revealed that this gene and its three homologs in *S. cerevisiae*, together with previously identified *S. pombe pkd2* and *N. crassa* calcium-related spray protein, belong to a fungal branch of TRP-like ion transporters related to human mucolipin and polycystin 2 calcium transporters. Moreover, disruption of *FLC2* gene confers severe sensitivity to Calcofluor white and hyper-activation of the cell wall integrity MAPK cascade, suggesting a role in cell wall maintenance as previously suggested for the fission yeast homolog. Perturbation in cytosolic resting calcium concentration and hyper-activation of calcineurin in exponentially growing cells suggest a role for this transporter in calcium homeostasis in yeast.

Abbreviations: $[Ca^{2+}]_i$, intracellular calcium concentration; HACS, high affinity calcium influx system; LACS, low affinity calcium influx system; SD, synthetic medium with glucose; YPD, yeast extract peptone glucose medium; HTS, hypotonic shock.

Keywords: budding yeast; mucolipin like calcium channels; polycystin 2 like calcium channels; PKD2 like calcium channels; osmotic shock; TRPML; TRPP

1. Introduction

Microorganisms and eukaryotic cells of multicellular organisms often experience mechanical stretch due to e.g. dehydration or injuries. Most cells respond to decrease in tonicity first by swelling and then by activating mechanisms that allow them to recover their original volume [1]. In several cell types, exposure to either hypotonic shock or mechanical stress produces a sustained increase in cytosolic calcium concentration [2-5], possibly originating from either or both influx from the extracellular solution and release of Ca^{2+} from intracellular stores. The relative importance of these mechanisms varies, likely according to the type of channels involved in Ca^{2+} transport in different cell types and species.

Stretch-activated channels (SACs) have been suggested as transducers in response to a variety of mechanical perturbations [6], such as hypotonic shock [7-9]. Mechanosensing channels are widely distributed from single-celled bacteria to animal and plant cells. They respond to a variety of mechanical stimuli, including shear stress, gravity, osmotic pressure, and stretch, by opening nanoscale protein pores that are selectively permeable to cations (mainly calcium and sodium) [10].

In yeast, cytosolic calcium concentration is typically maintained at low (sub-micromolar) levels by calcium homeostasis mechanisms, as happens in all eukaryotic cells. Stimulus-dependent opening of Ca^{2+}channels in plasma membrane and/or in internal compartments triggers a rapid increase in calcium concentration in the cytosol, which represents a versatile, universally utilized signal with specific spatial and temporal dynamics. In yeast, calcium signaling pathways are essential for survival during conjugation, ion stress resistance, cell cycle progression, osmotic shock, vacuoles fusion [11-14].

Hypotonic shock induces a transient rise in cytosolic Ca^{2+} levels in yeast [15, 16], which was reported to be mediated initially by a release of calcium from intracellular stores, and then sustained by extracellular calcium influx [15]. The patch-clamp technique allowes examination of the budding yeast *Saccharomyces cerevisiae* only after an enzymatic digestion of the cell wall. Analyses conducted with this technique revealed a 36-pS ion-nonspecific conductance of unknown molecular identity [8]. This conductance was mechanosensitive and occurred when membrane patches or whole-cell membranes were stretched. *MID1* was suggested to encode this channel [17], and evidence suggested that Mid1 might be a stretch-response activator of calcium influx, initiating calcium uptake in response to cell swelling [18]. However, no change in conductance was observed in *mid1*Δ protoplasts [19]. Mid1 appears to work in yeast mostly as a regulatory subunit of a high affinity, low capacity, calcium influx system (HACS), comprising Cch1, a homolog of the α1 catalytic subunits of L-type voltage-gated Ca^{2+} channels in mammals, and other regulatory subunits [20], primarily responsible for pheromone-induced and glucose-triggered calcium response in minimal media. In rich media, HACS is strongly inhibited by calcineurin, possibly through direct dephosphorylation, and other systems become active, namely LACS and GIC systems [21-23].

Very low gadolinium concentrations (10 μM) were sufficient to block the opening of SACs in patch-clamped yeast protoplasts [8]. Albeit only 10 mM gadolinium completely eliminates the hypotonic shock response of yeast intact cells, the sensitivity to gadolinium suggests that the calcium rise upon hypotonic shock could be mediated by SACs [15]. Nonetheless, a recent study reported that a different calcium influx is inhibited by this ion at micromolar concentration on intact cells and is activated by a stimulus distinct from membrane stretching, i.e. glucose refeeding [23].

In this work, the involvement of different known or putative calcium transporters in hypotonic shock-induced calcium transient is investigated, showing the existence of a peculiar calcium influx-inhibited calcium release from the budding yeast ER compartment, involving the activity of still poorly characterized proteins that we propose as members of a fungal specific TRPML (mucolipins) and TRPP (polycystin 2-related) calcium transporters family spanning calcium-related spray protein from *Neurospora crassa* and putative flavin transporters from other fungi.

2. Materials and Methods

2.1 Strains and cultural conditions

Strains used in this work are listed in Table S1.
Yeast strains were grown in YPD medium (2% glucose, 2% tryptone, 1% yeast extract and 2% agar for solid medium by Biolife, USA, supplemented with 50 mg/l adenine), with shaking, at 30°C, or in synthetic medium (SD), containing 2% glucose, 0.67% yeast nitrogen base w/o aminoacids (YNB, Difco, USA), supplemented with 50 mg/l adenine, 0.5 M sorbitol when indicated, 2.5% agar for solid media (Formedium, UK), and the appropriated drop out mixture of aminoacids and bases according to the manufacturer's instructions (CSM, BIO101). Calcofluor white was added after sterilization at a concentration of 20 µg/ml. Tunicamycin was added from a DMSO stock at the indicated final concentration.
Cell density was determined by measuring optical density at 600 nm (OD600) or by Coulter Counter (Coulter Electronics Z2).
Hypotonic shock was applied by diluting the cells suspension with 4 volumes of distilled water.

2.2 Plasmids and strains construction

CNB1, *CRZ1*, *ECM7*, *CLS2*, *YOR365C*, *FLC2*, *FLC1* and *FLC3* genes were deleted in wild-type or mutant strains indicated in Table S1 using a disruption cassette generated by PCR using pFA6a-His3MX4 plasmid, containing an expression cassette for the heterologous marker *his5*$^{+}$ from *Schizosaccharomyces pombe*, as a template [24]. The primers are listed in the Table S2. The deleted strains were selected on synthetic complete medium lacking histidine and the integration of the disruption cassette at the correct *locus* was verified by PCR.
Double, triple or quadruple deletions in His^{+} background were performed by using a disruption cassette generated by PCR using pFA6a-KanMX4 as a template, as described in Wach *et al*., 1997 [24], and the same primers listed in the Table S2. In this case the deleted strains were selected on YPD medium with 500 µg/ml G418 added, and the integration of the disruption cassette at the correct *locus* was verified by PCR.
RT1290 strain, expressing a Yor365c-eGFP fusion protein, was created by introducing the fusion encoding *ORF* in *YOR365C locus* by gene targeting in K601 strain as a PCR construct obtained with the following oligonucleotides: YOR365CeGFP-FOR and YOR365CeGFP-REV (see Table S2), driving PCR amplification on pYM28 plasmid (EUROSCARF).
YIplac204T/CSec7-7xDsRed [25] was kindly provided by B. Glick (University of Chicago, USA). The YIplac204T/CHmg1DsRED plasmid was previously described [26].
Yeast cells were transformed for luminescence assay by lithium acetate method with the multicopy pVTU-AEQ plasmid [27], while they were transformed, for β-galactosidase assays, either with multicopy pAMS366 plasmid (containing *4xCDRE::LacZ* reporter) [28], kindly provided by M. Cyert (Stanford University, CA), or with p1366 (carrying Mpk1 responsive *PRM5::LacZ* reporter) [29], kindly provided by D. Levin (John Hopkins University, MD), or with pMCZ-Y (carrying *UPRE::LacZ* reporter) [30], kindly provided by D. Eide (University of Winsconsin-Madison, WI).
In order to overexpress *FLC2* gene, a PCR was performed using FLC2GAP-REP For and FLC2GAP-REP Rev primers (see Table S2 in Supplementary Materials) as a template, to obtain a fragment of 125 bp. This fragment was digested with EcoRI and XbaI and inserted into the centromeric plasmid YCplac33, digested with the same enzymes, to obtain YCplac33-FLC2-GR. Yeast has been co-transformed with YCplac33-FLC2-GR digested with BamHI and a fragment of 3502 bp, obtained from a PCR performed on whole genome from the wild-type strain, using FLC2GenomeFor and FLC2GenomeRev primers (see Table S2 in Supplementary Materials); the result of the *in vivo* homologous recombination was recovered from yeast and called YCplac33-FLC2.

YCplac33-FLC2 and the multicopy plasmid YEplac112 were digested with PvuII and the fragment of 3849 bp, containing *FLC2* promoter, terminator and *ORF*, obtained from YCplac33-FLC2, was cloned in YEplac112 to obtain YEplac112-FLC2.

2.3 Bioluminescence assay

Cytosolic calcium concentration variation upon hypotonic shock (HTS) was evaluated as follows. Yeast strains were grown overnight at 30°C in YPD medium (6 OD/treatment). Cells were harvested while exponentially growing (5-6 x 10^6 cells/ml) by centrifugation at 4000 rpm for 10 min, and resuspended in the culture medium at a density of about 10^8 cells/ml. The cellular suspension was transferred to a microfuge tube and spinned at 7000 rpm for 2 min. 7.5 x 10^7 cells were resuspended in 20 µl of YPD medium for each treatment, 50 µM coelenterazine (stock solution 1 µg/µl dissolved in 99.5% methanol, conserved in the dark at -20°C, Molecular Probes) was added, and the suspension was incubated at room temperature in the dark for 20 min. Cells were collected by centrifuge at 7000 rpm for 2 min and washed three times with medium (200 µl/wash), then they were resuspended in 200 µl of medium.
The cellular suspension was transferred into luminometer tubes (200 µl each), in the presence of a solution of $CaCl_2$ and of 0.2 mM (final concentration) ethylene glycol tetraacetic acid (EGTA), giving Ca^{2+} final concentrations as indicated in the legends of the figures. Free calcium concentration in the medium was estimated by Maxchelator (http://www.stanford.edu/~cpatton/maxc.html), considering ion concentrations according to Loukin and Kung, 1995 [31]**.** Light emission was recorded with the luminometer at intervals of 5 s for at least 1 min before and for at least 3 min after the addition of 4 volumes of water, and converted into calcium concentrations according to Blinks *et al.* [32]. Water was added only when the signal was stable. At the end of each experiment aequorin expression and activity were tested by lysing cells with 0.5% Triton X-100 in the presence of 10 mM $CaCl_2$ (stock solution 100 mM $CaCl_2$), and then monitoring light emission for 24 min. This maximum intensity was used to normalize light emission according to the amount of aequorin expressed.
All experiments were performed at least in three biological replicates and a graph of the average emissions is presented with standard deviation as positive only error bars in order to avoid overlay of the lines.
Parameters estimation of the calcium transport was performed as previously described [23], with the only difference that light emission was recorded at intervals of 1 s.
For the experiments performed in the presence of 1 mM EGTA but without addition of $CaCl_2$ in the extracellular medium, variation in $[Ca^{2+}]_i$ in the 20 seconds immediately following hypotonic shock among different cellular strains was fitted by polynomial mixed effect models whereby the linear and the quadratic term of time were entered as covariates and cell strain as a fixed factor. The interactions between strain and both the linear and the quadratic terms of time were also included among predictors to account for differential patterns of $[Ca^{2+}]_i$ variation among cellular strains. The biologiacl replicate was entered as a random grouping effect to account for repeated measures of the same group of cells. A first order autoregressive form of the variance-covariance matrix of the polynomial mixed model, assuming a Gaussian error distribution, was also used to account for temporal autocorrelation of $[Ca^{2+}]_i$. We used a polynomial mixed model because it fitted the data significantly better than a linear model (Likelihood ratio test: $\chi^2_1=104.85$, $P < 0.001$). This means that $[Ca^{2+}]_i$ in each strain varied according to a second order polynomial equation:

$$[Ca^{2+}]_i = b_0 + b_1 \times Time + b_2 \times Time^2 \qquad \text{(Eq. 1)}$$

