UNIVERSITA' DEGLI STUDI DI MILANO-BICOCCA

Facoltà di Scienze Matematiche, Fisiche e Naturali
Dipartimento di Biotecnologie e Bioscienze
Dottorato in Biotecnologie Industriali XXVII ciclo

HYPOTONIC STRESS-INDUCED CALCIUM SIGNALLING IN *S. CEREVISIAE* INVOLVES A NEW FUNGAL SPECIFIC FAMILY OF TRP-LIKE TRANSPORTERS, HOMOLOGOUS TO HUMAN MUCOLIPIN AND POLYCYSTIC KIDNEY DISEASE RELATED (PKD2) CALCIUM CHANNELS

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Anno accademico 2014

Questa è sicuramente la parte più bella e difficile della tesi,

ringrazio il Prof. Martegani, per avermi dato la possibilità di lavorare nel suo laboratorio;

ringrazio infinitamente Fiorella e Renata, non solo per quello che mi hanno dato dal punto di vista lavorativo, ma soprattutto per quello che mi hanno regalato dal punto di vista personale. Non potevo sperare in nulla di meglio, mi mancherete!

Un grazie enorme anche a Loredana e Michela, anche grazie a voi alzarsi il lunedì per venire a lavorare era un atto più leggero!

Un pensiero speciale va anche a Silvia, che pur non essendoci stata in questi 3 anni è stata una parte integrante del nostro laboratorio, ed è parte integrante di questo lavoro!

Grazie anche a Sonia, Alessia, Serena e a tutte quelle persone che sono transitate nel nostro gruppo, chi per qualche mese o chi per più tempo, e che hanno dovuto in qualche modo sopportarmi.

Un grosso in bocca al lupo a Sara, che sta iniziando quello che io ho iniziato 5 anni fa!

Poi c'è anche Nic, un compagno eterno. Dopo quasi 9 anni pare che le nostre strade si divideranno (lavorativamente parlando), ma chissà, forse un giorno ci rincontreremo…dietro al banco del fresco dell'Esselunga magari.

Infine una dedica speciale a qualcuno che ancora non c'è, ma che già mi emoziona, e a una splendida donna che si sta impegnando per regalarmi qualcosa di unico, non vedo l'ora di conoscerti fra qualche mese!

AIM OF THE THESIS

The major goal of this work is to identify and characterize novel calcium transporters and to understand function and regulation mechanisms of calcium transport systems in *Saccharomyces cerevisiae*, in response to hypotonic shock.

Calcium serves many important and varied roles in all cell types, in particular in yeast this ion has an important role as a second messenger being involved in the regulation of many fundamental cellular processes, such as cell cycle, mating, glucose sensing, resistance to salt stress and cell survival.

The budding yeast actively maintains free cytosolic calcium concentration at extremely low levels, in a range of 50-200 nM, in spite of very steep gradients of this ion across the plasma membrane and across intracellular membranes. Different transporters located on the plasma membrane mediate calcium influx. Thethree identified systems responsible for Ca^{2+} influx from the extracellular environment are the High Affinity Calcium System (HACS), involving Mid1/Cch1 subunits, the low affinity system (LACS) involving Fig1 and the complex named GIC still unidentified at molecular level.

On the internal membranes we can find Yvc1, a vacuolar, mechanosensitive, calcium responsive transient receptor potential (TRP) channel, and Mid1, on the surface of endoplasmic reticulum.

S. cerevisiae cells growing in synthetic medium respond to hypotonic shock with a calcium pulse initially derived from intracellular stores and lately sustained by extracellular calcium. We tested the hypotonic shock induced calcium response in rich media, where it is clearly driven by two different component: a fast response (between 5 and 10 second after the stimulus), probably controlled by a transporter on the plasma membrane, which is dependent on extracellular calcium presence and which is inhibited by high concentrations of extracellular calcium, and a slow response (around 20-30 s after the stimulus), which is evident only after addition of EGTA, an extracellular Ca^{2+} chelator, suggesting a release from intracellular compartments.

Previous data on calcium-regulated genes suggested us to study *YOR365c* and its paralog *FLC2*, that is also homologous to *FLC1* and *FLC3*, encoding an endoplasmic reticulum protein previously reported as flavin carrier but also predicted as a homolog of *S. pombe* calcium transporter Pkd2.

Data based on our sequence alignments indicate that proteins of *FLC* family belong to a new fungal specific subfamily of TRP ion channels together with calcium related spray proteins and mucolipins.

By taking advantage of a bioluminescent assay in vivo allowing us to monitor cytosolic Calcium level changes, based on aequorin bioluminescent protein, the role of the known calcium channels and of the *FLC* family genes in hypotonic shock-dependent Ca^{2+} signalling, was here investigated.

Then, in order to better understand the role of *FLC* genes family in calcium homeostasis we tested the role of these proteins in cell wall integrity pathway, calcineurin activation and unfolded protein response.

INTRODUCTION

CALCIUM IN THE CELL

Calcium as a second messenger

Calcium serves many important and varied roles in all cell types. In mammalian non-excitatory cells, calcium acts as an important cellular signal and second messenger for a variety of signalling pathways, including secretion, transcriptional initiation and cell proliferation; in mammalian excitatory cells calcium can function as a trigger for muscle contraction, neurotransmitter release and signal propagation.
In yeast also, calcium plays a very important role as a second messenger being involved in the regulation of many fundamental cellular processes, such as cell cycle, mating, sensing of glucose, resistance to salt stress and cell survival.
The budding yeast actively maintains cytosolic free calcium concentration at extremely low levels, in a range of 50-200 nM, in spite of very steep gradients of this ion across the plasma membrane and intracellular membranes.
The major player in Ca^{2+} dependent signal transduction pathways is calcineurin, an eukaryotic Ca^{2+} and calmodulin dependent serine/threonine phosphatase involved in the regulation of a wide variety of cellular processes. Calcineurin in yeast is composed of two subunit: the regulatory subunit Cnb1 and the active subunit, encoded by *CNA1* and *CNA2* genes. When an increase of citoplasmatic calcium concentration occurs, the Ca^{2+}-calmodulin-calcineurin complex is formed and controls gene expression through the action of transcription factor Crz1.

Calcium homeostasis in *Saccharomyces cerevisiae*

Calcium is one of the most important second messengers in eukaryotic cells, where it plays essential roles in the regulation of several cellular functions, such as cell proliferation, muscle contraction, programmed cell death and nutrient sensing (Berridge *et al.,* 1998, 2000, 2003; Eilam and Othman, 1990; Eilam *et al.,* 1990; Putney, 2005; Tisi *et al.,* 2002). Many stimuli, such as membrane depolarization (Eilam and Chernichovsky, 1987), pheromone exposure (Iida *et al.,* 1990), glucose addition to derepressed cells (Nakajima-Shimada *et al.,*

1991), cell integrity response (Garrett-Engele *et al.,* 1995) or osmotic shocks(Batiza *et al.,* 1996; Bonilla and Cunningham, 2003; Cruz *et al.,* 2002; Denis and Cyert, 2002; Hirata *et al.,* 1995; Matsumoto *et al.,* 2002; Yu *et al.,* 2012a), cause a rapid and transient increase in cytosolic Ca^{2+} concentration in yeast cells, consisting both in an influx of Ca^{2+} from the extracellular environment and in its release from internal stores (Rispail *et al.,* 2009; Iida *et al.,* 1990; Kraus *et al.,* 2003; Burgoyne *et al.,* 2003; Groppi *et al.,* 2011). The yeast *Saccharomyces cerevisiae*, is able to maintain cytosolic [Ca^{2+}] in the range of 50–200 nM, even in the presence of environmental Ca^{2+}concentrations ranging from 1 µM to 100 mM, through an elaborate calcium homeostasis system (Bonilla & Cunningham, 2002; Cui & Kaandorp, 2006; Cui *et al.,* 2009). This intricate process is finely regulated in eukaryotic cells by the action on various pumps, channels and transporters located on both plasma and internal membrane.

In mammalians cells, calcium is mainly stored in endoplasmic reticulum (ER), that provides the most important source for a fast calcium mobilization(Berridge *et al.,* 2000). On the contrary, in yeast this role is accomplished by vacuole, even if correct calcium homeostasis in ER is very important for cell viability. More than 90% of total calcium in yeast is stored in the vacuole, albeit the free calcium concentration in this organel is quite low (30 µM); on the contrary, total Ca^{2+} concentration is estimated to be around 2-4 mM in rich medium growing cells, suggesting that calcium is probably stored as inorganic polyphosphate (Dunn *et al.,* 1994).Actually, Ca^{2+} vacuolar sequestration is the fungal physiological equivalent of detoxification by excretion into the extracellular space. Vacuolar Ca^{2+} uptake pathway is well known in yeast (Pittman, 2011): like in plants, two pumps are active for this purpose, a Ca^{2+}-ATPase and a Ca^{2+} exchanger, both able to restore basal Ca^{2+} level in the cytosol after a stimulus-induced Ca^{2+} raise, contributing to high Ca^{2+}concentration tolerance. Ca^{2+}-ATPases are high affinity Ca^{2+} transporters and can sense the smallest changes in cytosolic Ca^{2+}. They belong to the type II group of P-type ATPase superfamily (Brini *et al.,* 2012), which can be further separated into type IIA and type IIB Ca^{2+}-ATPases.

In the budding yeast *S. cerevisiae,* vacuolar Ca^{2+}-ATPase is codified by *PMC1*, whereas the Ca^{2+} exchanger is codified by the gene VCX1.

Albeit yeast Pmc1 is closely related to mammalian PMCA (plasma membrane Ca^{2+}-ATPase) family, it localizes on the vacuolar membrane; furthermore, Pmc1 lacks the domains necessary for regulation by calmodulin and by acidic

phospholipids typical of higher eukaryotes PMCA. Instead Pmc1 is regulated by calcium at transcriptional level by the action of Crz1 (Matheos *et al.,* 1997; Stathopoulos and Cyert, 1997), a transcriptional factor which is traslocated into the nucleus when it is dephosphorylated by calcineurin, theCa^{2+}/calmodulin-activated protein phosphatase (Boustany and Cyert, 2002; Cyert, 2003). Although Pmc1 is a high affinity transporter, it has anyway a quite low affinity for calcium if compared to other PMCAs (Takita *et al.,* 2001).

Yeast mutant strains impaired in Pmc1 activity show a reduction in Ca^{2+} uptake in the vacuole of more than 80% of the wild-type rate during growth in standard medium, and also display a severe sensitivity to high Ca^{2+} concentrations in the growth medium (Cunningham and Fink, 1994).

On the contrary, Ca^{2+} exchangers have low affinity and high capacity for Ca^{2+} and can efficiently transport Ca^{2+} when its cytosolic concentration is too high (Miseta *et al.,* 1999). They use the energy derived by the counter exchange of another cation (H^+ for the yeast or plant) for the transport of Ca^{2+}. Indeed, the strong acidification of yeast vacuoles through a V type H^+-ATPase provide a large proton gradient which can be efficiently exploited in Ca^{2+} uptake (Dunn *et al.,* 1994).

The role of Ca^{2+}/H^+ antiporter in yeast is accomplished by Vcx1, a vacuolar CAX (Ca^{2+}/H^+ exchanger), members of the CaCA (Ca^{2+}/cation antiporter) superfamily (Cai and Lytton, 2004) identified for its ability to confer resistance to high concentration of both calcium and manganese when overexpressed.

Vcx1 protein was biochemically characterized (Miseta *et al.,* 1999; Pozos *et al.,* 1996)as a high capacity transporter but with a lower affinity for Ca^{2+} if compared to Pmc1 (Forster and Kane, 2000; Miseta *et al.,* 1999).

Even if it was predicted that Vcx1 was responsible of the major Ca^{2+} transport into vacuole, its deletion causes neither a reduction of total calcium concentration in this organel nor an increased sensitivity to high extracellular calcium concentrations (Cunningham, 2011).

Opposite to Pmc1, whose expression is activated by calcineurin, Vcx1 is inhibited by calcineurin (Cunningham and Fink, 1994a; Pozos *et al.,* 1996), probably post-translationally (Kingsbury and Cunningham, 2000), in order to avoid an oversequestration of Ca^{2+} into the vacuole.

Vacuolar Ca^{2+} can be released in a regulated manner through the action of the vacuolar channel Yvc1, the only Transient receptor potential (TRP) channel identified hitherto in budding yeast, spanning the conserved

motif $_{448}$VILLNILIALY$_{458}$ which is conserved in the most mammalian TRPs channels (Chang *et al.,* 2010).TRP channels are conserved in mammals, flies, worms, yeast but not in plants (Venkatachalam and Montell, 2007)and they are composed of six transmembrane domains (TMs) probably working as tetramers (Denis and Cyert, 2002; Palmer *et al.,* 2001; Zhou *et al.,* 2003).