Finally, patterns of variation in $[Ca^{2+}]_i$ of different cellular strains were compared with those of the wild type by pairwaise comparison of model coefficients, whose significance was corrected for multiple statistical tests by the Bonferroni procedure.

2.4 β-galactosidase reporter assays

Exponentially growing cells (5-6 x 10^6 cells/ml) in YPD were harvested by centrifugation at 4000 rpm for 10 min and then resuspended in fresh medium at a cell density of 1.25 x 10^7 cells/ml. The method described by Kiechle *et al.* [33] was used to measure the β-galactosidase activity in exponentially growing cells. Briefly, 200 µl of the cell suspension ('exp', for exponentially growing treatment) were incubated at room temperature for 1.5 h and then collected by centrifugation at 13000 rpm for 2 min, resuspended in 550 µl of pre-cold Z-buffer (75 mM Na_2HPO_4, 50 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$) and quickly frozen in dry ice and conserved at -80 °C.

The remaining cell suspension was incubated at room temperature for 1.5 h either after the induction with 4 volumes of deionized water at room temperature in the presence of 0.2 mM ethylene glycol tetraacetic acid (EGTA) ('EGTA + w' treatment), or after mere addition of 0.2 mM EGTA. Then, 200 µl-aliquots were collected and processed as described above.

Detection of β-galactosidase activity was performed as follows: all samples were simultaneously thawed, a 100 µl-aliquot was removed and used to determine the OD_{600}. The remaining cells were incubated for 1 h at 37°C with 100 µL zymolyase solution (zymolyase T20, ICN, USA; 0.5 mg/ml in Z-buffer). To measure β-galactosidase activity, 100 µl of 4 mg/ml CPRG (chlorophenol-red-β-D-galactopyranoside, Sigma-Aldrich) in Z-buffer were added to each sample. After incubation for suitable time (t_{inc}) at 37 °C the reaction was stopped with 200 µl of 1M Na_2CO_3. Samples were then centrifuged at 13000 rpm for 15 min and the supernatant was read at 574 nm and at 634 nm. β-galactosidase activity was calculated as follows and expressed in Miller Units (MU): $MU=[(OD_{574} - OD_{634}) \times 1000] / (OD_{600} \times t_{inc})$. Each value reported throughout the paper is the average of at least three independent experiments on the same strain, each with at least double dosage. Student's t-test was used to assess significant differences with the respective control.

2.5 Epifluorescence microscopy

Cells were grown at 30 °C in the proper selective SD medium up to early exponential phase, incubated with 1.6 µg/ml DAPI for 90 min and observed with a Nikon Eclipse E600 microscope, fitted with either a 40x or a 60x immersion objective and a standard fluorescein isothiocyanate filter set (Nikon, EX 450-490, DM 505, BA 520) for eGFP-fluorescence, and a cy3 filter set for DsRed fluorescence. Images were recorded digitally using a Nikon FDX-35 camera and processed using MetaMorph 6.3r1 (Molecular Devices, Sunnyvale, CA) and MBF Image J (Mc Master Biophotonics).

3. Results

3.1 Hypotonic shock-triggered calcium signaling mainly involves calcium release from internal stores

Hypotonic shock (HTS) was reported to induce a transient calcium peak in yeast cells: in synthetic complete medium growing cells, calcium influx upon hypotonic shock was proposed to be initially derived from intracellular stores, while extracellular calcium would be involved in the maintenance of a transiently high level of $[Ca^{2+}]_i$ after the initial efflux from intracellular stores [15].
Since cultural conditions (and consequently calcium availability in the medium) are known to modulate functionality of calcium transporters in yeast [21, 23, 27], hypotonic shock was tested in cells growing in a rich medium (YPD).
In YPD growing cells, two different components contributing to the HTS-driven increase in cytosolic calcium concentration could be clearly observed: a fast response (between 5 and 10 s after

the stimulus), which is slightly inhibited by high concentrations of extracellular calcium but cannot be observed if external calcium is not available, and a slow response (around 20-30 s after the stimulus), which is evident only after addition of EGTA, an extracellular Ca^{2+} chelator (Fig. 1A). The same results were obtained when other calcium chelators, such as BAPTA or EDTA, were used instead of EGTA, indicating that, while the 5-10 s response is likely due to a calcium influx from the external medium, the 20-30 s peak could be due to a calcium release from intracellular stores, which is peculiarly inhibited by micromolar extracellular calcium availability.
In order to evaluate the contribution of extracellular calcium to $[Ca^{2+}]_i$ increase, the response was registered every second and the initial rate of $[Ca^{2+}]_i$ increase was estimated as the maximal value of the first derivative of the calcium concentration increase, that usually occurred between 3 and 6 s after the HTS stimulus. Since the calcium release from the intracellular stores is slowest than the calcium influx, the initial rate value obtained will give a good estimate of the calcium influx maximal rate, while it will surely underestimate the contribution of calcium release. The initial rate was plotted against extracellular free calcium concentration, drawing a quite complex graph. At submicromolar extracellular free calcium concentration, the graph could not be fitted by any Hill function, suggesting a more complex scenario than a simple calcium influx from the extracellular medium, while at micromolar extracellular calcium concentrations, the initial rate of $[Ca^{2+}]_i$ increase could be perfectly fitted by a Hill function with a K_M value of 1.1 ± 0.2 μM, actually suggesting the existence of a single transporter on the plasma membrane. Further rising of extracellular concentration induced an almost complete inhibition of calcium influx, with an IC_{50} of 20.6 ± 0.4 μM. This may explain why this transport has not been previously identified. Indeed, calcium content of growing media commonly used for budding yeast is slightly lower than millimolar, and thus dramatically inhibits both these transports.

3.2 HACS and calcineurin involvement in hypotonic shock-induced calcium response

To identify the calcium transporters involved in the observed response, mutants defective in HACS subunits Mid1 and Cch1 were challenged with HTS. In fact, this system was a good candidate for HTS-induced calcium signaling, since it is sensitive to gadolinium, which was reported to inhibit HTS-induced calcium signaling [15], and comprises Mid1 channel, which was previously reported to act as a mechanosensitive calcium channel in a heterologous system and to be sensitive to gadolinium itself [17].
In rich medium growing cells, hypotonic shock revealed a surprising pattern in mutants in HACS components. In *mid1*Δ *cch1*Δ mutant, defective for HACS voltage-gated channel pore forming subunit homolog, and for the stretch sensitive component too, both the intensity of calcium release from internal compartments (Fig. 2A) and of calcium influx (Fig. 2B) are even higher than in the wild type strain.
The main effector of cytosolic calcium level and regulator of calcium homeostasis is the Ca/calmodulin/calcineurin pathway [13]. Consequently, the effect of calcineurin inactivation was investigated, in order to assess if extracellular calcium availability could regulate HTS calcium response by activating calcineurin. In contrast with this hypothesis, albeit calcineurin inactivation in *cnb1*Δ mutant led to a slight increase in the calcium release in presence of EGTA (Fig. 2A), a dramatic reduction in the response could be observed when calcium chloride was added in the extracellular medium (Fig. 2B). This could be due to at least two different effects of calcineurin inactivation: first, Cch1 channel is no longer inhibited by calcineurin activity in rich medium [21]; second, calcium homeostasis system is defective due to the lack of calcineurin controlled circuit. In the first case, the deletion of HACS system would be expected to relieve calcium sensitivity. In fact, the triple mutant *mid1*Δ *cch1*Δ *cnb1*Δ showed a far less dramatic calcium sensitivity in HTS induced calcium influx (Fig. 2B), confirming that Cch1 mediated calcium influx was responsible for the inhibition of the HTS response, rather than being a significant component of the response itself. Consistently, deletion of calcineurin dependent transactivation factor encoding gene, *CRZ1*

[36], did not cause any effect on the HTS induced calcium signal (data not shown), confirming that the effect of calcineurin is due to post-transcriptional regulation, likely on Cch1 transporter.

3.3 Involvement of other known calcium transporters in HTS-induced calcium release from intracellular stores

Surprisingly, the single deletion of *MID1*, encoding the mechanosensitive component of HACS, was not associated to any appreciable effect on the response intensity (Fig. 3, data not shown). These different phenotypes may indicate a functional distinction of these two different subunits of HACS in YPD growing cells. Interestingly, *MID1* gene deletion affected the HTS-induced calcium response signature by delaying the calcium release from intracellular compartments (Fig. 3) but not the calcium influx (not shown). Anyway, a mutant strain disrupted in both *CCH1* and *MID1* genes behaved as the single *cch1*Δ mutant strain (compare Fig. 2A and 3), suggesting that Cch1 is the only HACS subunit involved in regulation of the HTS response.
The other known component of HACS, Ecm7, was not involved in HTS response either, since HTS calcium response in the *ecm7Δ* mutant was again comparable to that of the wild type (Fig. 3, and data not shown).
Only Fig1 protein has been identified up to now as a regulator or a component of LACS [34]. Fig1 promotes Ca^{2+} influx and elevation of cytosolic free Ca^{2+} concentration upon exposition to mating factor [34]. Furthermore, a genetic interaction was reported between Fig1 and GIC system [23]. *FIG1*gene was deleted together with *MID1* and *CCH1* genes, in order to get rid of HACS contribution. The triple mutant, *mid1*Δ *cch1*Δ *fig1*Δ, showed no difference in HTS response when compared to the double mutant *mid1*Δ *cch1*Δ (Fig. 3). So, LACS system seems not to be involved in HTS response, or at least not to require Fig1 to be activated by HTS.
Hypertonic shock was reported to activate calcium release from the vacuole, through the vacuolar calcium channel Yvc1 [37]. In yeast, the vacuole serves as a major store for Ca^{2+}, for the purposes of both detoxification and signaling. The Yeast Vacuolar Channel, Yvc1, is a homolog of the constitutively active inwardly rectifying calcium channels in mammals known as TRP (Transient Receptor Potential) channels, and likely represents a calcium-activated calcium channel, which has been shown to release Ca^{2+} from the vacuole into the cytosol in response to hyperosmotic shock both by mechanical activation and Ca^{2+}-induced calcium release [37]. Furthermore, a role was previously proposed for Yvc1 in glucose refeeding-induced calcium signaling [38].
In the *yvc1*Δ mutant, HTS response in presence of the extracellular calcium chelator EGTA was comparable to the wild type response (Fig. 4). Moreover, synthetic deletion of HACS and LACS components in *mid1*Δ *cch1*Δ *fig1*Δ *yvc1*Δ strain produced a response similar to *mid1*Δ *cch1*Δ *fig1*Δ strain (data not shown). This suggests that the HTS-induced calcium release should not involve Yvc1-driven calcium release from the vacuole major calcium storage.
Conversely, deletion of *CLS2/CSG2*, encoding for an endoplasmic reticulum protein that is important for the regulation of intracellular Ca^{2+} in secretory intracellular compartments [39-41], stimulated HTS-induced calcium release (Fig. 4), suggesting an involvement of ER or Golgi in this response.

3.4 An unidentified TRP-like protein family of putative calcium transporters exists in fungi with similarity to TRPP and TRPML mammalian ion transporters

YOR365C gene product was previously identified as a 703 amino acids putative ionic transporter on the basis of a phylogenetic classification of different unknown membrane proteins in yeast [42]. Yor365c is somewhat similar to the mammalian TRP Ca^{2+} transporters superfamily: by performing a search for conserved domains on NCBI Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) on Yor365c sequence, conservation of

TRP-like domains was evident when aligned to a consensus obtained with other TRP family members from different species. By performing a more detailed bioinformatics research on the whole yeast genome, focusing on still uncharacterized membrane proteins, YOR365C gene was found to have a paralog, *FLC2/YAL053W*, that arose from the whole genome duplication [43] and was recently proposed as a member of the *fungi* specific FLC family, required for importing FAD into the endoplasmic reticulum [44], but was previously proposed also as a homolog of the fission yeast TRP-like calcium channel *pkd2* [45].
By performing alignments among calcium transport proteins in fungi, a fungal specific family of calcium transporters was identified, comprising yeast proteins proposed as flavin transporters, homologous to *FLC* genes from *S. cerevisiae*, and fungal calcium-related spray proteins, homologous to spray protein from *N. crassa*. No significant distinction is possible among the members of this family based on sequence similarity, meaning that these proteins all belong to an unique family of proteins that we will call *spray* proteins hereafter. These proteins are all distantly related to the TRP proteins of higher eukaryotes, and span a TRP-like domain in the middle-C-terminal part of the protein, which is the most conserved region. The C-terminal tail is on the contrary quite divergent, although generally characterized by a high presence of acidic residues, sometimes in stretches, suggesting a functional meaning that has evolved within the different organisms and even within the same organism. Since *S. pombe* spray homolog was proposed as a homolog of human polycystic kidney disease 2/polycystin 2 calcium channel, an alignment of this region was performed among budding yeast Flc2 and *N. crassa* spray proteins, TRPP and TRPML protein family members (see Fig. S1 in *Supplementary Materials*). The fungal proteins appear to be quite conserved along the pore forming region (spanning transmembrane domains TM4 to TM6 and the large loop between TM5 and TM6), and actually show some of the features typical of the PKD channels, with an aminoacid identity above 20% between Flc2 and human polycystin 2 in this region, but spray proteins appear to be more similar to the TRPML branch of TRP proteins when conservative amino acids substitutions are considered.

3.5 FLC proteins are involved in HTS induced-calcium response

Deletion of *FLC2* dramatically affected the signal at low external calcium concentration (Fig. 5), suggesting that this protein was involved in external calcium independent HTS-induced calcium release from internal stores. In contrast, HTS-induced calcium influx from extracellular environment seemed not to be impaired, but rather increased, by *FLC2* disruption (see Fig. S2 in *Supplementary Materials*). Hence, Flc2 seems at least to be involved in the regulation of calcium release from internal stores, if it is not the transporter itself.
The effect of Flc2 deprivation was not reversed either in the *mid1*Δ *cch1*Δ *fig1*Δ *flc2*Δ (see Fig. S3) or in the *cls2*Δ *flc2*Δ strain (data not shown), indicating that this effect was not related to a Flc2 indirect function either on HACS calcium transporters regulation or in calcium homeostasis in the ER. Furthermore, the presence of *FLC2* gene on a multicopy plasmid dramatically perturbed both the calcium level in exponentially growing cells and the response to HTS (see Fig. S4 in *Supplementary Materials*), supporting the hypothesis that Flc2 protein would be directly involved in calcium transport.
A polynomial mixed model was used to quantitatively investigate the different behavior of *flc2*Δ mutant strain compared to the wild-type strain as far as HTS-driven calcium release from intracellular stores is concerned. In order to evaluate this phenomenon, the analysis was performed on the curves obtained in the presence of 1 mM EGTA without $CaCl_2$ addition, condition that, at least in the wild-type strain, minimizes the contribution of external calcium influx (see Fig. 1B). Resting $[Ca^{2+}]_i$ immediately before the shock did not differ significantly among cellular strain, as indicated by the non-significant effect of strain factor (Table 1). $[Ca^{2+}]_i$ then increased with non-linear patterns that were significantly different among the *flc2*Δ and the wild-type strain, as

indicated by the significant interactions between the strain factor and both the first and the second order polynomial terms of time (b_1 and b_2 coefficients in Table 1).
Conversely, deletion of *YOR365C* only slightly, albeit significantly (see Table 1), affects HTS-induced calcium release either alone (Fig. 5 and Table 1) or in combination with the *mid1*Δ *cch1*Δ *fig1*Δ deletion background (see Fig. S3 in *Supplementary materials*), confirming a marginal role, if any, of this hypothetic transporter in HTS-induced calcium release. Yor365c was produced as an eGFP fusion protein and was observed mainly in the mitochondria in YPD exponentially growing cells (data not shown), as predicted by WoLF PSORT software [46] too, while Flc2 was reported to localize in late Golgi, bud neck and cell periphery by the Yeastgfp database (http://yeastgfp.yeastgenome.org/getOrf.php?orf=YAL053W) [47] and to the ER and probably cell membrane by membrane fractionation [44]. Thus, the localization of the two proteins is also not identical, suggesting that the two homologs could have diverged in function.
FLC2 gene is also a homolog of FLC family genes *FLC1* and *FLC3*, albeit it is less similar to them than to *YOR365C*. A redundant role was proposed for Flc2 and Flc1, since double deletion of *FLC1* and *FLC2* genes is lethal [44]. In contrast, effect on HTS-induced calcium release is not redundant: *flc1*Δ and *flc3*Δ strains showed almost the same slight reduction in the HTS-induced calcium release, that is less evident than in the *flc2*Δ mutant, albeit significant, as revealed by the application of the polynomial mixed model (see Table 1). Post-hoc tests also indicated that $[Ca^{2+}]_i$ variation pattern of all strains differed significantly from that of the wild type. Close inspection of the coefficients of the model indicated that $[Ca^{2+}]_i$ increased more quickly and reached higher maximum value in the wild-type than in all other cellular strains, confirming that all the mutants defective in one of the *FLC* genes have significant inhibition in HTS-induced calcium release from internal stores. Obviously, the model did not take into account the small contribution of the calcium influx, which was considered almost negligible when only 0.2 mM EGTA was added in the medium.
The defect in HTS-induced calcium release is additive in the *flc1*Δ *flc3*Δ double mutant (Fig. 6), with a slower calcium mobilization but no decrease in response intensity. Surprisingly, the effect was quite similar to that observed in the *mid1*Δ strain (see Fig. 3), and could therefore be an indirect effect related to calcium homeostasis in the ER compartment. Surprisingly, HTS-driven calcium release in the *mid1*Δ *cch1*Δ *fig1*Δ *flc1*Δ strain was similar to the response observed in the *mid1*Δ *cch1*Δ *fig1*Δ strain, while the ER calcium release observed in the *mid1*Δ *cch1*Δ *fig1*Δ *flc3*Δ strain was more similar to that observed in the *flc3*Δ strain (see Fig. S3 in *Supplementary materials*), suggesting a more direct involvement of Flc3 in Cch1-dependent regulation of HTS-driven calcium release.
Similarly to what we observed in *flc2*Δ strain, the HTS-triggered calcium influx of *flc1*Δ and *flc3*Δ strains was not defective (see Fig. S2), albeit inhibition of the influx when extracellular calcium concentration was higher became less evident than in the wild-type strain (data not shown).
In order to better analyze the HTS-induced calcium influx and also to observe if the complex behavior not associated with calcium influx from extracellular medium, that was reported for the wild-type (Fig. 1B), was still observed in the *flc2*Δ strain, the initial rate of $[Ca^{2+}]_i$ increase in the presence of different external calcium concentrations was assayed in this mutant strain. The pattern of the HTS-triggered $[Ca^{2+}]_i$ increase initial rate in absence of Flc2 activity largely differed from that of the wild-type strain (Fig. 7). Indeed, the initial rate graph in the *flc2*Δ strain was almost perfectly fitted by a Hill curve with a k of 0.92 ± 0.04 μM and a cooperativity coefficient n of 2.9 ± 0.5. Albeit the overall response of the *flc2*Δ mutant was lower at 0.48 μM than that of the wild-type, this was not related to any defects in calcium influx rate, which in contrast was higher even in scarcity of external calcium. Indeed, the fact that the initial rate, even at the lower external calcium concentration, is higher in the *flc2*Δ mutant does not imply that the total response should be higher, since this maximum rate may be maintained for only a very short time, and, when external calcium is scarce, its contribute to the observed variation in $[Ca^{2+}]_i$ is by far lower than that of calcium

release from ER. Consistently with this interpretation, in conditions of low external calcium, the wild-type strain showed the highest increase in internal calcium concentration (Fig 1A), despite that the initial rate was lower (Fig 1B), suggesting that the two parameters are evidently not correlated. This steep increase in calcium concentration was actually maintained in the *flc2*Δ mutant for a very short time (1-2 s) (data not shown), and did not compensate for the disappearance of the additive component in the submicromolar range of external calcium concentration (likely due to internal calcium release, mediated by Flc2), resulting in an overall lower response in the mutant in conditions of external calcium shortage.
Surprisingly, the dramatic inhibition occurring at free external calcium concentrations above 5 μM also disappeared, leaving only a residual inhibition, reaching half of the maximal rate only when free extracellular calcium concentration is very high, i.e. in the millimolar range. This suggests that not only Flc2 is the intracellular calcium transporting protein or at least a subunit of the transporting system, but also that it is probably the calcium responsive one. Indeed, Flc2 is also involved in the high sensitivity to calcium of the HTS-responsive plasma membrane calcium transporter, since in the absence of Flc2 protein the calcium influx rate of this transporter was generally higher than in the wild-type strain, suggesting an inhibitory role for Flc2 on the transporter activity. This is consistent with the absence of a significant increase in the response in the *mid1*Δ *cch1*Δ *fig1*Δ *flc2*Δ (see Fig. S3 in *Supplementary* materials) when compared to the *flc2*Δ strain, suggesting that the Cch1-mediated inhibition requires the presence of Flc2.