Yvc1 was identified by electrophysiological studies on isolated *S. cerevisiae* vacuolar membrane. Yvc1 is a voltage gated channel and its activity is strongly linked to the cytosolic Ca^{2+} concentration, in a process commonly known as Ca^{2+}-induced Ca^{2+} release, and seems to be also activated by other cations such as magnesium and manganese (Groppi *et al.,* 2011; R. Tisi, unpublished results). The activity of Yvc1 in vitro is known to depend on a high and un-physiological calcium concentration, whereas if reducing agents are present in the cytosolic side, in order to recreate the strong reducing environment present in the cell, the calcium concentration needed for Yvc1 opening is lower (Palmer *et al.,* 2001).

This Ca^{2+}-dependent regulation of channel activity can be explained by the presence of a cluster of four acidic residues ($_{573}$DDDD$_{576}$) in the C-terminus of Yvc1, that can be important for direct calcium binding(Su *et al.,* 2009).

Yvc1 mediates calcium release after hyperosmotic shock (Denis and Cyert, 2002) thanks to a mechanical activation: calcium efflux induced by hyperosmolarity is independent from cytosolic calcium concentration but is directly due to a deformation of vacuolar membrane (Su *et al.,* 2011; Zhou *et al.,* 2003). Recent studies also suggest that Yvc1 is an important component of the signal transduction pathway activated in response to glucose addition leading to a raise in Ca^{2+} concentration and the activation of plasma membrane H^{+}-ATPase (Bouillet *et al.,* 2012).

Even if the vacuole is the most important calcium store, the Golgi apparatus and the ER are also fundamental for maintaining proper intracellular Ca^{2+} homeostasis in yeast, which is also required in order to ensure the proper folding and processing of proteins transported through the secretory pathway.

Pmr1 (for Plasma membrane ATPase related) is a member of SPCA family (Secretory Pathway Ca^{2+} ATPases) localized on the Golgi membrane, where it plays an important role in the maintenance of luminal calcium concentration required for proper protein glycosylation (Rudolph *et al.,* 1989; Antebi and Fink, 1992; Halachmi and Eilam, 1996; Strayle *et al.,* 1999).

A strain carrying the deletion of *PMR1* shows typical phenotypes that can be linked to a misregulation of calcium homeostasis such as increased Ca^{2+} uptake from the extracellular environment and a greater sensitivity to elevated extracellular calcium levels. This increase in calcium uptake is mediated by specific calcium transporters located on the plasma membrane; this behaviour is similar to mammalian capacitative calcium entry (CCE) response generated by a signal caused by efflux of calcium from ER causing store depletion (Aiello *et al.,* 2004).

Pmr1 also seems to have a marginal role in controlling calcium concentration in ER lumen that is reduced in *pmr1* mutant strain. However, ER seems to play a less important role in calcium storage if compared to mammalian cells, since lumenal free calcium concentration was estimated to be 10µM, a very low level if compared to free calcium concentration in mammalian ER (100-400µM) (Strayle *et al.,* 1999).

It is most likely that yeast ER is not important for calcium storage but may play an important role as a dynamic actor involved, together with Golgi, in calcium signalling and homeostasis.

Despite its marginal role in calcium storage a putative calcium transporter has been identified in yeast on ER membrane. CLS2/CGS2 was identified in a screening for mutants with a high sensitivity to extracellular calcium concentration in order to find new genes involved in calcium homeostasis (Beeler *et al.,* 1994).

Cls2 is a hydrophobic protein characterised by the presence of an EF-hand type domain, in the cytoplasmic loop, that is able to bind Ca^{2+}.

Even if it was proposed a role for Cls2 as a calcium channel (Tanida *et al.,* 1996) recent data demonstrated that it is required for mannosylation of inositolphosphorylceramide and for protein abundance increases in response to DNA replication stress (Tkach *et al.,* 2012; Stock *et al.,* 2000). Its role in calcium homeostasis is still to be cleared.

Calcium passive transport in *Saccharomyces cerevisiae* (HACS, LACS, and GIC)

Conversely, to mammalian cells, where cytoplasmatic calcium raise depends on release of Ca^{2+} from the ER, yeast calcium signalling is mostly controlled by calcium influx from extracellular environment towards plasma membrane.

In budding yeast, the best characterised calcium transporter located on the plasma membrane is HACS (High Affinity Calcium System), composed by Mid1 and Cch1; recently also Ecm7, a member of PMP-22/EMP/MP20/Claudin superfamily of transmembrane proteins, was proposed as a component of HACS (Ding *et al.,* 2013; Martin *et al.,* 2011).

HACS is normally inhibited by calcineurin in rich medium, but it is fully functional in synthetic media. It can be activated by several stimuli like pheromones (Iida *et al.,* 1990; Nakajima-Shimada *et al.,* 2000), hypotonic shock (Batiza *et al.,* 1996), salt stress (Matsumoto *et al.,* 2002; Peiter *et al.,* 2005), glucose addition (Tökés-Füzesi *et al.,* 2002) or depletion of calcium stores (Locke *et al.,* 2000).

High affinity calcium system was first identified in a study on pheromone induced calcium signalling. *S. cerevisiae* can exist both in a haploid or diploid form; haploid cells can be of two different mating types, a and α, and can conjugate generating a diploid cell. In order to find the correct mating type for conjugation haploid cells produce a peptidic pheromone called a and α factors secreted by a and α mating type cells respectively.

The presence of the pheromone induces a great variety of responses in yeast cells with opposite mating type: changes in gene expression, exposition of agglutinine on cellular surface to facilitate cells anchorage and arrest of cells in G1 phase of cell cycle.In the latest stages of conjugation yeast cells also undergo the development of a particular cellular morphology, called shmoos, characterised by the presence of one or more projections on their surface with the aim of facilitate the contact between cells.

It is known that pheromone exposure causes an increase in cytosolic free calcium concentration in the late stage of conjugation (Iida *et al.,* 1990 Nakajima-Shimada *et al.,* 2000). This signal is necessary to maintain viability of cells during this stage and it is generated by an influx of Ca^{2+} from extracellular environment (Iida *et al.,* 1990).

MID1 gene product is a stretch-activated, Ca^{2+}-permeable channel, component of HACS required for Ca^{2+} influx and for the maintenance of viability of cells exposed to the mating pheromone. When exposed to pheromones, cells of the *mid1* mutant will die because of low Ca^{2+} uptake, showing that the Mid1 protein has a crucial role in supplying Ca^{2+} during the mating process (Iida *et al.,* 1990). Mid1 localize on plasma membrane and ER (Ozeki-Miyawak *et al.,* 2005) and is composed of 548-amino-acid residues with four hydrophobic region.

Moreover, the amino acid sequence of two of its putative transmembrane segments shows a strong similarity to those of the membrane-spanning domains of voltage-sensitive ion channels (Kanzaki *et al.,* 1999).

Mid1 had been previously described as having no animal homologs (Iida *et al.,* 1994), and the similarity was weak, but several bioinformatics-based validations support a one-to-one homologous relationship of the insect and fungal proteins with Mid1 domains (Ghezzi *et al.,* 2014)

CCH1 is a gene, conserved in fungi, that has been discovered thanks to Yeast genome project gene and encodes for a homolog of the pore-forming α_1 subunit of mammalian voltage-gated calcium channels. Voltage-gated calcium channels (VGCCs) in animals sense a membrane potential change and drive a transient and rapid increase in cytosolic free Ca^{2+}concentrations (Catterall, 2000). VGCCs are composed of the pore-forming subunit α_1 and auxiliary subunits α_2/δ, β and γ, which play prominent roles in the function of the α_1 subunit (Catterall *et al.,* 2005; Jarvis, Zamponi, 2007). Like the animal α_1 subunit, Cch1 contains four structurally similar domains (I–IV), each of which has six transmembrane (TM) segments (S1–S6) that tetramerize to form the core of the Ca^{2+} channel (Fig. 1). In calcium channels, a Ca^{2+}-binding site that contributes to ionic selectivity is formed by four acidic residues (EEEE), one from the selectivity filter region of each of the four domains. Cch1 has a similarly placed acidic motif but with three, rather than four (Cibulsky and Sather, 2000; Catterall, 2000). The fungal homologues of have several regularly spaced basic residues in the TM4 region of each domain. This suggests that these regions may act as voltage sensors similar to those of mammalian voltage gateg calcium channels (Catterall, 2000; Prole and Taylor, 2012)

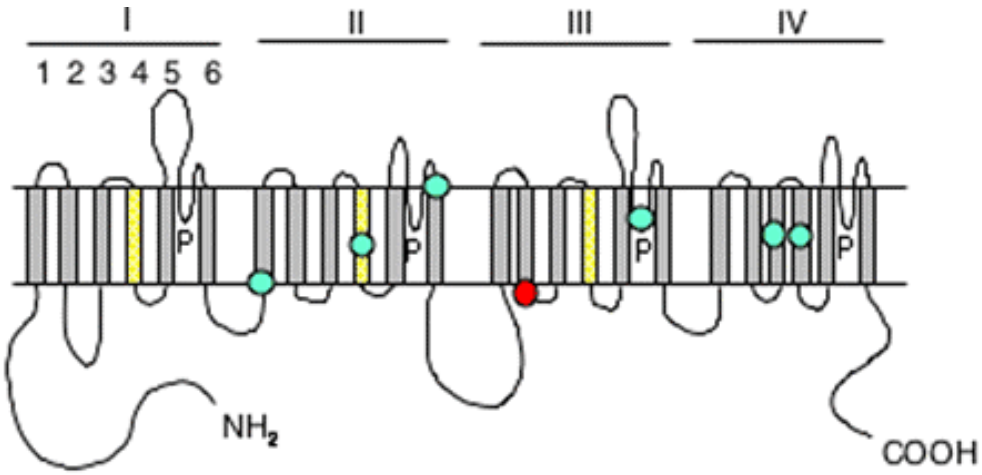


Fig. 1: *Structure of the Cch1 protein (I-IV represent putative domains and 1-6 putative transmembrane segments)* (Iida *et al.,* 2007).

Mid1 and Cch1 seems to cooperate in the same pathway since the deletion of this genes, either alone or together, results in an identical decrease in cell viability after pheromone exposure (Paidhungat, Garrett, 1997; Fischer *et al.,* 1997)

As a confirm of the effective cooperation of these two proteins, the overexpression of both *MID1* and *CCH1*, but not single overexpression is able to increase calcium influx activity (Iida *et al.,* 2007).

ECM7 is a gene identified thanks to a screening aimed to find knockout mutants that showed abnormal uptake of Ca^{2+} after exposure to mating pheromone or to tunicamycin. Ecm7 was proposed as a subunit of HACS; in fact it physically interacts with Mid1/Cch1 and in non signalling condition it's stabilised by Mid1 (Martin *et al.,* 2011).

HACS is also responsible for the activation of a CEE-like mechanism. The depletion of calcium storage, observed for example in a strain with impaired activity of Ca^{2+} pump Pmr1, causes a raise in intracellular Ca^{2+}concentration that is dependent on HACS activity (Locke *et al.,* 2000).

Again, growth of mutants in the Mid1/Cch1/Ecm7 complex is reported to be hypersensitive to low temperature and to high iron concentration in the medium. Both phenotypes were partially suppressed by high Ca^{2+}concentration

suggesting the presence of an alternative unidentified Ca^{2+} influx pathway independent on HACS activity (Peiter *et al.,* 2005).

The most famous and industrially important characteristic of *Saccharomyces cerevisiae* is its ability to produce ethanol as a metabolic product and to survive high concentrations of this toxic compound. Exposure to ethanol above 15% causes immediate inhibition of growth and metabolism, and may result in death, but calcium ions seems to be protective for concentrations of ethanol in the range of 8-10%.Ca^{2+} fluxes in yeast have been observed after cells exposure to ethanol: the increase in cytosolic Ca^{2+} level is rapid and is dependent on HACS. In fact, *mid1Δ* and *cch1Δ* strains accumulate significantly lower levels of Ca^{2+} after ethanol exposure, compared to wild-type strains, reducing their Ca^{2+} mobilization by more than 90% (Courchesne *et al.,* 2011).

During the mating process of yeast cells, two distinct Ca^{2+} influx pathways seems to become activated. In fact, when pheromone-triggered Ca^{2+} signal was observed in rich medium-growing cells, mutant strains impaired in HACS activity showed no decrease in survival relative to wild-type. In these conditions, a calcium influx is observed that is not dependent from HACS, and the apparent K_M estimated for calcium in strains carrying the deletion of both *MID1* and *CCH1* was in the range of 3mM, suggesting the presence of a calcium transporter with a low affinity for calcium. This system was named LACS (Low affinity calcium system) and involves the role of Fig1 (Muller *et al.,* 2001).

Fig1 is an N-glycosylated protein that localizes to the plasma membrane via its four predicted transmembrane helices (TM). Cells impaired in Fig1 activity displayed a cell fusion defect, which can be suppressed by high Ca^{2+} conditions. These cells mated at rates similar to wild-type strains suggesting that Fig1 does not solve any important role in cell cycle arrest after pheromone exposure even if this increases*FIG1* expression (Muller *et al.,* 2003).

Fig1 does not resemble any known calcium channels, but it seems to work as a regulative subunit of LACS and may act like connexins or tetraspanins in animals. Connexins are a superfamily of four transmembrane segments-containing proteins that function as Ca^{2+} channels at GAP junctions, sites of cell-cell contact and communication in higher eukaryotes, whereas tetraspanins are unrelated to connexins, but also contain four transmembrane segments and are well known to associate with integrins, receptors and signalling complexes to coordinate processes such as fertilization. By the way, the role of both LACS and Fig1 remains to be better investigated especially at the molecular level.