3.6 FLC2 gene disruption confers severe sensitivity to Calcofluor white but not to tunicamycin

Mutants in several calcium channels in yeast suffer from hypersensitivity to cell wall-perturbing agents, such as Calcofluor white, which binds to chitin and glucan in the cell wall, thus interfering with the synthesis and cross-linking of these major cell wall components. Coping with this kind of stress involves a functional Cell Wall Integrity (CWI) pathway, which, in turn, is activated by calcium [48]. Thus, defects in calcium signaling can also involve a certain fragility in the response to cell wall damage [49].
Cells defective in *FLC2* were reported to be sensitive to Calcofluor white [44], consistently with its role in calcium transport. In order to assess genetic interactions of *FLC2* deletion, sensitivity to Calcofluor white was assayed on a YPD plate for mutants lacking this putative transporter, alone or together with other known calcium transporters (Fig. 8A).
As expected, disruption of *FLC2* gene conferred a very high sensitivity to Calcofluor white, either alone or together with *MID1* encoded HACS component. In contrast, deletion of *YOR365C* gene did not imply a different sensitivity to Calcofluor white than that of the wild type strain (data not shown), confirming again that these two genes, although paralog, have diverged in function.
Since *flc2*Δ mutant was previously reported to suffer from ER stress [44], sensitivity to tunicamycin of the mutants in *FLC2* was also tested. Single deletion in *FLC2* did not confer higher sensitivity to 0.25 μg/ml tunicamycin in YPD than in the wild-type strain, but interestingly the *mid1*Δ *flc2*Δ double deletion mutant was extremely sensitive to tunicamycin, suggesting a genetic interaction between these two genes impinging on ER integrity (Fig. 8A). Furthermore, *mid1*Δ *cch1*Δ *fig1*Δ *flc2*Δ mutant was also more sensitive to tunicamycin than the *mid1*Δ *cch1*Δ *fig1*Δ triple mutant (data not shown).
In order to better evaluate ER stress in these mutant strains, a UPR (Unfolded Protein Response) responsive reporter was used. UPR-dependent transcription was not higher in any of the mutants tested than in the wild-type (Fig. 8B), and was even lower in the *flc2*Δ strain, confirming that the defect in calcium signaling is not due to a general stress in the ER.

3.7 FLC2 gene disruption induces calcineurin and Mpk1 activation in exponentially growing cells

The main effector of calcium signaling in eukaryotes is the calmodulin/calcineurin circuit, which in budding yeast has Crz1 as a main responsive transcription factor. A reporter, based on four Crz1 binding CDRE (Calcineurin Dependent Responsive Elements) driving transcription of *E. coli LacZ* gene, was used to investigate calcineurin activity in YPD exponentially growing cells and in HTS challenged cells. HTS induced only a marginal increase in HTS-exposed cells in the wild-type strain (Fig. 9A). In the *flc1*Δ and *flc2*Δ strains, exponentially growing cells showed an evident hyper-activation of calcineurin when compared with the wild-type strain, which is consistent with the higher basal calcium concentration in the cytosol observed in *flc1*Δ and *flc2*Δ strains (see Fig. 5 and 6A). No further significant increase in calcineurin dependent transcription was revealed after HTS. In the *cls2*Δ strain, calcineurin dependent transcription did not differ from that of the wild-type strain.
A reporter carrying the *LacZ* gene under control of Rlm1 responsive elements [50] was used to monitor the activation of the Cell Wall Integrity Mpk1 kinase cascade, which is known to be activated by hypotonic shock in budding yeast [51]. Consistently, a 3-fold increase in the β-galactosidase activity was reported when the wild-type strain was exposed to hypotonic shock (Fig. 9B). Interestingly, *flc2*Δ strain revealed a hyperactivity of the Rlm1-dependent reporter even in exponentially growing cells, suggesting a deregulation of the CWI pathway which would tally with a cell wall perturbation, as evidenced by the observed extreme sensitivity to Calcofluor white. HTS treatment was able to induce further β-galactosidase production, indicating that *flc2*Δ strain has no defects in the activation of Mpk1 pathway by HTS. Conversely, *cls2*Δ strain showed a significant reduction in Rlm1-dependent transcription, both in exponentially growing cells or in HTS-exposed cells. This suggests that cytosolic calcium increase would not be related to Mpk1 activation, but that ER calcium homeostasis could have an effect on CWI pathway, which is also confirmed by the similar effect observed in *flc1*Δ strain. Again, Flc2 and Flc1 do not appear to exert a redundant function.

4. **Discussion**

Calcium is a second messenger involved in different signals in yeast, mostly for stress triggered signaling, such as response to hypotonic or hypertonic shock [15, 52], but also to nutrients [23]. Hypotonic shock was proposed to impose a mechanical stretch on the plasma membrane, thus probably activating a transport system that has mechanosensitive properties [53].
Genes encoding MscS homologues have been found in the genomes of prokaryotes and cell-walled eukaryotes, for example, plants, algae and fungi [54], but not in the *Saccharomyces cerevisiae* genome [55]. Recently several TRP channels with mechanosensitive properties have been identified in higher eukaryotes [56] and in yeast [57].
In budding yeast, hypotonic shock stimulates phospholipase C activity [58], albeit this is not required to generate the calcium signal ([59] and data not shown), which was previously supposed to be directly elicited by mechano-sensitive calcium channels on cell surface. Indeed, electrophysiological studies of ion channels in the plasma membrane of the yeast *Saccharomyces cerevisiae* described channels that were activated by, and adapted to, stretching of the membrane, passing both cations and anions, showing voltage-dependent adaptation to mechanical stimuli [8]. Furthermore, Mid1 channel showed mechanosensitive features in a heterologous system [17].
The two kinetically distinct components of HTS calcium signaling in YPD medium growing cells (Fig. 1) suggest that different systems could transport calcium in response to HTS: the fast response requires external calcium presence, although it is severely inhibited by free calcium concentration above the micromolar range in the wild-type strain. A working model could comprise a stretch-activated channels with high affinity for calcium, whose gating then promotes calcium release from internal calcium stores, and which is inhibited at high calcium concentrations, pointing out to HACS as a good candidate. However, in our hands *mid1*Δ or *cch1*Δ mutants did not show any

defects in responding to hypotonic shock (Fig. 3). Furthermore, Cch1 rather seemed to exert an inhibitory role on this signal, while Mid1 seemed to be involved in the regulation of the response only in extracellular calcium shortage conditions. This is particularly interesting since the budding yeast *S. cerevisiae* employs a CCE-like mechanism to refill Ca^{2+} stores within the secretory pathway, involving HACS system [60], suggesting the existence of a regulatory loop between the ER and Golgi intracellular calcium compartments and HACS system.

Recently, in *N. crassa* staurosporine treatment was reported to trigger both Ca^{2+} uptake from the extracellular milieu by a novel fungal influx system resembling a TRP channel, which also seems to be up-regulated in the absence of the HACS, and an IP_3-mediated cytosolic recruitment of organelle-stored Ca^{2+} [61]. Moreover, hyphal growth in *N. crassa* has been reported to rely on a tip-high $[Ca^{2+}]_i$ gradient that is internally derived by means of Ca^{2+} channels activated by inositol-1,4,5-trisphosphate (IP_3) [62-64], though no IP_3 receptor seems to exist in fungi [65, 66]. IP_3 is generated by a stretch-activated tip-localized phospholipase C that senses tension due to hyphal expansion [63, 64], promoting the release of Ca^{2+} through a large conductance channel, associated with the vacuolar membrane and a small conductance channel, associated with endoplasmic reticulum (ER)- and Golgi-derived vesicles.

This is in contrast with the observation that a *plc1*Δ strain in budding yeast shows a higher calcium influx than a wild-type strain when insulted with a hypotonic shock ([59] and data not shown).

As mentioned above, in presence of free calcium concentrations (less than 1 μM is enough) in the extracellular medium, the HTS-response slows down with the peak in calcium concentration shifting from nearly 10 s to 20-30 s after the stimulus, suggesting the presence of a slower but stronger calcium release, which was completely inhibited when free extracellular calcium was in the micromolar range. This HTS-triggered calcium release likely originates from the secretory compartments and involves putative ion transporters of the FLC protein family.

Flc2 was recently proposed as a member of the FLC family required for importing FAD into the endoplasmic reticulum [44], but it was also previously proposed as a homolog of a TRP-like calcium channel identified in *S. pombe* [45]. The *FLC* genes belong to a conserved fungal gene family of integral membrane proteins, spanning a TRP-like domain. The *S. cerevisiae* genome contains three homologues of *C. albicans FLC1*, *YPL221W*, *YAL053W*, and *YGL139W*, which exhibited 48, 36, and 47% amino acid identity to *CaFLC1* and were designated *FLC1*, *FLC2*, and *FLC3*, respectively [44]. *YOR365C* exhibits slightly less homology to *CaFLC1* (28% identity) but higher similarity to *FLC2* than *FLC1* or *FLC3*. Hsaing and Baillie [67] recently identified *YOR365C* and the *FLC* gene family as one of 17 "core fungal" genes that are represented in all fungal species but not in prokaryotes or non-fungal eukaryotes. Actually, as mentioned above, *FLC2* was previously reported to be the *S. cerevisiae* homolog of a *S. pombe* calcium channel, encoded by *pkd2* gene, which belongs to the TRP (transient receptor potential)-like ion channel family and has low similarity to polycystic-kidney-disease (PKD)-related ion channel genes [45]. A member of these genes, in tubular epithelial cells, encodes for a Ca^{2+} permeable ion channel, PKD2 *alias* polycystin-2 (PC2 or TRPP2), responsive to a mechanical stimulus triggered by ciliary action, which increases the intracellular Ca^{2+} levels causing cell cycle arrest [68, 69].

S. pombe pkd2 is essential for cellular viability, and appears to be a key signaling component in the regulation of cell shape and cell wall synthesis through an interaction with a Rho1-GTPase in the plasma membrane [70]. The gene *pkd2* plays a critical role in cell proliferation and cell viability. As with human PKD2, the fission yeast Pkd2 protein is localized mainly to Golgi and, to a lesser extent, to plasma membrane when endogenously expressed, similarly to FLC proteins in budding yeast [44].