Recently Groppi and co-workers proposed the existence of a still unidentified calcium channel on the plasma membrane that control calcium influx from extracellular environment when glucose is added to derepressed cells. This flux also involves the activity of HACS system, but in yeast cells growing in rich media it is implemented by a channel, named GIC (Glucose Induced Calcium) channel, that binds calcium with an apparent K_M of 43.8 ± 10.3 μM.

Even if GIC transporter has not been characterised at molecular level it has been well characterised at pharmacological level: this channel is rather resistant to nickel and to verapamil, which even acts as an agonist at high concentration. Conversely, GIC channel is sensitive to magnesium, with an apparent IC_{50} of 1.04 ± 0.05 mM, to gadolinium (apparent IC_{50} 16.7 ± 1.7 μM) and to nifedipine (apparent IC_{50} 0.36 ± 0.13 μM).

GIC transporter is very sensitive to nutrients since it is active only in rich media. HACS system was already reported to be negatively regulated by calcineurin in rich media while GIC transporter seems to be positively regulated by calcineurin in these cultural conditions (Groppi *et al.,* 2011). Surprisingly this regulation seems not a transcriptional control, since is not dependent on Crz1 (Rigamonti and Tisi, unpublished data).

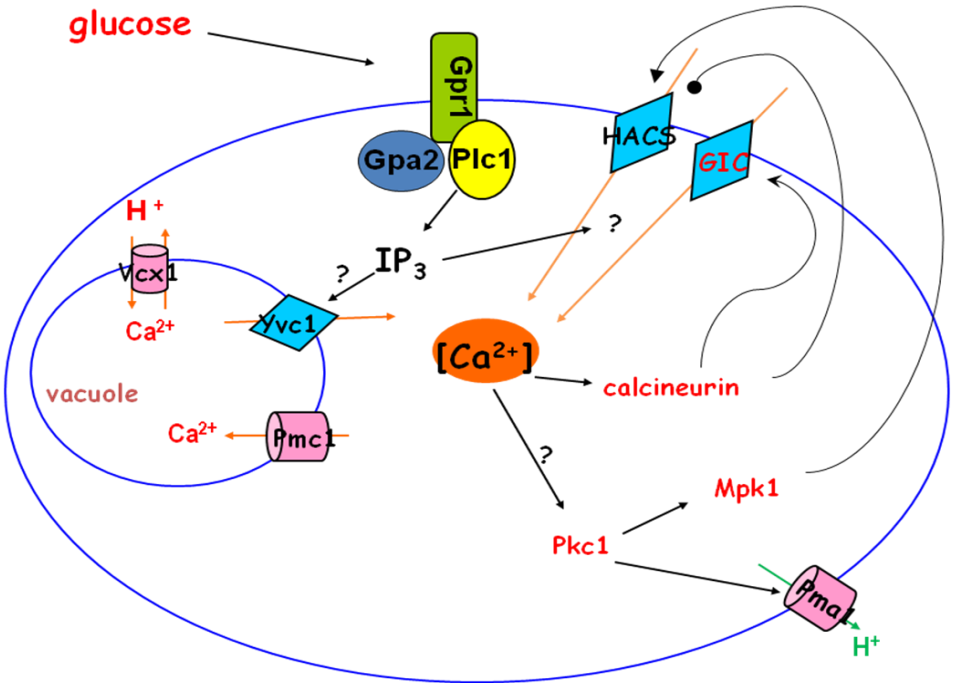


Fig. 2: *Glucose-induced calcium signalling pathway*

Hypotonic Shock

The cell membrane of virtually all organisms is highly permeable to water, and hence the cell volume will be determined by the cellular content of osmotically active solutes and by osmolarity of extracellular environment. Cellular swelling occurs in response to a hypotonic shock and all cells seem to have the capacity to counteract this swelling with a mechanism that regulates the intracellular concentration of some ions and organic molecules called osmolytes. These osmolytes usually are K^+, Na^+, Cl^-, free aminoacids and quaternary ammonium compounds, but also Ca^{2+} solves a very important role in the regulating volume decrease responses to hypotonic shock.
A rapid and transient peak of intracellular calcium concentration was observed both in epithelial and endothelial cells challenged with hypotonic shock.

Additionally, this increase of calcium concentration in human umbilical vein endothelial cells was found to derive initially from intracellular sources whose depletion potentiates an intake of extracellular Ca^{2+} (Rothstein *et al.,* 1990; Bagnasco *et al.,* 1993; Oike *et al.,* 1994).

However, the origin of calcium rise and its regulatory effect controlled by hypotonic shock seems to be tissue specific. Since cellular swelling is a strong mechanic perturbation, stretch-activated channels (SACs) have been proposed to be responsible for ion flux after hypotonic shock (Hoffmann *et al.,* 1989; Pierce *et al.,* 1990; Oike *et al.,* 1994; Guharay *et al.,* 1984; Christensen, 1987).

Skeletal muscle, for example, swells as a consequence of intense activity and gradually returns to its original volume after cessation of exercise (Sjogaard *et al.,* 1985; Peeze Binkhorst *et al.,* 1990; Watson *et al.,* 1993; Raja *et al.,* 2006). Swelling occurs due to an accumulation of metabolites within the cytosol and a consequent increase in osmolarity, which drives the entry of water from the extracellular space (Nagesser *et al.,* 1992; Sejersted and Sjogaard, 2000; Lannergren *et al.,* 2002; Raja *et al.,* 2006). Changes in skeletal muscle volume induce localized sarcoplasmic reticulum Ca^{2+} release events, which are sustained for many minutes, suggesting a possible signaling role in plasticity or pathology.

TRPC5, a poorly characterized calcium channel widely expressed in the central and peripheral nervous system, has been recently proposed as a potential osmosensory protein. This TRP channel is activated by swelling and pressure-induced membrane stretch. The calcium raise activated by hypotonic stimulation is dependent on extracellular Ca^{2+} and is also prevented by intracellular Ca^{2+}buffering, suggesting that Ca^{2+} elevation and basal Ca^{2+} levels are needed to sustain activation of the channel (Gomis *et al.,* 2008).

The budding yeast *S. cerevisiae* in synthetic medium responds to hypotonic shock with a calcium pulse initially derived from intracellular stores and lately sustained by extracellular calcium. In fact, the extracellular calcium chelator BAPTA only affects the later stages calcium response of yeast cells to the hypotonic treatments (Batiza *et al.,* 1996).

This particular stimulus is reported to activate the Pkc1/MAP kinase pathway in yeast (Kamada *et al.,* 1995; Davenport *et al.,* 1995). Surprisingly the intensity of the shock positively regulates both the phosphorylation level of Mpk1 MAP kinase (Davenport *et al.,* 1995) and the intensity of calcium peak (Batiza *et al.,* 1996), suggesting a possible role of transient calcium raise in the activation of

PKC1 MAP kinase pathway, although direct activation of yeast Pkc1 by calcium is still under discussion.

Batiza and coworkers suggested that transient calcium raise in response to hypotonic shock is controlled by a stretch activated channel (SACs), since the signal is strongly inhibited by gadolinium (Gd^{3+}), a trivalent ion of the lanthanide series, which has a high charge density, a similar ionic radius to Ca^{2+} and is able to block SACs activation (Bourne *et al.,* 1982).

It has also been reported that hypotonic shock induced calcium response is dependent on carbon source, suggesting that, even in this case, the whole transport pathway could be differentially regulated in rich or minimum and synthetic media (Batiza *et al.,* 1996).

CALCINEURIN

Structure and function

Calcineurin, also called protein phosphatase 2B (PP2B), is a serine/threonine protein phosphatase very important in the coupling of calcium signalling with downstream pathways, in order to regulate various cellular responses. In addition to calcineurin, the serine/threonine protein phosphatase family members include protein phosphatases 1 (PP1), 2A (PP2A), and 2C (PP2C), phosphatases essential for a large number of signal transduction pathways in eukaryotic cells (Cohen *et al.,* 1989; Shenolikar *et al.,* 1991). These proteins differs one from the other for their substrate specificity, sensitivity to various inhibitors and also in divalent metal ions that are required for their activity; e.g., only PP2B class needs calcium for activation.

Calcineurin sequence is highly conserved in all the living organisms. This holoenzyme is composed of two polypeptides: the active part of the complex is the A subunit, which, in mammals, is 57–59 kDa, depending on the isoform. The size of the catalytic subunit can be up to 20% larger in lower eukaryotic species (Cyert *et al.,* 1991; Liu *et al.,* 1991; Ye *et al.,* 1992).

Nevertheless, calcineurin is strictly conserved through all eukaryotic organisms since all calcineurin A genes codify for a protein characterized by the presence of a catalytic domain, homologous to other Ser/Thr protein phosphatases, and three regulatory domain at the C terminus of the polypeptide (Fig. 3).

These domains are: the calcineurin B binding domain (CNB) (Clipstone *et al.,* 1994; Guerini *et al.,* 1992; Husi *et al.,* 1994; Sikkink *et al.,* 1995; Watanabe *et al.,* 1995), the calmodulin binding domain (CaM) (Guerini *et al.,* 1992; Kinkaid *et al.,* 1988) and an autoinhibitory domain that blocks the active domain in absence of calcium and calmodulin complex, inhibiting the enzyme (Hashimoto *et al.,* 1990; Kissinger *et al.,* 1995; Perrino *et al.,* 1995) .

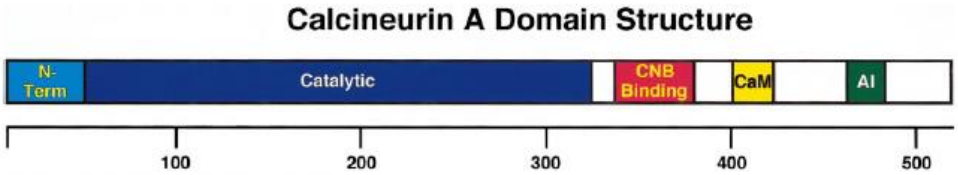


Fig. 3: *structure of Calcineurin A.*

The calcineurin B subunit is highly conserved throughout evolution. The calcineurin B of mammalian cells is encoded by two different genes, showing 86% amino acid sequence identity with insect calcineurin B (*Drosophila*) and 54% identity with calcineurin B from *S. cerevisiae*. These two calcineurin B genes are differently expressed: one is ubiquitarious and one is found only in testes. Calcineurin B gene in mammals encodes a protein of 170 amino acids containing four EF-hand motifs necessary for calcium binding (Aitken *et al.,* 1984).

The four EF-hand motifs in calcineurin do not have the same affinity for calcium: the one localized in the C-terminus of the protein has the higher Ca^{2+} affinity, while Ca^{2+} binding at the others sites is likely to be structural and less avid (Feng *et al.,* 1999).

Some of the most interesting work to investigate biological roles of calcineurin have been made using *S. cerevisiae* as a model system. The catalytic subunit of calcineurin in *S. cerevisiae* is encoded by two genes named *CNA1* and *CNA2* while, for the B subunit, only one gene is present (*CNB1*).

Most of the structural features of the mammalian calcineurin catalytic subunit are conserved in Cna1 and Cna2, even if yeast subunits are larger than in mammals, displaying a molecular mass of 63 and 69 kDa respectively. In addition, Cnb1, a 16kDa peptide, is a Ca^{2+}-binding protein carrying four Ca^{2+}-binding EF-motifs, which shows an aminoacidic identity of 56% with mammalian regulatory subunit of calcineurin.

The activity of calcineurin can be highly inhibited by a great number of molecules. The most powerful, specific and well-known inhibitors are the immunosuppressant drugs cyclosporin A and FK506, which inhibit calcineurin when complexed with their respective cytoplasmic receptors cyclophilin and FKBP (Rusnak and Mertz, 2000).

Calcineurin calcium binding cofactor: Calmodulin

Calmodulin, a small, ubiquitous Ca^{2+}-binding protein, regulates a wide variety of proteins and processes in all eukaryotes. *CMD1*, the single gene encoding calmodulin in *S. cerevisiae*, is essential and modulates mitosis, through its regulation of components of the spindle pole body, and in bud growth, by binding myosin required for polarized secretion. *S. cerevisiae* contains a single

calmodulin gene that is required for viability and encodes a protein 60% identical to vertebrate calmodulins (Davis, 1986).

Calmodulin serves as a major intracellular Ca^{2+}receptor and mediates many of the effects of this ion. At resting levels of Ca^{2+}, calmodulin exists in the Ca^{2+}-free, or apo-calmodulin, form. In response to a Ca^{2+}signal, calmodulin binds $Ca^{2+}$and consequently undergoes a conformational change that allows it to bind to and activate a great variety of target enzymes.

The mechanism of Ca^{2+}/calmodulin-dependent regulation of target enzymes has been characterized extensively through in vitro biochemical and structural analyses. Calmodulin contains four copies of a Ca^{2+}-binding motif known as EF-hand, each of which binds one Ca^{2+}ion. An EF-hand is made up of a 12-residues Ca^{2+}-binding loop, flanked by two α-helices and, within the loop, $Ca^{2+}$is coordinated by oxygens on six different amino acid residues (Kretsinger, 1980). EF-hand–containing proteins typically undergo a structural change upon bindingCa^{2+}; however, this conformational change differs substantially for each class of EF-hand proteins. Structural analyses have established that calmodulin is a dumbbell-shaped molecule with two similar domains, each containing two EF-hand Ca^{2+}-binding motifs (Fig. 4).

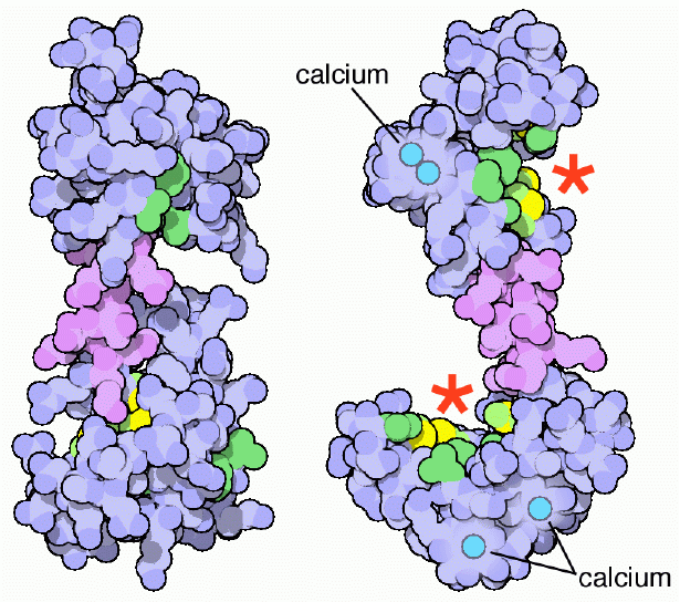


Fig. 4: *3D structure of calmodulin (PDB)*

In the absence of Ca^{2+}, the EF-hands are in a "closed" conformation. This Ca^{2+}-free form of calmodulin is able to bind to a subset of target proteins. Ca^{2+}binding causes a change to an "open" conformation, which also results in exposure of two hydrophobic surfaces that allow calmodulin to bind to its Ca^{2+}-dependent target proteins (Yap *et al.,* 1999).
The primary structure of yeast calmodulin is similar to its vertebrate counterpart, having four predicted helix-loop-helix EF-hand domains distributed similarly in the protein sequence. However, there are significant differences in the structure and Ca^{2+}-binding properties of yeast and vertebrate calmodulins: vertebrate calmodulin binds four molecules of Ca^{2+}, whereas yeast calmodulin binds a maximum of three (Luan *et al.,* 1987; Matsuura *et al.,* 1991; Starovasnik *et al.,* 1993).
Examination of conditional calmodulin mutants identified multiple distinct essential functions for this protein in vivo: inactivation of calmodulin causes an accumulation of cells with doubled DNA and with defects in spindle and bud morphology (Davis., 1992; Sun *et al.,* 1992). Again, these mutants show defects in mitosis, bud emergence and actin localization. Indeed, calmodulin localizes to sites of bud formation, bud tips, and the bud neck in vivo and this cellular distribution overlaps in part with that of actin patches and reflects calmodulin role in polarized growth (Brockerhoff *et al.,* 1992; Spang *et al.,* 1996).

Calcineurin and conjugation

Haploid cells of *S. cerevisiae* produce one of two mating pheromones, a-factor and α-factor. Exposure of haploid strains to the opposite mating pheromone prepares cells for mating by inducing cell cycle arrest in G1. This is mediated by a complex signal transduction pathway involving the activation of calcineurin, following an increase in citoplasmatic calcium (Buttini *et al.,* 1995; Heitman *et al.,* 1993).
Escape from α-factor-induced cell cycle arrest involves three metabolic processes that have been referred as recovery, adaptation, and survival (Moser *et al.,* 1996). We talk about recovery when cells are able to resume growth after removal of the pheromone, whereas adaptation involves the capacity of cells to resume growth even in presence of pheromone; on the contrary, for survival we mean the capacity of cells to remain viable after α-factor exposure.

Strains carrying the deletion of both calcineurin active subunit are viable, but fail to recover from α-factor-induced growth arrest. Cells containing a single *CNA1* or *CNA2* gene are twice as sensitive as wild type to α-factor-induced growth arrest, whereas the double mutant *CNA1/CNA2* is four times as sensitive (Cyert *et al.,* 1991-1992; Wang *et al.,* 1995). Furthermore, once arrested, the double mutant failed to resume growth. In contrast, the *cnb1* mutant does not show any increased sensitivity compared with wild type, but it fail to recover from growth arrest as well as the *cna1*/*cna2* double mutant, confirming a clear role of calcineurin in the regulation of this phenomena. Again, also the levels of the transcripts of both the *CNB1* and the *CNA2* genes increased significantly after cells are exposed to pheromone (Cyert and Thorner, 1992).
These results taken together suggest that calcineurin is involved in the pathway that controls yeast cells response to mating pheromone exposure by inducing cell cycle arrest, in agreement with the previously described role for Ca^{2+} influx in the late stages of the mating pathway and for survival after exposure to α-factor.

Calcineurin controls homeostasis of calcium and other ions.

Calcineurin is responsible for calcium homeostasis in yeast and mammalian cells by playing an essential role in regulation of calcium channels, pumps, and exchangers. These proteins actively maintain cytoplasmic Ca^{2+} in the range of 100–300 nM (Cunningham *et al.,* 1994), while other ion transporters indirectly influence intracellular calcium.
A clear example of a calcineurin-regulated transporter is the vacuolar H^+-ATPase, encoded by *VCX1* gene, which allows the calcium sequestration in the vacuole by taking advantage of the high H^+ gradient between the organel and cytosol (Garrett-Engele *et al.,* 1995; Hemenway *et al.,* 1995; Tanida *et al.,* 1995).
The depletion of calcium from cytosol is also ensured by the action of two Ca^{2+}-ATPases, Pmc1 and Pmr1, respectively localized on vacuole (Cunningham *et al.,* 1994) and on Golgi membranes. The latter also plays a very important role in the secretory pathway (Rudolph *et al.,* 1989). Strains carrying the deletion of either *PMR1* or *PMC1* cannot grow in media containing a high concentration of calcium. Anyway, the growth can be restored by the addition of cyclosporine A

or FK506, two molecules able to block calcineurin. The mechanism by which external Ca^{2+} inhibits the growth of *pmc1* and *pmr1* mutants was then investigated by characterizing spontaneous revertants able to proliferate in high Ca^{2+} media: the identified revertants carried additional mutations in *CNB1*, *CNA1* and *CNA2* genes indicating a clear involvement of calcineurin activity (Cunningham and Fink, 1994). The large increase of calcium sequestration observed in *pmc1cnb1* double mutant strain seems to be dependent on Vcx1p activity, since *vcx1 pmc1cnb1* triple mutants only accumulated low levels of calcium in the internal compartments.

These results indicate that calcineurin activity can positively regulate Pmc1 and Pmr1 while it inhibits Vcx1 function, probably in a post-translational manner (Cunningham and Fink, 1995).On the contrary, calcineurin leads to the activation of Pmr1 and Pmc1 by regulating the transcription of these two genes through the activation of calcineurin-dependent transcriptional factor Crz1 (Matheos *et al.,* 1997; Stathopoulos *et al.,* 1997).

Calcineurin-deficient cells show high sensitivity to monovalent cations Na^+ and Li^+, but not to bivalent cations K^{2+}, Ca^{2+} and Mg^{2+} (Mendoza *et al.,* 1994; Nakamura *et al.,* 1993). Tolerance mechanism to high salt stress requires the presence of Pmr2, a plasma membrane Na^+-ATPase that control Na^+ and Li^+ efflux. The deletion of *CNB1* and the consequent inactivation of calcineurin function allow an accumulation of both sodium and lithium due to a decreased expression of Pmr2 (Mendoza *et al.,* 1994). The activity of Pmr2 is also stimulated by Ca^{2+}/calmodulin, resulting in both transcriptional and posttranslational regulation of Na^+ efflux mediated by Ca^{2+} (Rudolph *et al.,* 1989; Weiland *et al.,* 1995).

In addition, calcineurin has been shown to regulate the high/low-affinity state of the plasma membrane K^+ channel, Trk1.Cells lacking *CNB1*are unable to convert the K^+ transport system (Trk1) to a high-affinity state. In the high-affinity state, this pump has increased affinity for K^+, but the K_M for Na^+ and Li^+ is not affected, resulting in increased K^+ uptake. The mechanism of this regulation has been hypothesized to be direct or indirect dephosphorylation of Trk1 by calcineurin (Mendoza *et al.,* 1994).

Other roles of Calcineurin inside the cell

Calcineurin is responsible for transcriptional regulation of *FKS2*, one of two genes encoding β-1,3-glucan synthase (Eng *et al.,* 1994; Zhao *et al.,* 1998). The Fks1 protein is the predominant synthase expressed during optimum growth, but expression of Fks2 is induced upon treatment of cells with mating pheromone, high Ca^{2+}, or growth on poor carbon sources and is actively controlled by calcineurin. Deletion of the *FKS1* and *CNB1* genes results in lethality due to the inability to induce *FKS2* (Garrett-Engele *et al.,* 1995).Again the defect in Pkc1-Mpk1/Slt2 MAPK cascade in a*cnb1Δ* strain, suggests that calcineurin plays a role in regulating cell wall structure.
Beside the roles described above, calcineurin also plays a pivotal role in cell cycle progression. Calcium is known to regulate mainly G_1 events in mammalian cells, while in yeast this ion seems to be fundamental mainly for G_2/M transition (Iida *et al.,* 1990). Calcineurin appears to up-regulate Swe1, an inhibitor of Cdc28/Clb complex, both at a transcriptional and post-transcriptional level, by dephosphorylating Hsl1, a Swe1inhibitory kinase, and deactivating Zds1, a transcriptional repressor of *SWE1*. Again, calcineurin promotes Swe1 accumulation by down-regulating the proteasome activity through the action on Yap1 transcription factor. Yap1 level really diminished in presence of external $CaCl_2$ in a calcineurin-dependent manner, through a direct dephosphorylation (Miyakawa and Mizunuma, 2007). Calcineurin also controls bud emergency together with HOG (High Osmolarity Glycerol) pathway, which responds to high osmolarity, coupling the high osmolarity signal to glycerol accumulation.
Calcineurin and HOG pathway seem to be antagonistic in the regulation of bud emergence. In presence of concentration of calcium above 50mM, cells show a delay both in bud emergence and actin polarization that is reverted by deletion of *CNB1,* indicating that this phenotype is directly controlled by calcineurin activity. Moreover, the deletion of *HOG1*, in the same condition of high calcium concentration, causes a longer bud emergence but no delay in actin polarization, suggesting that Hog1 activates bud emergence only after actin is correctly localised at the bud site. The delay of bud emergence observed in the *hog1*Δ strain grown on high calcium media was only partially suppressed by *CNB1* deletion, suggesting that calcineurin inactivation can suppress the defect in actin polarization, but not in the bud emergence. Thus, while calcineurin

seems to negatively regulate actin polarisation at the bud site, Hog1 controls bud emergence a step after the localisation of actin (Shitamukai *et al.,* 2004).
More recently, calcineurin activity has been associated also to response to nutrient. Actually, in a genome wide-transcriptional study on yeast cells exposed to a severe alkaline pH stress, that is known to activate calcineurin, an induction of most genes involved in hexoses transport and sugar metabolism has been observed after the stimulus (Ruiz *et al.,* 2008). For example the expression of *HXT2*, a gene encoding for the high affinity hexoses transporter, is greatly reduced after alkaline pH stress in cells lacking calcineurin activity (Ruiz *et al.,* 2008).
HXT2 expression is normally enhanced in low glucose in a Snf1p-dependent manner; however, the expression of *HXT2* is not fully abolished in a *snf1Δ* strain after high pH stimulus, indicating the presence of additional regulatory events not triggered by external glucose (Ruiz *et al.,* 2008). Again, alkaline pH-dependent expression of *HXT2* is 50% of the wild type expression in cells treated with FK506 or in *cnb1Δ* strain, and a role of calcineurin in *HXT2* transcription is also confirmed by the fact that its activation is strongly induced by addition of $CaCl_2$. Many other genes related to glucose metabolism were found to be induced in a calcineurin-dependent manner, confirming calcineurin involvement in a regulatory mechanism required for survival under certain conditions involving impaired glucose utilization.
Furthermore, Groppi and co-workers pointed out that glucose-induced calcium influx in starved cells, mediated by the yet uncharacterised calcium channel GIC, is also controlled by calcineurin in rich media (Groppi *et al.,* 2011) in a Crz1-indipendent manner (unpublished data).