This gene family, has representatives in several fungal species, formerly identified as members of a calcium-related spray proteins family. The paradigm of this family is the *N. crassa* spray protein [71], an ER-localized calcium channel regulating thigmotropism, i.e. the ability of this organism to respond to a topographical stimulus by altering the axis of growth of the hyphae [72].

Sequence alignment reveals that fungal *spray* proteins are more similar to the TRPML (for mucolipin) branch of TRP channels, actually the channels most closely related to TRPP class of calcium transporters. They mostly reside in membranes of organelles of the endolysosomal system such as early and late endosomes, recycling endosomes, lysosomes, or lysosome-related organelles. Mutations in the gene coding for TRPML1 (*MCOLN1*) were found in patients affected by mucolipidosis type IV disease, an autosomal-recessive neurodegenerative lysosomal storage disorder. TRPML1 is a calcium and iron permeable intracellular channel in lysosomes, and thus a loss of function of this channel impairs endosomal/lysosomal function and autophagy [73]. Consistently with the observed hyper-sensitivity to Calcofluor white stain, Flc2 was also previously reported to be regulated by the cell wall biogenesis and maintenance pathway and to be regulated by Rlm1, an important cell integrity maintenance factor. Moreover, hypotonic shock induces the Pkc1 signal transduction cascade [75], which activates the Cell Wall Integrity (CWI) MAP kinase cascade, with components functionally conserved in mammalian cells [76].
Probably the previously described effect of *FLC1* and *FLC2* deletion on FAD transport could be due to an indirect effect of ER stress [44]. Actually, the single *flc2*Δ mutant did not suffer from hypersensitivity to tunicamycin, nor showed an induction of UPR dependent transcription, suggesting that ER stress was not an issue in the single mutant. The similarity with channels permeable to calcium and iron may explain the previously reported involvement of *S. cerevisiae* FLC proteins overexpression in yeast survival on hemin [44]: for instance, it is unclear if their overexpression could allow yeast cells to grow with traces or without any source of iron at all for longer periods than the wild-type strain.
Interestingly, Flc2 seems to be involved in calcium homeostasis. Genetic interaction with *MID1* or *FLC1* genes could be merely due to the additive effect on deregulation on ER calcium homeostasis, but further investigation are necessary to clarify this point. Given the existing relation among the budding yeast *FLC* gene family, the spray proteins and the TRPP and ML branches of ion transporters in higher eukaryotes, renaming the *FLC1*, *FLC2*, *FLC3* and *YOR365C* genes as *TPR* (for TRP Related) *1*, *2*, *3* and *4* seems appropriate.

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LEGENDS TO FIGURES

Fig. 1. Hypotonic shock-induced calcium response is regulated by calcium availability in extracellular medium. Panel A, hypotonic shock-triggered calcium peak was observed as described in Materials and Methods section in K601 strain cells exponentially growing in YPD medium additioned with 0.2 mM EGTA and different concentrations of $CaCl_2$ giving the indicated free calcium ion concentrations. Panel B, initial rate in cytosolic calcium increase was plotted against free calcium concentration in the extracellular medium. Bars represent standard deviations and in Panel A are reported on the upper side of each curve only, to avoid overlap.

Fig. 2. HACS components are involved in inhibition of calcium response to HTS by calcineurin. Hypotonic shock triggered calcium peak was observed as described in Materials and Methods section in *mid1*Δ *cch1*Δ strain, *cnb1*Δ strain and *mid1*Δ *cch1*Δ *cnb1*Δ strain exponentially growing in YPD medium, at the free calcium concentration of 0.48 μM (panel A) or 5.9 μM (panel B) in the extracellular medium. Bars represent standard deviations and are reported on the upper side of each curve only, to avoid overlap.

Fig. 3. HACS or LACS calcium transport systems components are not required for calcium release induced by HTS. Hypotonic shock triggered calcium release was observed as described in Materials and Methods section in *mid1*Δ strain, *cch1*Δ strain, *ecm7*Δ strain and *mid1*Δ *cch1*Δ *fig1*Δ strain cells exponentially growing in YPD medium at the presence of 0.48 μM free extracellular calcium concentration. Bars represent standard deviations and are reported on the upper side of each curve only, to avoid overlap.

Fig. 4. Involvement of intracellular calcium stores in HTS response. Hypotonic shock-triggered calcium release was observed as described in Materials and Methods section in *yvc1*Δ strain and *cls2*Δ strain cells exponentially growing in YPD medium at the presence of 0.48 μM free extracellular calcium concentration. Bars represent standard deviations and are reported on the upper side of each curve only, to avoid overlap.

Fig. 5. Involvement of Flc2 and Yor365c paralog proteins in HTS-induced calcium release. Hypotonic shock-triggered calcium peak was observed as described in Materials and Methods section in *flc2*Δ strain, *yor365c*Δ strain and *flc2*Δ *yor365c*Δ strain cells exponentially growing in YPD medium at the presence of 0.48 μM free extracellular calcium concentration. Bars represent standard deviations and are reported on the upper side of each curve only, to avoid overlap.

Fig. 6. Involvement of Flc2 homologous proteins in HTS-induced calcium release. Hypotonic shock-triggered calcium peak was observed as described in Materials and Methods section in the indicated strains exponentially growing in YPD medium at the presence of 0.48 μM free extracellular calcium concentration. Bars represent standard deviations and are reported on the upper side of each curve only, to avoid overlap.

Fig. 7. Flc2 is the calcium responsive protein in HTS induced calcium response. Initial rate in cytosolic calcium increase in the *flc2*Δ strain was plotted against free calcium concentration in the extracellular medium.

Fig. 8. Panel A. Disruption of *FLC2* gene confers Calcofluor white hyper-sensitivity but has not any effect on sensitivity to tunicamycin. Indicated strains were grown in YPD medium until exponential phase and then diluted to appropriate cell density to spot 10^4, 10^3, 10^2 and 10 cells. Spots were laid on YPD medium either additioned with DMSO (left) or with 0.25 μg/ml tunicamycin (middle) or with 0.5M sorbitol and 10 μg/ml Calcofluor White (right). Panel B. Exponentially growing cells of the indicated strains, transformed with pMCZ-Y plasmid (UPR-responsive reporter) were assayed for β-galactosidase activity as described in Materials and Methods section. *, P=0.001 *vs.* wt.

Fig. 9. Disruption of *FLC2* gene impinges on calcineurin and Mpk1-dependent transcription. Wild-type (closed rectangles), *flc2*Δ (light grey rectangles), *flc1*Δ (dark grey rectangles) or *cls2*Δ (open rectangles) strains cells transformed either with pAMS366 (Crz1-responsive reporter) plasmid (panel A) or with p1366 (Rlm1-responsive reporter) plasmid (panel B) exponentially growing in YPD medium (exp), exposed for 1.5 h to 0.2 mM EGTA (EGTA) or to 0.2 mM EGTA and HTS (EGTA + HTS) were collected and assayed for β-galactosidase activity as described in Materials and Methods section. *, P<0.01 *vs.* wt (for exp) or EGTA (for EGTA+HTS).

Table 1. Results from the polynomial mixed model of variation in $[Ca^{2+}]_i$ between zero and 20 seconds after hypotonic shock in different cellular strains in presence of 1 mM EGTA in the extracellular medium. χ^2, df and P respectively report the value of the Likelihood Ratio Test for the main effect under focus, the relative degrees of freedom, and the associated P-value. Coef. and SE report the coefficients and the relative standard erros as estimated by the polynomial mixed model. For the wild type coefficients report the actual value of the coefficient in Eq. 1, while for the other strains they report the difference with the wild type (e.g. actual b_1 value for strain *flc1*Δ is therefore 35.59). df reports the degrees of freedom assessed by the between-within method, and t the value of the Student's t statistic. P_{Bonf} reports for the wild type the significance of the difference of coefficients from zero, while for the other strains it reports the significance of the Bonferroni-corrected post-hoc test between the strain under scrutiny and the wild type.

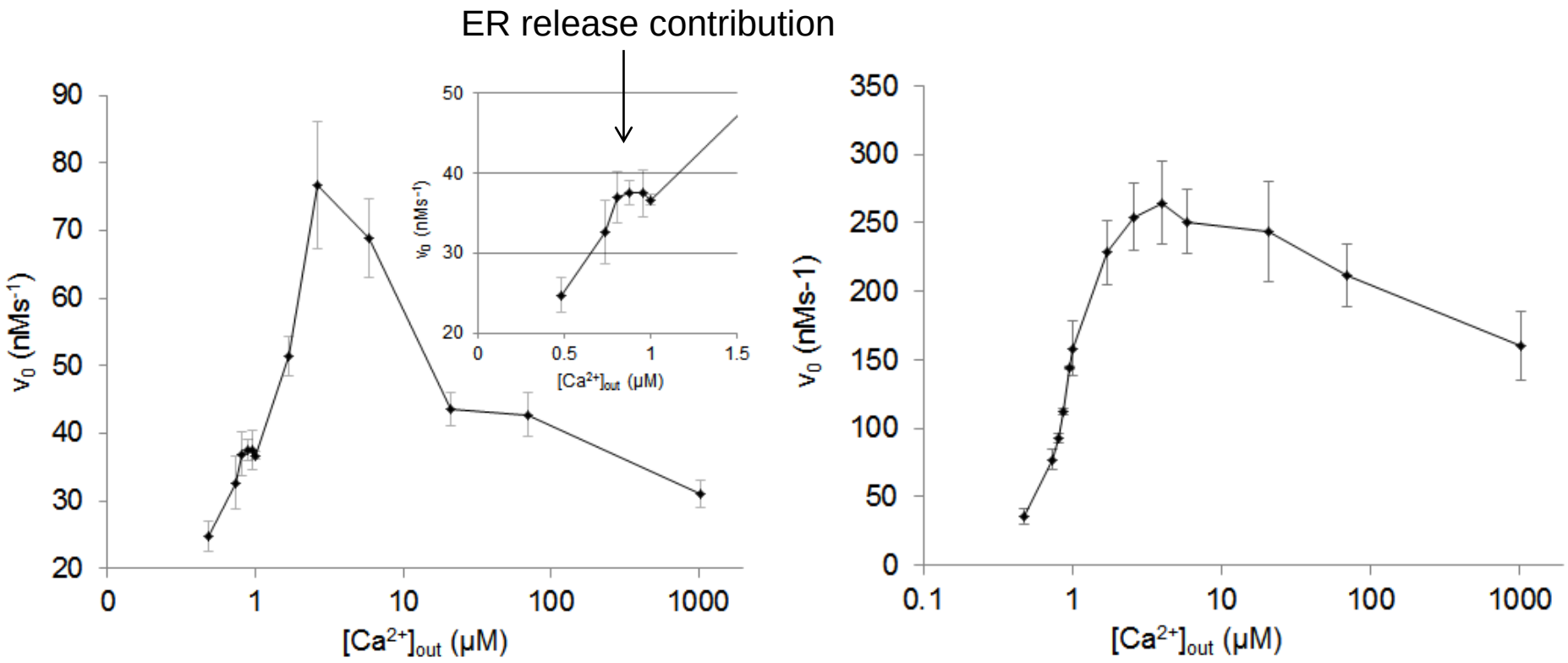
	χ^2	df	P	Strain	Coef.	SE	df	t	P_{Bonf}
Strain	8.20	5	0.146	wt	192.63	31.18	52	6.178	< 0.001
(b_0)				*flc1*Δ	66.07	47.63	10	1.387	0.196
				flc2 Δ	58.67	47.63	10	1.232	0.246
				*flc3*Δ	-11.65	54.01	10	-0.216	0.833
				*yor365c*Δ	-61.35	54.01	10	-1.136	0.282
				*yvc1*Δ	66.07	47.63	10	1.387	0.546
Strain x	84.49	5	< 0.001	wt	72.12	3.74	52	19.301	< 0.001
Time				*flc1*Δ	-36.53	5.71	52	-6.400	< 0.001
(b_1)				*flc2* Δ	-46.26	5.71	52	-8.105	< 0.001
				*flc3*Δ	-40.54	6.47	52	-6.264	< 0.001
				*yor365c*Δ	-30.61	6.47	52	-4.730	< 0.001
				*yvc1*Δ	-23.38	6.47	52	-3.612	0.011
Strain x	35.20	5	< 0.001	wt	-1.78	0.17	52	-10.559	< 0.001
$Time^2$				*flc1*Δ	0.93	0.26	52	3.627	0.011
(b_2)				*flc2* Δ	1.16	0.26	52	4.493	< 0.001
				*flc3*Δ	1.45	0.29	52	4.962	< 0.001
				*yor365c*Δ	1.05	0.29	52	3.585	0.011
				*yvc1*Δ	0.96	0.29	52	3.279	0.028