Crz1, the main effector of calcineurin activity

One role of the calcineurin phosphatase under stress conditions is to activate gene expression through the regulation of the Crz1 transcription factor. This phosphatase is able to turn on a very specific program of gene expression by dephosphorylating and thus activating Crz1 in a way similar to mammalian NFATc transcriptor factor activation leaded by calcineurin (Yoshimoto *et al.,* 2002; Stathopoulos-Gerontides *et al.,* 1999).
Crz1 contains a zinc-finger motif for DNA binding and binds specifically to the calcineurin-dependent response element (CDRE). This sequence has been

identified in *FKS2* promoter, a gene encoding for a β-1,3-glucan synthase transcription, is regulated by Ca^{2+} and mating pheromone in a calcineurin-dependent fashion. The promoter of *FKS2* contains a 24 bp-region that is necessary and sufficient for calcineurin-dependent transcriptional induction and that was named CDRE.In a genetic screening Crz1 resulted to be necessary for induction of *FKS2* transcription, and for physical interaction with its promoter, in a CDRE-dependent manner. Again, its overexpression is fundamental to bypass calcineurin activation of a CDRE-containing reporter gene in *cnb1* mutant cells (Stathopoulos *et al.,* 1997).

Calcineurin and Crz1-regulated genes belong to several functional classes, most of which encode products that are known or predicted to be integral membrane proteins (Mewes *et al.,* 2002; Frishman *et al.,* 2001) or components of the plasma membrane and cell wall (Dwight *et al.,* 2002). Other calcineurin-regulated genes encode for proteins that participate in vesicle trafficking, lipid/sterol synthesis, and protein degradation. Thus, in response to stress, calcineurin and Crz1 seems to activate a specific program of gene expression that promotes remodeling of the cell surface. Consistently with that, calcineurin/Crz1 are known to control gene expression when cell wall is perturbed or stressed (Lagorce *et al.,* 2003). Obviously, numerous calcineurin/Crz1-dependent genes encode for proteins involved in ion and/or small molecule transport, controlling some the most important aspects of ion homeostasis (Mendoza *et al.,* 1994; Cunningham *et al.,* 1996).

The capability of calcineurin and Crz1 to control transcription of numerous key factors of various signaling pathways allows to hypothesize the presence of potential cross-talks between calcineurin and other transduction cascades. These key regulators are for example *RCN1*, a regulator of calcineurin itself, *CMK2*, a Ca^{2+}/calmodulin-dependent kinase, *YPK1*, a regulator of protein kinase C that control Cell Wall Integrity (CWI) pathway and also several transcription factors like *CUP2*, *SMP1* and *NDT80* (Kingsbury *et al.,* 2000, Cyert 2003).

Calcineurin can directly control Crz1 localization by modulating its phosphorylation state. During growth under optimal conditions, Crz1 is phosphorylated and distributed throughout the cell. However, after a rise in intracellular calcium and a consequent calcineurin activation, it's dephosphorylated and rapidly accumulates in the nucleus (Stathopoulos-Gerontides *et al.,* 1999). This nuclear accumulation is readily reversible, as inhibition of calcineurin by FK506 leads to rapid re-distribution of Crz1 to the

cytosol. The phosphorylation state of Crz1 is fundamental for the interaction with the importin Nmd5, which is responsible of shuttling Crz1 in the nucleus. *In vitro*, only the dephosphorylated form of the transcription activator can correctly bind with Nmd5, while *in vivo* the NLS recognized by the importin has been identified as sufficient and necessary for a correct nuclear localization (Polizotto *et al.,* 2001).

The exportin Msn5, which is known to translocate several yeast protein outside in the cytoplasm, controls Crz1 nuclear export.Msn5 transport only phosphorylated proteins. The nuclear exporting sequence of Crz1 physically interacts with Msn5 but this interaction is disrupted when putative phosphorylation sites near Crz1 NES are mutated to non-phosphorylable amino acids, indicating a clear role of Crz1 phosporylation state in its export (Boustany *et al.,* 2002).

Thus, when calcineurin activity is low, Crz1 is phosphorylated and accumulates in the cytosol because of a low rate of nuclear import and a high rate of nuclear export. Active calcineurin dephosphorylates Crz1, which then rapidly accumulates into the nucleus thanks to the interaction with Nmd5.

Furthermore, calcineurin probably controlsCrz1 function not only regulating its cellular localization, but also with a different mechanism probably linked to protein stability. Thus, a mutant strain carrying a version of Crz1 fused with SV-40 T-antigen NLS and constitutively retained in the nucleus by the deletion of Msn5, shows an induction of CDRE-LAC-Z reporter gene still dependent on intracellular calcium concentration (Cyert 2003).

Using a high-throughput protein chip-based method, some putative kinase for Crz1 have been identified (Zhu *et al.,* 2000). One of these kinases is Hrr25, homolog of mammalian casein kinase 1 (CK1) and most similar to the δ and ε isoforms of this class (De Maggio *et al.,* 1992). Hrr25 controls both activity and localization of Crz1 since the overexpression of *HRR25* counteracts Crz1-dependent transcriptional activity and decreases its nuclear accumulation induced by Ca^{2+}.On the contrary, lack of Hrr25 results in a higher basal level of Crz1-dependent gene transcription, showing a great increase in Crz1 activity after calcineurin activation. Similarly, lower concentration of calcium are required for Crz1 nuclear localization in *hrr25Δ* cells (Cyert 2003).

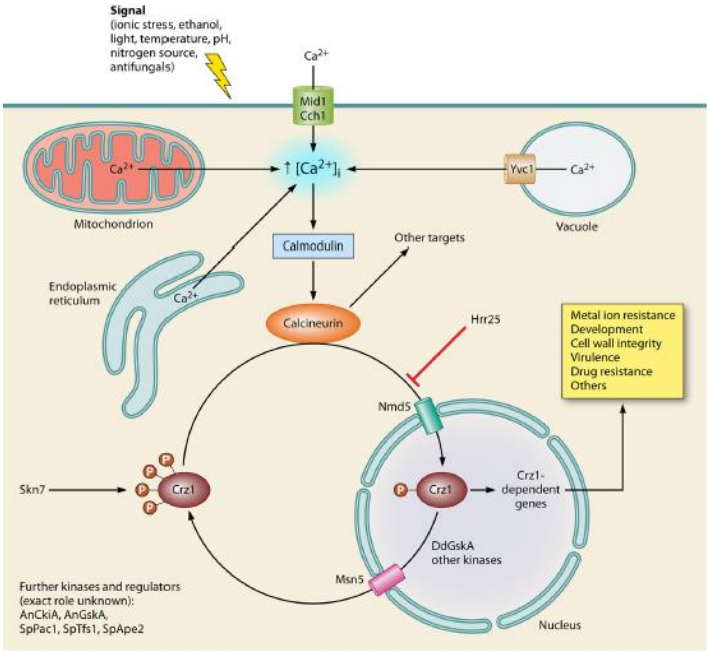


Fig. 5: *Calcineurin/Crz1 signalling pathway in yeast*

In mammals calcineurin activates the family of nuclear factor of activated T-cells (NFAT) transcription factors, and through regulation of NFAT activity, calcineurin modulates a range of processes including T-cell activation, neuronal differentiation and activity, cardiac development and hypertrophy, and skeletal muscle fiber-type specification.

The mechanism by which calcineurin regulates NFAT activity is analogous to calcineurin-mediated regulation of Crz1 in yeast. NFAT is composed of a cytosolic protein, NFATc, which when dephosphorylated, rapidly accumulates in the nucleus and combines with various nuclear component to activate transcription of a specific set of genes.

As observed for Crz1, calcineurin causes rapid changes in NFAT localization by concerted regulation of nuclear import and export.

However, although Crz1 and NFAT have remarkably similar modes of regulation, they display very little sequence similarity, except for the PxIxIT-

related calcineurin docking domain, suggesting that Crz1 and NFAT clearly evolved independently (Fig. 6) (Crabtree *et al.,* 2001).

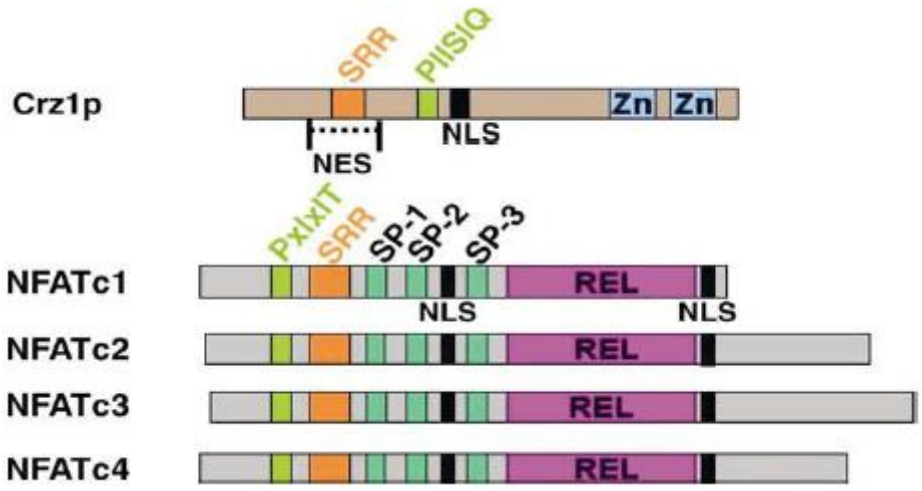


Fig. 6: *Schematic representation of sequence motifs in Crz1 and NFATc family members (NES, nuclear export sequence; NLS, nuclear localization sequences; SRR, serine-rich region; SP-1, SP-2, SP-3, Serine–proline repeats; ZN, C2H2 Zn2þ finger motifs; REL, REL-related DNAbinding domain.).*

CALCIUM RELATED PATHWAYS

Unfolded Protein Response (UPR)

The unfolded protein response is an adaptive stress response that is usually activated by the imbalance between the entrance of newly synthesized unfolded proteins and the inherent folding capacity in the endoplasmic reticulum (ER). Various environmental stresses and changes in physiological conditions can result in the accumulation of unfolded proteins in the ER, which is sensed through ER transmembrane protein sensors.
UPR signalling is involved deeply in numerous physiological processes besides protein quality control. In fact, several diseases such as diabetes, NAFLD (non-alcoholic fatty liver disease), cystic fibrosis, Parkinson's disease, Huntington's

disease, Alzheimer's disease, inflammation, cancer and liver failure are associated with the UPR pathways (Hetz *et al.,* 2012-2013).

In yeast and mammalian cells all the plasma membrane proteins as well as those who belongs to secretory pathway are folded and assembled in ER before being delivered to target organelles or to cellular surface via the Golgi apparatus. Most of the newly synthetized proteins enters in the endoplasmic reticulum and undergo glycosylation or disulphide bond formation, in order to obtain a correct folding and assembly (Gething and Sambrook 1992; Ellgaard *et al.,* 2003). Again, in ER a particular family of proteins, called chaperones, is present, which promotes the correct tertiary and quaternary structures by preventing aberrant proteins aggregation.

However the capacity of ER to assembly, folding and modifying new proteins could be alterated by cellular exposition to unphysiological condition or stresses including nutrient starvation, calcium depletion from the ER, strong reducing conditions, viral infection, hypoxia or even in the course of normal development and differentiation.

When one or more of these condition are present, the cell interrupts the normal protein folding and assembly, leading to an accumulation of misfolded proteins in ER and a consequent stress overall the organel. In response to this situation the cell activates a specific signaling pathway, which ends in a massive regulation of gene transcription, collectively named the unfolded protein response (UPR).

The principal goal of UPR is to restore the normal folding and protein modification capacity of the ER in order to allow adaptation to new condition (Ron and Walter 2007; Schroder and Kaufman 2005; Hetz and Glimcher 2009).

In mammalian cells, three independent branches can sense ER stress. Ire1 (inositol-requiring enzyme-1) branch is the most highly conserved among eukaryotes and is the only pathway identified in yeast, while metazoans and higher eukaryotes utilize two additional pathways, double-stranded RNA-activated PERK (protein kinase-like ER kinase) and ATF6 (activating transcription factor 6) (Cox *et al.,* 1993; Mori *et al.,* 1993; Harding *et al.,* 1999; Shi *et al.,* 1998; Haze *et al.,* 1999).

IRE1, the first ER stress receptor to be discovered, encodes an ER-localized type I transmembrane protein with a predicted serine/threonine kinase domain and an RNAse domain on its cytoplasmic side (Cox *et al.,* 1993; Mori *et al.,* 1993).

The first reported structure of this UPR transactivator was the cLD (core lumenal domain, amino acids 111–449) that also exhibits a groove similar to MHC (major histocompatibility complex). From this observation, it was proposed that the direct binding of unfolded proteins to Ire1 lumenal domain was sufficient to activate the UPR in yeast.

Before acquisition of the Ire1 domain structure in *S. cerevisiae*, it was accepted that Ire1 was activated by the release of Hsp70 molecular chaperone BiP from its lumenal domain. Moreover, overexpression of BiP is sufficient to attenuate the UPR (Dorner *et al.,* 1997), suggesting that this protein could serve as a negative regulator of the UPR (Okamura *et al.,* 2000). BiP keeps Ire1 inactive through direct binding of its lumenal domain, but during ER stress BiP dissociates to bind unfolded proteins, resulting in Ire1 activation.