Note. The overall effects of Time and $Time^2$ were respectively $\chi^2_1 = 372.53$, $P < 0.001$ and $\chi^2_1 = 111.49$, $P < 0.001$, as assessed by Likelihood Ratio Tests.

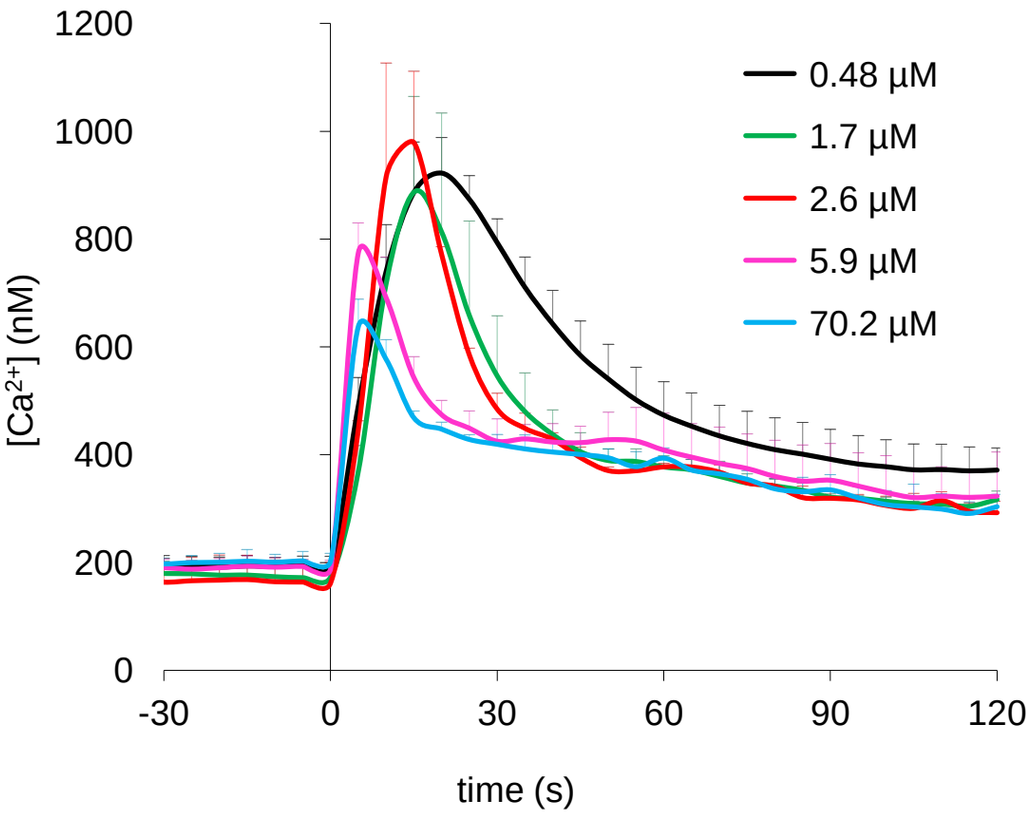
Highlights

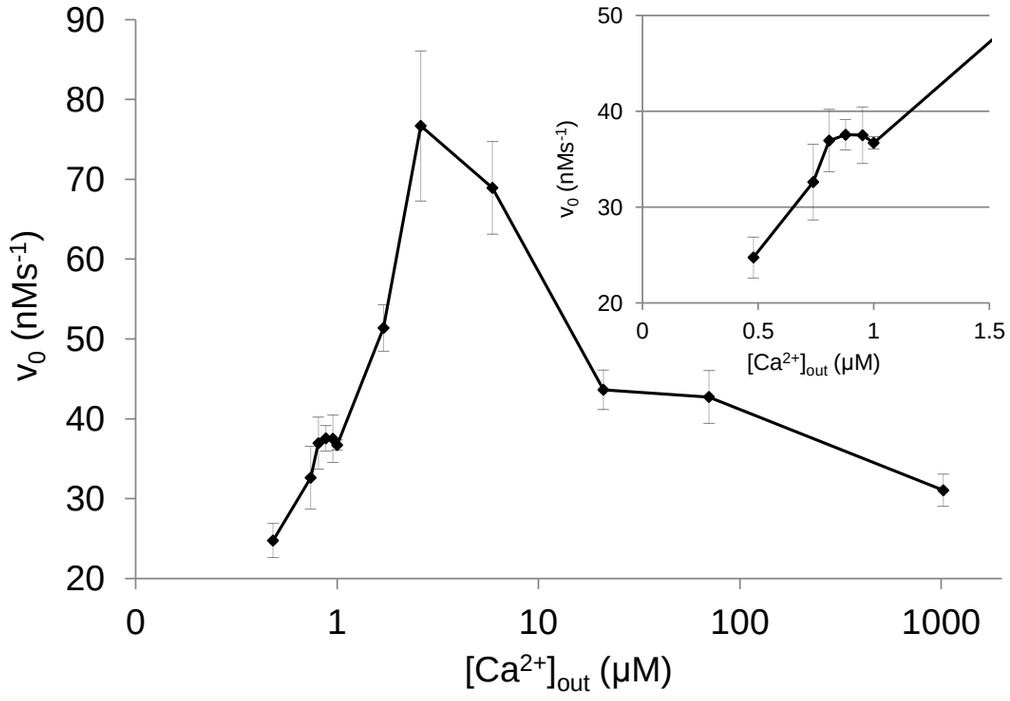
- Hypotonic shock induces both calcium influx and calcium release from secretory compartments in budding yeast
- Yal053w/Flc2 is required for calcium release from intracellular stores, upon hypotonic shock in YPD growing budding yeast cells
- Flc2 is a member of a fungal specific branch of TRP-like calcium transporters spanning calcium-related spray protein from *Neurospora crassa* and Pkd2 from *S. pombe* with significant similarity to human polycystin 2 and mucolipin