Indeed, several other studies contradict this model. The deletion of Ire1 lumenal domain is sufficient to disrupt BiP binding (Kimata *et al.,* 2004), but in this mutant the UPR activation level appeared normal during ER stress, indicating that interation between BiP and Ire1 is not required to activate UPR pathway (Oikawa *et al.,* 2007).

A direct activation model was proposed involving a two-step sensor activation: first, upon ER stress, BiP dissociates from Ire1 due to its interaction with accumulated unfolded proteins, and/or by other mechanisms, resulting in formation of Ire1 clusters. Second, binding of unfolded proteins to the Ire1 core causes a conformational change in the luminal domain that leads to the reorientation of the cytosolic domain, resulting in the activation of the RNase activity.

In fact, inactive human Ire1 adopts a face-to-face orientation that inhibits protein activity (Ali *et al.,* 2011); in contrast, dimerized Ire1 RNase domain holds a back-to-back orientation when activated (Fig. 7) (Pincus *et al.,* 2010).

Ire1 core contains a domain responsible for the recognition of ER stress.

Analysis of the crystal structure showed that Ire1 dimer forms higher order oligomers, and point mutations that interfere with the peptide-binding groove of Core-oligomer result in a loss in sensing activity, suggesting that this higher order oligomer formation is required for the recognition of unfolded proteins (Credle *et al.,* 2005). This higher-ordered oligomer of Ire1 was observed in yeast in vivo and the relocalization of Ire1 during ER stress has been demonstrated (Kimata *et al.,* 2007). In the absence of ER stress, Ire1 is located in the ER

membrane, but this pattern rapidly changes to a punctate pattern under conditions of ER stress.

Interestingly, several studies reported that Ire1 directly binds unfolded proteins and prevent proteins aggregates formation (Promlek *et al.,* 2011; Kimata *et al.,* 2007; Welihinda *et al.,* 1997). In addition, Ire1 preferentially binds to peptides enriched in basic and hydrophobic residues supporting the hypothesis of a high affinity for misfolded proteins (Gardner and Walter 2011). However, studies on the active site of human ire1 suggested that protein core is too small to accommodate unfolded proteins. Actually, Gln105, which forms hydrogen bond to adjacent residues, prevents direct binding of unfolded proteins. Thus, these studies support the conventional indirect UPR activation model.

Based on its motif folding, RNase domain was redefined as the KEN (kinase-extension nuclease) domain. KEN domain is similar to mammalian RNase L, which cleaves endogenous and viral RNA, and shows high ribonuclease activity after *trans*-autophosphorylation of its adjacent kinase domain. Interestingly, there is no known protein other than Ire1 with both kinase and RNase activities.

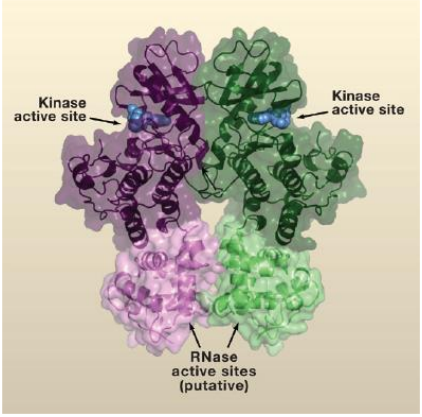


Fig. 7: *Structure of cytosolic domain of Ire1 (Ron, Hubbard. 2007)*

The oligomerization of Ire1 cytosolic domain is essential to its enzymatic activity, and is driven by the oligomerization of Ire1 lumenal domain that ends in the formation of MHC-like grooves. The kinase activity of Ire1 is not required during the UPR activation cascade but essential in Ire1 deactivation, while the

RNase activity is essential for the whole process (Korennykh *et al.,* 2009; Rubio *et al.,* 2011; Zhou *et al.,* 2006).

When activated, the cytosolic ribonuclease domain of yeast Ire1 cleaves the intron of *HAC1* pre-messenger RNA to initiate the synthesis of Hac1 transcription factor (Cox *et al.,* 1993-1996; Mori *et al.,* 1993). Hac1 then translocates into the nucleus to regulate the expression of nearly 400 target genes (Thibault *et al.,* 2011) including ER chaperones, lipid biosynthesis enzymes and ERAD (ER-associated degradation) machinery.

In mammals, the presence of three different ER stress transducers facilitates the activation of the UPR (Walter and Ron 2011). Mammalian cells have two different forms of *IRE1*: *IRE1α*, that is expressed ubiquitously, and *IRE1β* that is present only in the intestinal and lung epithelium (Bertolotti *et al.,* 2001; Martino *et al.,* 2013).

The branch of the UPR regulated by Ire1*α* has been well studied. The transcription factor downstream of Ire1*α*, *XBP1*, share some differences with Hac1 but its RNA maintains the unconventional splicing activation site recognized by Ire1, while the protein also shares the basic leucine zipper motif conserved in Hac1. *XBP1* activates similar downstream target genes as Hac1 in *S. cerevisiae* with the induction of genes involved in protein folding as well as in the secretory pathway (Yoshida *et al.,* 2001).

The other UPR activating mechanism in mammals involve the activity of PERK, which is an ER transmembrane kinase that mediates transcriptional and translational control of the UPR program (Harding *et al.,* 1999).During ER stress, PERK oligomerizes and phosphorylates itself and eIF2*α* (eukaryotic initiation factor 2*α*) resulting in temporary attenuation of the overall protein translation and up-regulation of the transcription factor ATF4. This translation inhibition decreases the influx of proteins that enters the ER, reducing ER protein folding load and alleviating ER stress.

ATF4 again activates the transcription of both CHOP (C/EBP homologous protein) and GADD34 (growth-arrest and DNA-damage-inducible 34). The first promotes ER stress-induced apoptosis while the second is involved in a negative feedback loop to counteract PERK activity by dephosphorylation of eIF2*α*. This ends in the resume of protein synthesis and in an increase of cellular apoptosis (Zinszner *et al.,* 1998; Marciniak *et al.,* 2004)

In addition, another study has shown that activation of PERK could lead to down-regulation of anti-apoptosis protein XIAP, which could lead to increase in apoptosis (Hiramatsu *et al.,* 2014).
The last protein involved in UPR in mammals is ATF6, a type II transmembrane protein, containing a cytosolic N-terminal region composed by a basic leucine zipper (bZIP) transcription factor region (Haze *et al.,* 1999).After accumulation of unfolded protein in ER, ATF6 is packaged into vesicles and transported to the Golgi. Into these vesicles ATF6 N-terminal cytosolic fragment is cleaved and released by the action of S1P and S2P proteases, and shuttled to the nucleus, where it can regulate transcription of UPR related genes (Schindler *et al.,* 2009; Yoshida *et al.,* 2001; Adachi *et al.,* 2008; Okada *et al.,* 2002).

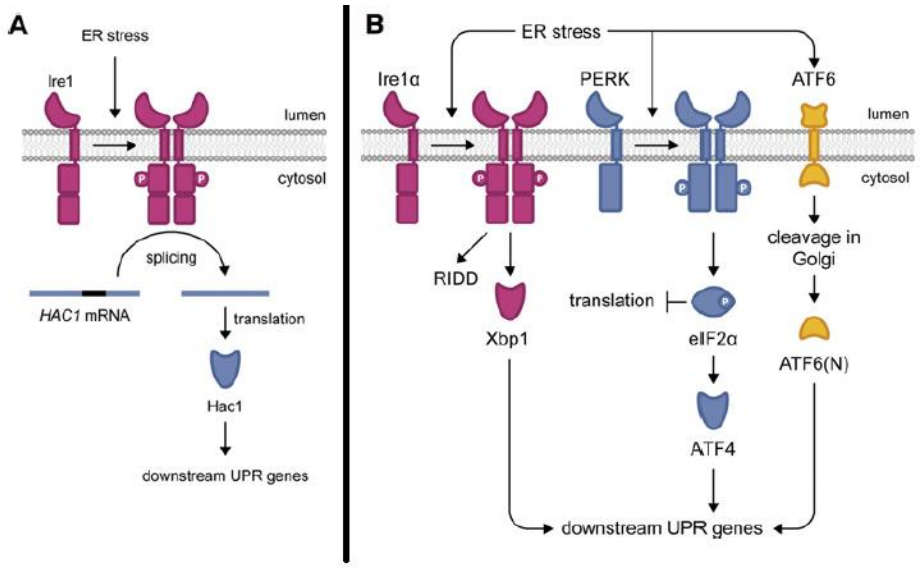


Fig. 8: *Schematic representation of UPR signaling pathway both in Saccharomices cerevisiae (A) and mammalian (B)*

The Cell Wall integrity pathway

The yeast cell wall is a strong, but elastic, structure that is essential not only for the maintenance of cell shape and integrity, but also for progression through

the cell cycle. During growth and morphogenesis, and in response to environmental challenges, the cell wall is remodeled in a highly regulated and polarized manner, a process that is principally under the control of the cell wall integrity (CWI) signaling pathway.

One of the principal functions of yeast cell wall is to provide protection from osmotic shock. In order to survive to rapid and extreme changes in extracellular osmolarity the cell must limit the influx of water to maintain an appropriate intracellular environment for biochemical reactions (Smits *et al.,* 1999; Hohmann 2002).

Budding yeast invests a considerable part of its energy in cell wall synthesis, which comprises 10-25% of cell mass depending on growth media (Orlean 2012; Smits *et al.,* 1999; Aguilar-Uscanga and François 2003). The cell wall is a layered structure with an electron-transparent inner layer and an electron-dense outer layer (Cappellaro *et al.,* 1994). The inner layer is composed principally of glucan polymers and chitin (β-1,4-N-acetylglucosamine polymers). This layer is constructed mainly (80–90%) of β-1,3-glucan chains branched through β-1,6 linkages. Polymers of β-1,6-glucan chains make up most of the remainder of the inner layer (8–18%) with chitin chains representing the smallest fraction (1–2%). The principal role of this layer is to maintain mechanical strength and elasticity of the cell wall, thanks to the helical nature of β-1,3-glucan chains (Rees *et al.,* 1982; Smits *et al.,* 1999).

The outer cell wall layer is a lattice composed of two major classes of cell wall glycoproteins (CWPs). Glycosylphosphatidylinositol (GPI) proteins are directed through the secretory pathway to the extracellular face of the plasma membrane by lipid anchors at their C-termini that are cleaved living a lipidless GPI. The remnants of GPI-CWPs become linked to the external surface of the β-1,3-glucan network indirectly through β-1,6- glucan chains (Klis *et al.,* 2006).

The other class of CWPs is composed by five polypeptides named Pir1-5 (Proteins with internal repeats) (Toh-e *et al.,* 1993; Kapteyn *et al.,* 1999; Mrsa and Tanner 1999; Ecker *et al.,* 2006) that are not essential for cell viability but, if lacking, cause defects in growth rate, cell morphology and high sensitivity to cell wall stress agent (Mrsa and Tanner 1999). The Pir proteins are attached directly to β-1,3-glucan chains through a linkage that involves their repeat sequences, DGQΦQ (where Φ is any hydrophobic residue) (Castillo *et al.,* 2003). Because most members of this class of proteins contain several repeat motifs, they may provide sites for cross-linking of multiple β-1,3-glucan chains. In

contrast to GPI–CWPs, Pir proteins are distributed uniformly through the inner glucan network, consistent with their attachment to β-1,3-glucan (Kapteyn *et al.,* 1999).

Under nonstress condition the percentage of chitin chains found in cell wall is very little but can rise up to 20% of total wall polymer in response to cell wall stress. Chitin chains are attached to the internal surface of the β-1,3-glucan network in the lateral wall after cytokinesis (Kollar *et al.,* 1995), but can also be attached to β-1,6-glucan chains associated with GPI–CWPs (Popolo *et al.,* 1997; García-Rodriguez *et al.,* 2000; Valdivieso *et al.,* 2000).

The CWI pathway responds to cell wall stress signals through a family of cell surface sensors coupled to a small G protein, Rho1, the functional orthologous of mammalian RhoA (Qadota *et al.,* 1994), which is considered the master regulator of CWI signaling. Indeed Rho1 integrates signals from the cell and controls a variety of outputs involved in cell wall biogenesis, actin organization, and polarized secretion.

Rho1 is localized to sites of polarized growth (Yamochi *et al.,* 1994; Qadota *et al.,* 1996) where it activates different targets. These collectively regulate processes including β-glucan synthesis, gene expression related to cell wall biogenesis, organization of the actin cytoskeleton, and secretory vesicle targeting to the growth site. Both the β-1,3-glucan synthase (GS) encoded by the *FKS1* and *FKS2* genes and the β-1,6-glucan synthase, are regulated by Rho1. Finally, the transcriptional output of the CWI pathway is under the control of a MAPK cascade headed by a Rho1-activated protein kinase C (Pkc1). Disruption of the MAPK cascade signaling pathway compromises the integrity of the cell wall, heading to cell lysis (Levin 2011).

A linear protein kinase cascade is responsible for amplification of the CWI signal from Rho1. This MAPK cascade for CWI signaling is composed of Pkc1 (Levin *et al.,* 1990), a MEKK (Bck1) (Costigan *et al.,* 1992; Lee and Levin 1992), two redundant MEKs (Mkk1/2) (Irie *et al.,* 1993), and a MAPK (Mpk1/Slt2) (Lee *et al.,* 1993; Martín *et al.,* 1993). Genetic and biochemical studies have established that Pkc1 activates Bck1, which activates Mkk1 and Mkk2, which in turn activate Mpk1 (Levin *et al.,* 1994; Levin 2005).

Among the various Rho1 effector pathways identified, the Pkc1-activated MAPK cascade has been studied in detail. The *S. cerevisiae* genome encodes only a single homolog of mammalian protein kinase C, designated Pkc1 (Levin *et al.,* 1990).

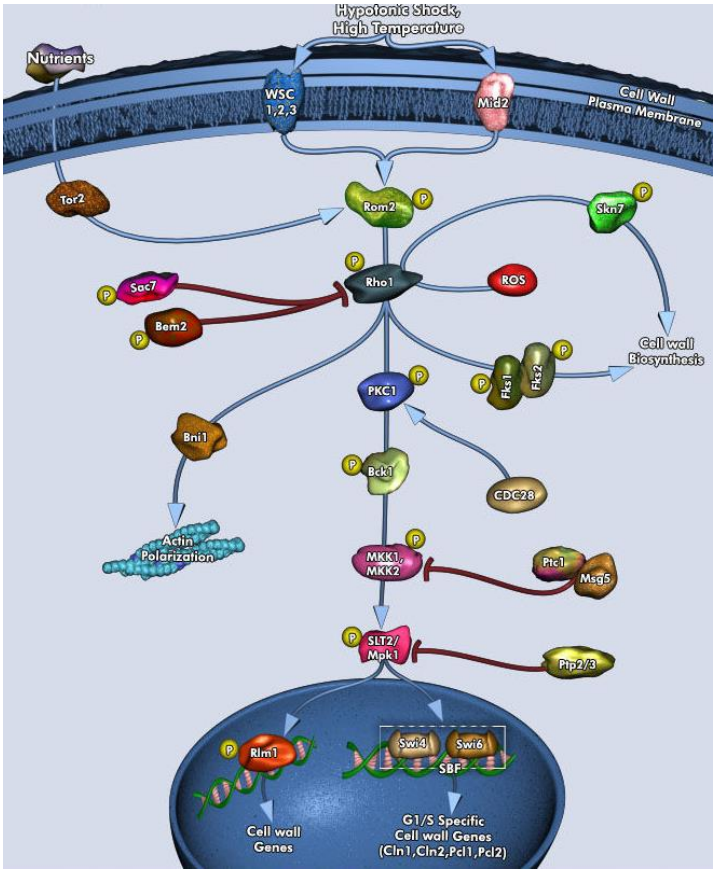


Fig. 9: *Scheme of cell wall integrity MAPK cascade (Qiagen)*

Deletion of PKC1 is lethal under normal growth conditions because cells undergo cell lysis, but the growth defect of a *pkc1Δ* mutant can be suppressed by addition of sorbitol or other osmotic protectors in growth media (Levin and Bartlett-Heubusch 1992; Paravicini *et al.,* 1992).

The principal defect due to the lack of Pkc1 is the presence of a thinner layer of glucan and mannoprotein, revealing a pleiotropic set of cell wall defects (Levin *et al.,* 1994; Roemer *et al.,* 1994). Loss of *PKC1* results in a more severe growth defect than that observed in strains carrying the deletion of any of the members of the MAPK cascade under the control of Pkc1, indicating that Pkc1

regulates at least one additional pathway (Lee and Levin 1992). GTP-bound Rho1 activates Pkc1 (Nonaka *et al.,* 1995; Kamada *et al.,* 1996), conferring upon the protein kinase the ability to be stimulated by phosphatidylserine (Kamada *et al.,* 1996). The ability to bind Rho1 is conferred by two regions of Pkc1 localized in the regulatory N-terminal domain named C1 (cys-rich domain) and HR1 (homology region 1) (Nonaka *et al.,* 1995; Schmitz *et al.,* 2002b). Classic cofactors that can activate PKCs protein, such as diacylglycerol (DAG) or calcium, do not activate Pkc1 even in the presence of the active form of Rho1 (Antonsson *et al.,* 1994; Watanabe *et al.,* 1994; Kamada *et al.,* 1996).

Pkc1 is also a target of the Pkh1 and Pkh2 protein kinases (Inagaki *et al.,* 1999; Friant *et al.,* 2001). Pkh1 and -2 serve an essential but overlapping function in the maintenance of cell wall integrity, and their function is required for full activation of Pkc1 in response to heat shock. In G1 and S phase, Pkc1 resides at the pre-bud site and at bud tips, a pattern that is very similar to that of Rho1 (Yamochi *et al.,* 1994; Qadota *et al.,* 1996). Pkc1 delocalizes during G2 phase and finally relocalizes at the mother-bud neck during mitosis, a transition that requires an intact septin ring (Denis and Cyert 2005).

As said before the most important component of cell wall are linear polymers of β-1,3-linked glucan and the enzymatic complex that catalyzes this reaction have been well studied in yeast (Inoue *et al.,* 1996; Douglas 2001; Klis *et al.,* 2006). *S. cerevisiae* Fks1 and Fks2 (for FK506 sensitive), a pair of closely related genes encoding alternative catalytic subunits of the glucan synthase, are large membrane proteins with a cytoplasmic central domain, either one of which is sufficient for glucan synthetase activity and cell viability (Douglas *et al.,* 1994; Mazur *et al.,* 1995; Ram *et al.,* 1995).

Functional analysis of *FKS1* revealed that its central domain, which is predicted to be cytoplasmic, is necessary for enzyme activity and, unlike loss of Pkc1, loss of Fks1/2 is not suppressed by increased osmotic support. This is probably due to a total shut down of cell wall biosynthesis in an *fks1Δ* or *fks2 Δ* mutant (Okada *et al.,* 2010). The principal regulatory subunit of glucan synthase complex is Rho1 itself, which serves to stimulate enzyme activity in a GTP-regulated manner. Indeed Fks1 colocalize with Rho1 in the plasma membrane and more in detail colocalizes with cortical actin patches and moves on the cell surface in a manner dependent on actin patch mobility (Yamochi *et al.,* 1994; Qadota *et al.,* 1996; Utsugi *et al.,* 2002). Surprisingly, as demonstrated in a study on *rho1* conditional mutant, the two essential function of this protein are

separated: some mutants resulted defective only in glucan synthase activity and the others were defective only in activation of Pkc1 (Saka *et al.,* 2001).
The main difference between *FKS1* and *FKS2* is at level of expression control. During growth in optimal condition, *FKS1* is predominantly expressed even if its mRNA fluctuate through cell cycle with a peak in late G1 (Ram *et al.,* 1995; Igual *et al.,* 1996). The regulation of *FKS1* during cell cycle is dependent on Swi4 and Swi6, that form the SBF transcription factor, while under stress condition the expression is regulated by CWI pathway through the Mpk1-activated transcription factor Rlm1 (Andrews and Herskowitz 1989; Mazur *et al.,* 1995; Ram *et al.,* 1995; Igual *et al.,* 1996; Spellman *et al.,* 1998; Jung and Levin 1999). On the contrary, expression of *FKS2* is low in normal condition, but is induced in response to several stimuli like exposure to pheromone, cell wall stress, high extracellular calcium concentration, glucose starvation, entry in stationary phase or lack of *FSK1* function (Mazur *et al.,* 1995; Zhao *et al.,* 1998).
The induction of *FKS2* expression in presence of pheromone, calcium, or in case of *FSK1* deletion is known to require calcineurin function (Garrett-Engele *et al.,* 1995; Mazur *et al.,* 1995). Thus, sensitivity to FK506 of *fsk1Δ* cells is explained with calcineurin regulation of *FKS2* (Foor *et al.,* 1992; Liu 1993). In response to cell wall stress, the immediate transcriptional induction of *FKS2* is mediated by the calcineurin-activated transcription factor Crz1, which binds to a calcineurin-dependent response element within the *FKS2* promoter (Stathopoulos and Cyert 1997; Zhao *et al.,* 1998). The CWI pathway drives maintenance of high levels of *FKS2* expression under chronic cell wall stress (Zhao *et al.,* 1998; Jung and Levin 1999), through the noncatalytic activation of the Swi4/Swi6 (SBF) transcription factor by Mpk1 (Levin. 2011). Under stress condition Rho1 regulates Crz1, possibly through the action of its target Skn7, a transcriptional factor that stabilizes Crz1, thus creating a crosstalk between MAPK cascade and Ca^{2+}-calcineurin signaling pathway (Fig. 10) (Williams and Cyert 2001).

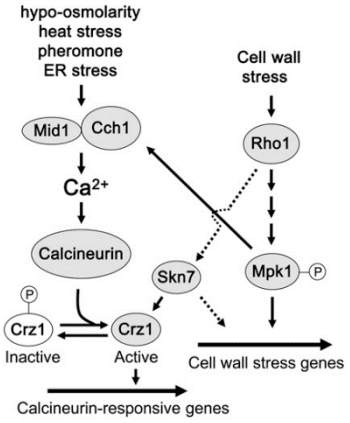


Fig. 10: *Crosstalk between CWI MAPK cascade and Ca^{2+}-calcineurin pathway (Levin 2011)*

Another important crosstalk between these two pathways has been observed during ER stress. Several data confirm that ER stress induced by tunicamycin or dithiothreitol activates CWI signaling and that cell survival after ER stress depends on Mpk1 activity (Bonilla and Cunningham 2003; Chen *et al.,* 2005; Babour *et al.,* 2010). The activation of cell wall integrity pathway under ER stress is very different and independent from UPR pathway, indeed CWI cascade is not controlled by Ire1 or Hac1 (Chen *et al.,* 2005). Surprisingly, activation of CWI signaling by cell wall stress also activates the UPR (Scrimale *et al.,* 2009), revealing the existence of cross-regulation between these two systems. Krysan (2009) hypothesized that activation of CWI signaling by cell wall stress may increases the total protein flux through the ER requiring increased ER capacity. Conversely, ER stress may result in the delivery of misfolded proteins to the cell surface, which consequently induces cell wall stress. Anyway, the mechanism by which the CWI signaling pathway interfaces with the Ire1 protein kinase in the ER membrane is still unclear (Levin 2011).
Tunicamycin treatment leads to Mpk1 activation principally by the Wsc1 sensor (Babour *et al.,* 2010), but in some manner is also dependent on the Hos2/Set3 histone deacetylase complex with an unknown mechanism (Cohen *et al.,* 2008). Activation of CWI signaling by ER stress drives the Rlm1-mediated transcriptional program (Cohen *et al.,* 2008; Babour *et al.,* 2010), indicating that response to this stress involves enhanced cell wall biogenesis.

The Rlm1 transcription factor is responsible for the bulk of the CWI signaling transcriptional program and is regulated by Mpk1 that actively control its nuclear localization (Watanabe *et al.,* 1997; Jung *et al.,* 2002). A genome-wide analysis of genes induced by mutations affecting the cell wall identified a group of more than 80 upregulated genes whose promoters possess sites for Rlm1, Swi4, and Crz1, as well as for Msn2/4. These data confirm that under these conditions CWI signaling, Ca^{2+} signaling, as well as with general stress signaling are co-activated (Bermejo *et al.,* 2008; Garcia *et al.,* 2009).
The Cch1-Mid1 plasma membrane Ca^{2+} channel is also activated in response to ER stress, which causes elevation of cytosolic Ca^{2+} and activation of calcineurin (Bonilla *et al.,* 2002). The activation of HACS the plasma membrane complex is clearly dependent on Mpk1, but whether the control is direct or indirect is still unknown (Bonilla and Cunningham 2003). Nevertheless, it is suggestive that, in addition to activation by ER stress, Cch1-Mid1 and calcineurin are activated by all conditions that activate CWI signaling, such as pheromone treatment (Cyert and Thorner 1992; Foor *et al.,* 1992; Moser *et al.,* 1996), heat shock (Zhao *et al.,* 1998), and hypo-osmotic shock (Batiza *et al.,* 1996).

TRP CHANNELS

Transient receptor potential channel family of ion transporter
Transient receptor potential (TRP) channels constitute a large and functionally versatile family of cation-conducting channel proteins, which have been mainly considered as polymodal unique cell sensors. The first TRP channel gene was discovered in *Drosophila melanogaster* (Montell and Rubin 1989) in the analysis of a mutant fly whose photoreceptors failed to retain a sustained response to maintained light stimuli. So far, more than 50 TRP channels have been identified with representative members in many species. The 28 mammalian members of the super-family of transient receptor potential channels are cation channels, mostly permeable to both monovalent and divalent cations, and can be divided into six subfamilies: the TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), and the TRPA (ankyrin) groups (Fig. 11). They are involved in a great number of physiological functions, ranging from pure sensory functions, such as pheromone signaling, taste transduction, nociception, and temperature sensation, over homeostatic

functions, such as Ca^{2+} and Mg^{2+} reabsorption and osmoregulation (Gees, Colsoul, and Nilius. 2010).