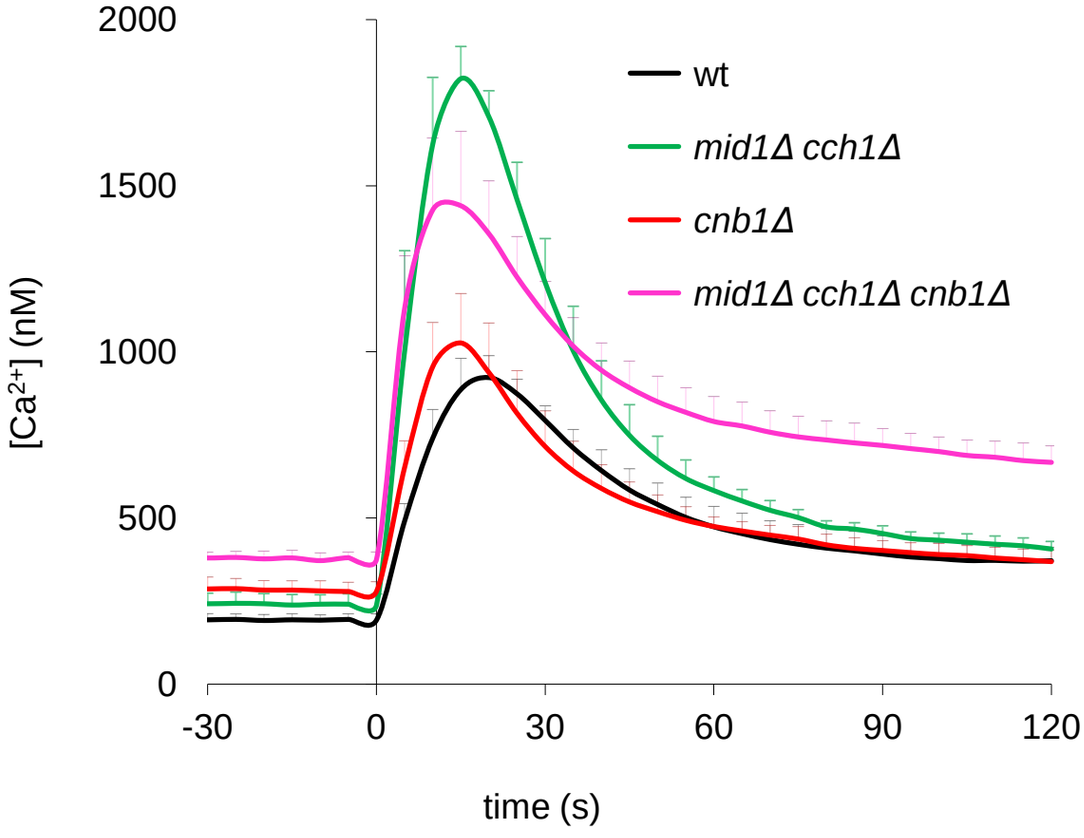
***Graphical Abstract**

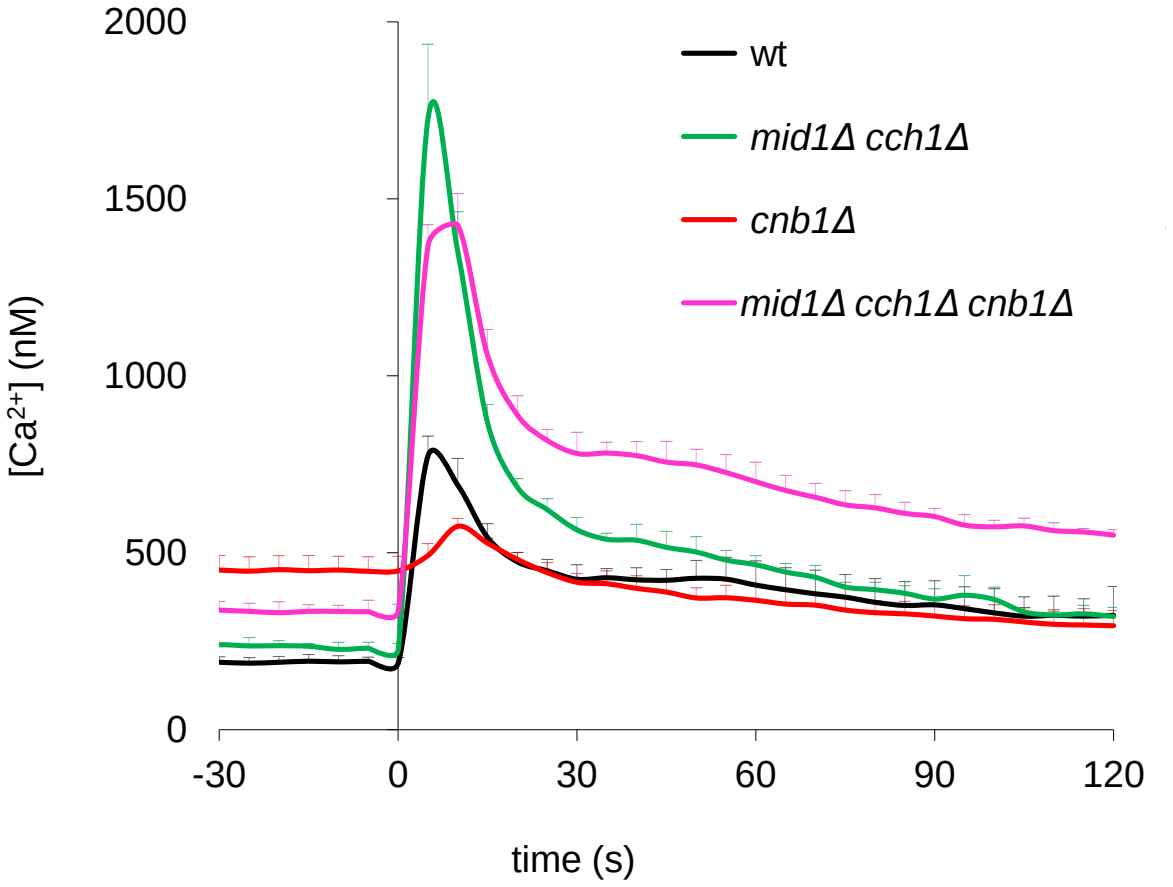


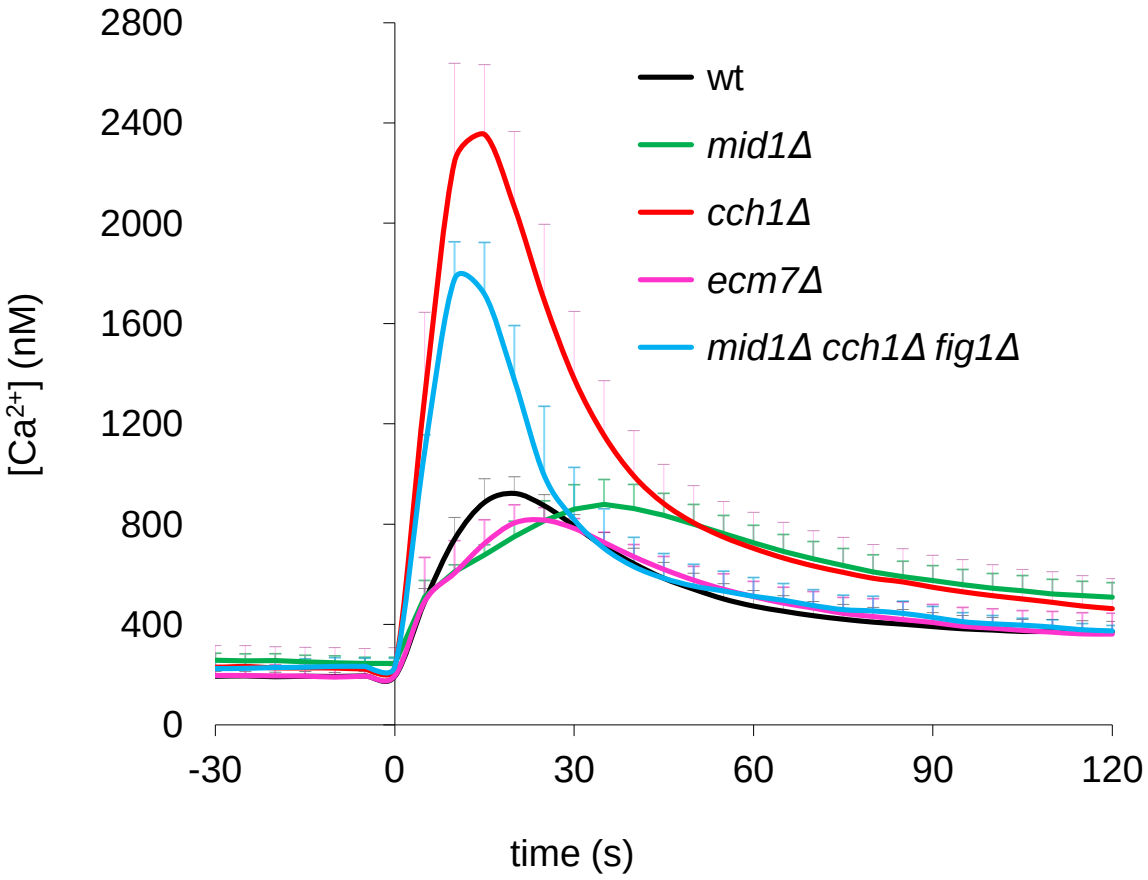
Dependance of the initial rate of hypotonic shock-induced (HTS) cytosolic calcium concentration increase upon extracellular free calcium concentration in the wild-type (left) or in the *flc2*Δ strain (right). Flc2 is a TRP-like channel localized in the ER and plasma membrane with close similarity to *N. crassa* spray protein and far similarity to mammalian mucolipin 2 and polycystic kidney disease/polycystin 2 calcium channels. Flc2 is required for HTS-induced ER calcium release and contributes to regulation of HTS-induced calcium influx from extracellular medium.

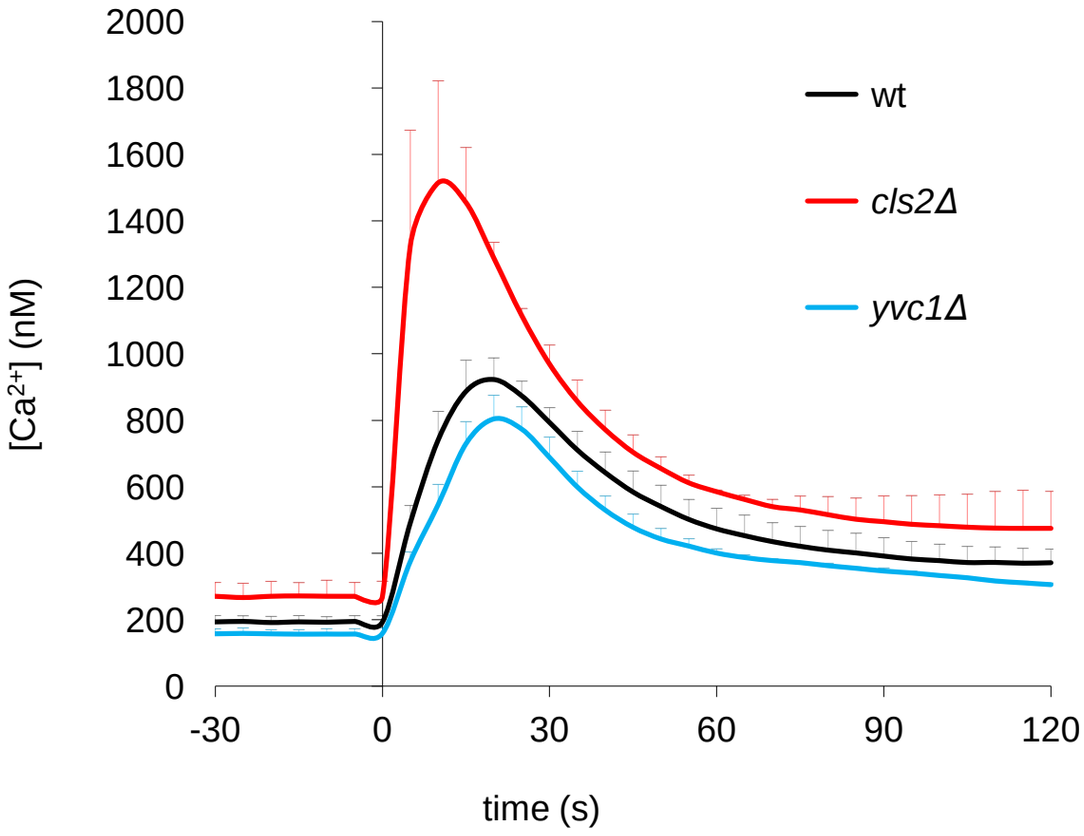


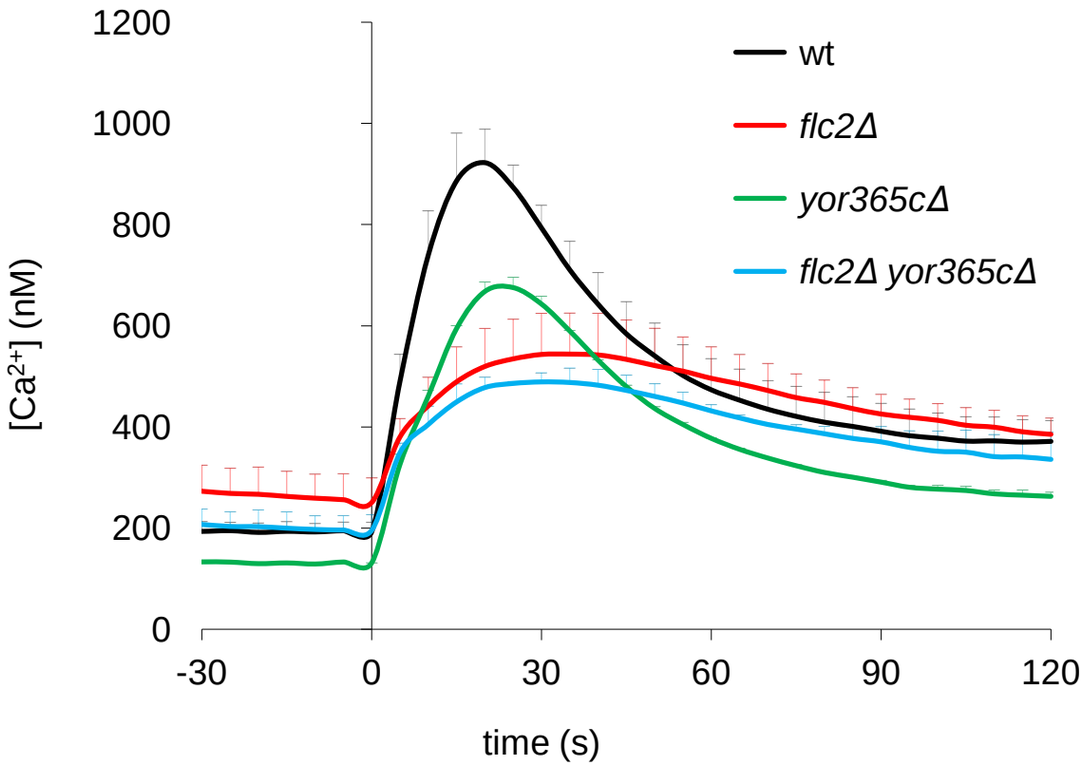


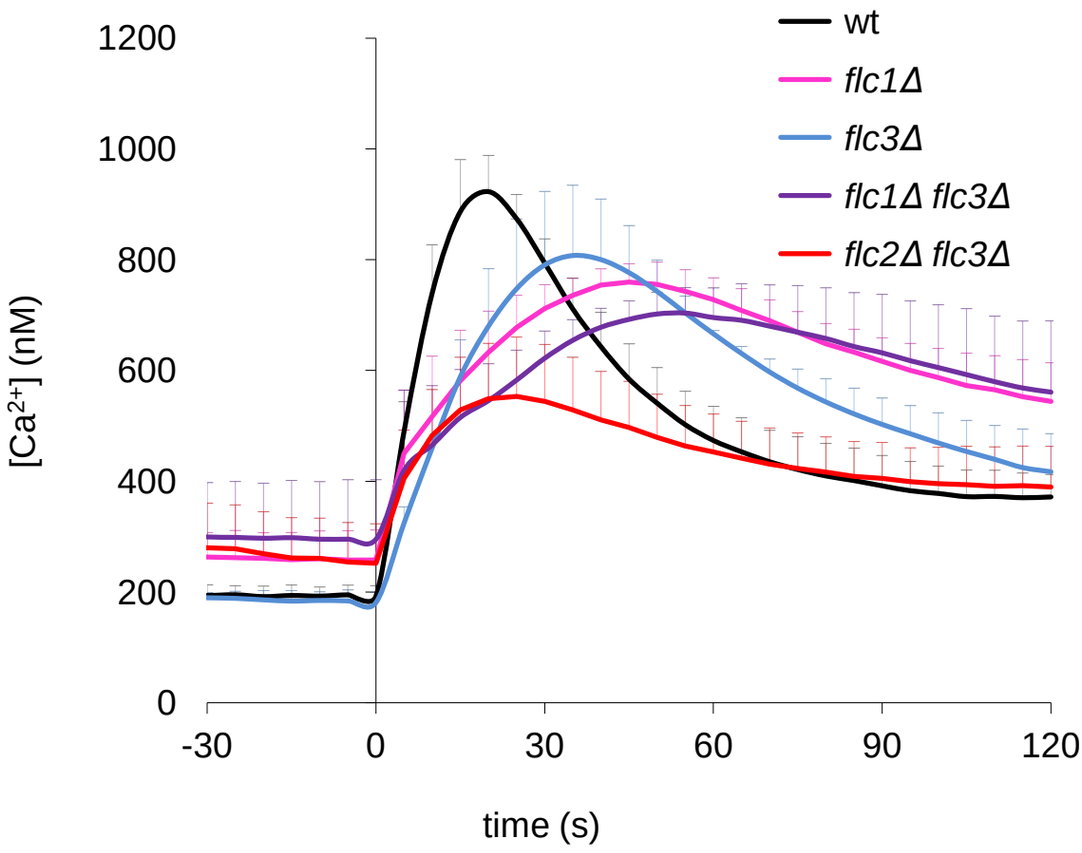












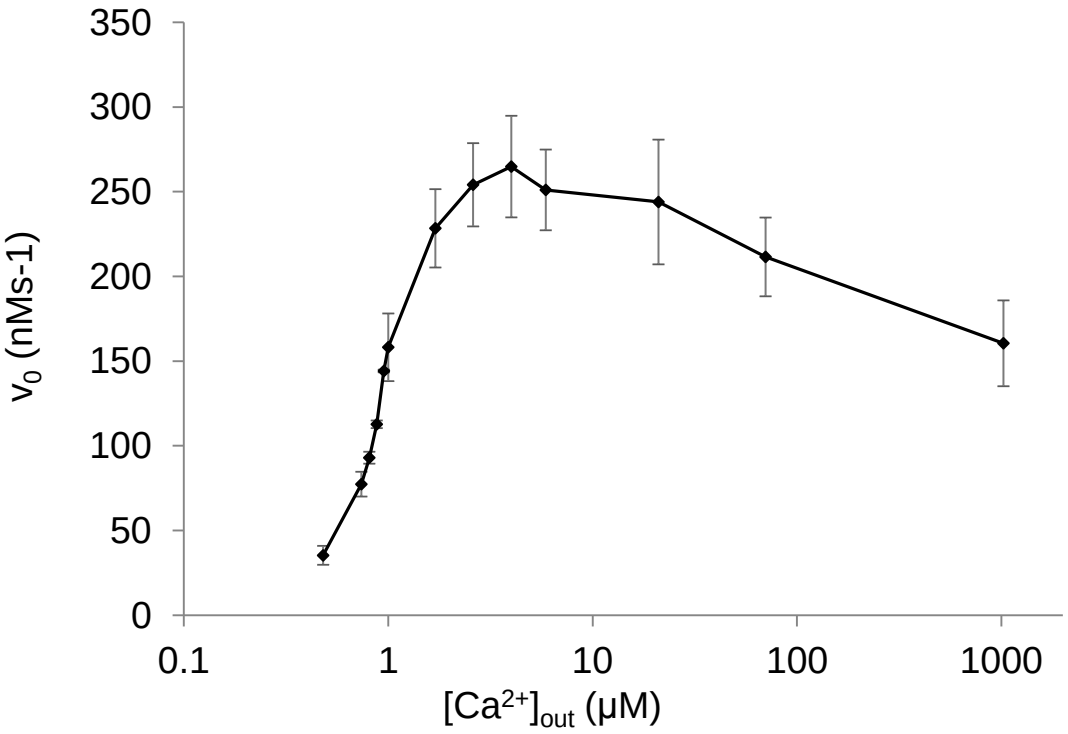
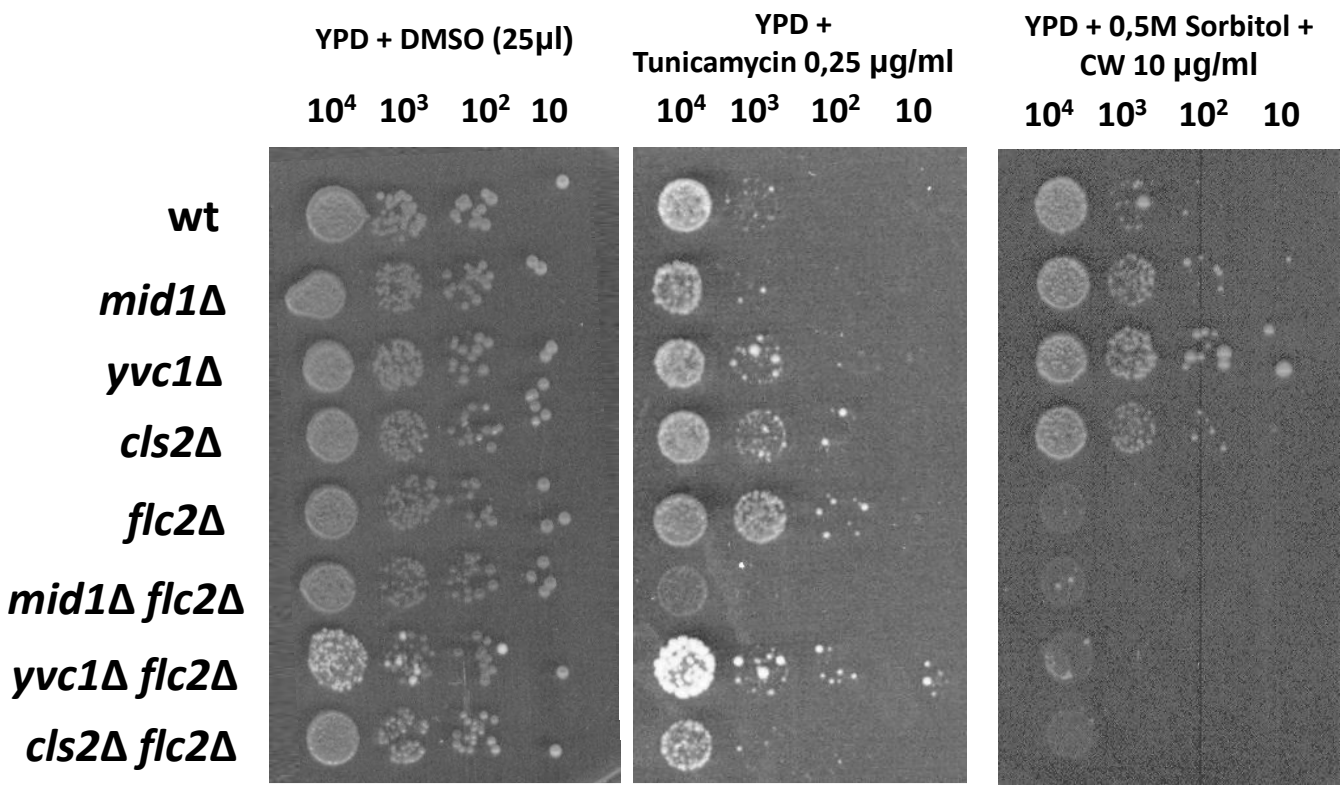


Figure 8A


YPD + DMSO (25µl)
YPD + Tunicamycin 0,25 µg/ml
YPD + 0,5M Sorbitol + CW 10 µg/ml
10⁴ 10³ 10² 10
10⁴ 10³ 10² 10
10⁴ 10³ 10² 10
wt
mid1Δ
yvc1Δ
cls2Δ
flc2Δ
mid1Δ flc2Δ
yvc1Δ flc2Δ
cls2Δ flc2Δ