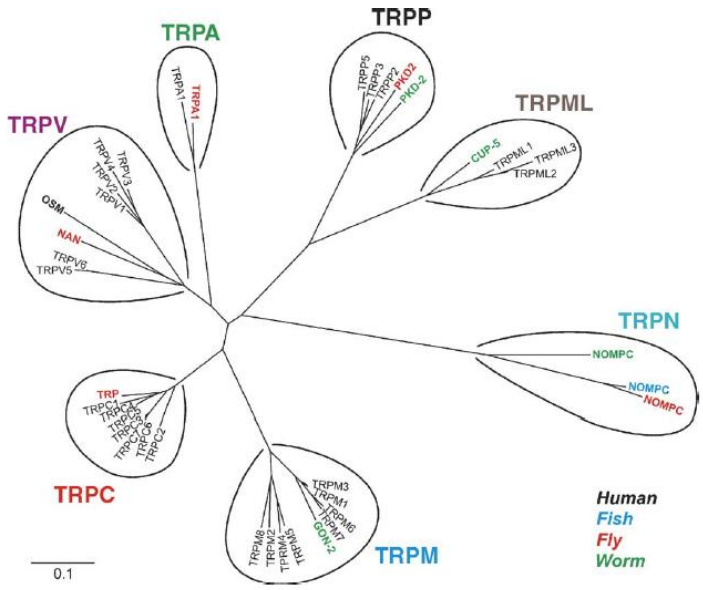


Fig. 11: *Phylogenetic tree of TRP subfamily of ion transporter (Pedersen et al., 2005)*

TRP channels are widely expressed in many tissues and cell types, with a number of them characterized by a constitutive expression in all cells, whereas others with more restricted expression patterns (Zheng 2013).
TRP channels are usually found on the plasma membrane, but some subfamilies, like TRPML for example, are localized on ER membrane and internal membrane (Dong *et al.,* 2010).

TRP: Structure

In order to have more detailed information regarding activation and functions, the structure of TRP ion channels would be fundamental. Unfortunately, structural information for TRP ion channels is very scarce, albeit data based on sequence similarity tell us that TRP channels are expected to look like voltage-

gated potassium channel, with four subunits surrounding a central pore that allows ion permeation (Zheng 2013).
TRP channels share six transmembrane segment, with both C- and N-termini probably located inside the cell, and, in most of them, the ion selectivity filter region can be identified on the base of their similarity with potassium channels. For example most of TRPV are characterized by the sequence TIGXGD (X = M/L). Glycosylation sites are suggested in a number of TRP channels in the pore region, after the fifth transmembrane segment, as well as a number of protonation sites that can be identified in TRP channels. Again, on the base of their sensitivity to pH changes either on the intracellular side or on the extracellular side, the transmembrane location of these protonation sites can be determined (Zheng 2013).
TRP channels discovered so far are mostly functional homotetrameric proteins, but some of that, for example TRPP2 subunits, form a trimeric channel that then tetramerizes with Pkd1 protein to form a functional complex (Storch *et al.,* 2011; Yu *et al.,* 2009). In addition, there are ample examples of heteromeric channels formed by different TRP subunits (Cheng *et al.,* 2010).
The N-terminal part of TRP protein is characterized by the presence of ARD domain, that contains a high number of helix-turn-helix repeats able to bind ATP and represents one of the most commonly found protein-protein interaction motifs (Mosavi *et al.,* 2004; Sedgwick *et al.,* 1999). However, ARDs domains seem not to interact between TRPs proteins, since they remain monomers in crystal form, indicating that they probably recognize some unknown motifs in TRP ion channels (Lishko *et al.,* 2007; Arniges *et al.,* 2006; Zhang *et al.,* 2011).
In addition to the pore forming subunits TRP tetramers are often characterized by the presence of auxiliary or modulator proteins. For example, calmodulin is known to modulate TRPV1 channel either in absence of Ca^{2+} or when intracellular calcium concentration rises. Surprisingly this association is affected by ATP concentration that can compete with calmodulin for interaction with N-terminal ARD (Phelps *et al.,* 2010).

TRP as Ca^{2+} entry channels

The family of transient receptor potential (TRP) channels contributes to changes in $[Ca^{2+}]_i$ by providing Ca^{2+} entry pathways, by modulating the driving

force for the Ca^{2+} entry, and very likely also by providing intracellular pathways for Ca^{2+} release from cellular organelles. Even if most of TRP channels are calcium permeable the permeability ratio between calcium and sodium (P_{Ca}/P_{Na}) can considerably vary through TRP subfamily, ranging from 0.3 for TRPM2 to >100 for TRPV5 and TRPV6, indeed only TRPV5, TRPV6, TRPA1 and TRPM3 show a high affinity for calcium (Turner *et al.,* 2003; Zhang *et al.,* 2004). This variance depends on differences in pore structures and obviously also in the dynamic pore behavior (Chung *et al.,* 2008; Karashima *et al.,* 2010).

Data based on aminoacidic sequence alignments show that there is not a high similarity among TRP subfamilies for what concern selectivity filter regions (Owsianik *et al.,* 2006b). For example, in TRPV5 and TRPV6 the capacity to transport calcium depends on residue D^{542} of TRPV5 that correspond to D^{541} in TRPV6 (Nilius *et al.,* 2001). Since these two channels are known to form homo and heteromultimeric complexes it appears that calcium selectivity depends on a ring of four aspartate in the pore region, which is very similar to the ring of four negatively charged residues (aspartates and/or glutamates) of voltage gated Ca^{2+} channels (Ellinor *et al.,* 1995; Hoenderop *et al.,* 2003).

In TRPV1 is important for calcium permeability the residue D^{546} that corresponds to D^{682} of TRPV4, since their mutation highly reduces calcium flux. Moreover, for TRP4V the additional mutation of D^{672} reduces the divalent selectivity, while introducing a negative charge instead of M^{680} completely abolishes the Ca^{2+} permeability (Garcia-Martinez *et al.,* 2000; Voets *et al.,* 2002).

Neutralization of the negative charges in the pore forming region between TM5 and TM6 of TRPC5 (E543, E595, E598) and TRPC1 (all seven, D to N and E to Q) resulted in a decreased La^{2+} and Ca^{2+} permeability (Jung *et al.,* 2003; Liu *et al.,* 2003). These data suggests that even if negative charges in pore region are fundamental for calcium permeability, they can be located also in the distal parts of the putative pore entrance (Gees, Colsoul and Nilius. 2005).

Even if TRP pore structures are relatively stable, is evident that for some TRP channels the pore diameter as well as Ca^{2+} permeation depend on the mode of activation. Thus, depending on the pore structure, TRP channels vary in their pore size and in the permeation for Ca^{2+}, that obviously also varies under different experimental conditions since it is influenced by associated proteins and cofactors (Gees, Colsoul and Nilius. 2005).

Activation Mechanisms of TRP ion channels

Voltage

One of the best understood forms of ion channel gating is the voltage dependent activation, which involves a series of positively charged amino acids in the fourth transmembrane segment, S4, in voltage-gated K^+, Na^+ and Ca^{2+} channels. The S4 segment is able to sense changes in the electric field across the plasma membrane and to modify channel form in response to this stimulus (Bezanilla, 2008; Sigworth, 1994; Yellen, 1998). A large number of TRP channels are sensitive to voltage differences, however the activation process of those TRP exhibits some unique features not present in other voltage-gated channels. Indeed, the voltage sensitivity of TRP is quite low if compared to other K^+, Na^+ and Ca^{2+} channels (Voets *et al.,* 2004; Matta and Ahern, 2007). Then, most of TRP channels lack charged residues in S4 necessary for voltage sensing, that also explains the low voltage sensitivity. This suggests that the molecular mechanism underlying the observed voltage dependence in most TRP channels may have a different structural basis. Finally, the voltage range in which TRP channels exhibit sensitivity often falls far beyond the physiological range a cell normally experiences. Despite their low voltage sensitivity that often falls in non physiological range, response to change in membrane potential seems to be important for TRP functions. This happens because voltage response can be highly affected by other stimuli sensed by the channel, shifting the voltage range at which the channel is responsive, making a voltage change in the physiological range relevant to channel function (Voets *et al.,* 2004; Zheng, 2013).

Mechanical force

Transmembrane differences in osmolarity lead to cell swelling or shrinkage, resulting in a great variation in membrane tension, which can be also generated by direct pressure applied on the cells. In the cells there are a great number of ion channels able to sense mechanical stimuli, comprehending also some TRP ion channels. TRP channels appear to respond not to the mechanical force per se, but instead to intracellular signaling molecules generated by mechanical

stimuli to the cell (Blount *et al.,* 1997; Kloda *et al.,* 2008; Sachs *et al.,* 1990; Spencer *et al.,* 1999).
The most studied osmosensitive TRP channel responsive to hypotonic shock is TRPV4, which is expressed in kidney, liver, heart, lung and neurons (Strotmann *et al.,* 2000; Liedtke *et al.,* 2000; Wissenbach *et al.,* 2000). Again, it seems that activation of TRPV4 after cell swelling is achieved biochemically, mediated by intracellular molecules produced by membrane bound mechanosensitive proteins (Xu *et al.,* 2003).

Calcium and calmodulin

Most TRP channels are permeable to Ca^{2+}. Opening of TRP channels initiates Ca^{2+} translocation into the cytosol, leading to a transient increase in intracellular Ca^{2+} concentration, probably only near to the channel site. Ca^{2+}-triggered signaling events are important for many signal transduction processes, such as the PLC-mediated pathways, the release of intracellular Ca^{2+} store, and the PIP_2-mediated pathways (Numata *et al.,* 2007).
A large number of TRP channels are known to be regulated by calmodulin and this represents an important link between intracellular Ca^{2+} dynamics and channel activity. The calmodulin dependent regulation is very interesting for what concern those TRP channels that also permeate calcium ions. In these cases, Ca^{2+} cross the membrane activates calmodulin in the cytosol that in most case exerts an inhibitory effect on channel activity (Liu *et al.,* 1994).
In addition, activation of CaM by Ca^{2+} influx following TRP channel activation can affect channel function indirectly through the binding of CaM to other proteins such as CaM-sensitive kinases (Tokumitsu *et al.,* 2000). Furthermore, binding of CaM to a TRP channel can occur in the absence of Ca^{2+}. Indeed apo-CaM may be constitutively associated with certain TRP channels, such as TRPV1 (Rosenbaum *et al.,* 2004). In this case, binding of Ca^{2+} to CaM serves as a molecular switch to initiate the regulation process.
A calmodulin binding site is often present In the N- and C-terminus of TRP channel (Rhoads and Friedberg 1997).
In TRPC4α sequence many calmodulin binding sites have been observed, one of which is known to also bind the IP_3 receptor (IP_3R). This particular site is called CIRB (Calmodulin and IP_3R binding) site and is also present in several other TRP channels (Birnbaumer *et al.,* 1996).

Binding of calmodulin can also disrupt intracellular interactions that exist at low Ca^{2+} concentrations, as is the case for cyclic nucleotide-gated channels (Varnum and Zagotta 1997). As the C-terminal calmodulin binding sites are often found to overlap with the proposed PIP_2-binding sites, it is likely that competitive binding of calmodulin and PIP_2 underlies at least some of the observed calmodulin effects on channel gating (Gordon-Shaag *et al.,* 2008).

TRP subfamilies

TRPML family and TRPML1, TRPML2, TRPML3

The TRPML ("Mucolipin") family consists of three mammalian proteins (TRPML1–3) of which TRPML1 (or MCOLN1) was identified as the product of the gene that, if mutated, causes a severe lysosomal storage disease called mucolipidosis type IV (Sun *et al.,* 2000; Bach, 2001). Analysis of the protein encoded by the *MCOLN1* gene immediately revealed significant homology to the TRP superfamily of cation channels (Bargal *et al.,* 2000; Bassi *et al.,* 2000; Sun *et al.,* 2000; Slaugenhaupt *et al.,* 2002), thus MCOLN1 was also named TRPML1. Most mammals express two additional TRPML isoforms called TRPML2 and TRPML3 (Montell *et al.,* 2001). In contrast, a single yeast gene, called *YVC1/TRPY* has been proposed up to now as homologous of TRPML transporters (Venkatachalam *et al.,* 2007; Denis *et al.,* 2002; Dong *et al.,* 2010).
TRPML proteins are relatively small proteins (less than 600 a.a.) and share a distant homology with other TRP channels.
Early studies relying on the activity of TRPML1 present on plasma membrane revealed non-selective ion conductance with permeability to Ca^{2+} (La Plant *et al.,* 2002,2004).
TRPML1 is widely expressed, and appears to reside in late endosomes/lysosomes and mediates control of lysosomal Ca^{2+} levels that plays an important role in proper lysosome formation and recycling. This channel is permeable to Ca^{2+} as well as to Na^{+} and K^{+}, and is probably activated by increases in $[Ca^{2+}]_i$ (La Plant *et al.,* 2002,2004; Piper *et al.,* 2004). TRPML1 also contains a lipase domain in the intracellular loop between TM1 and TM2, a nuclear localization signal, and a putative late endosomal–lysosomal targeting signal (Sun *et al.,* 2000).

TRPMLs can also be trafficked from endolysosomes to the plasma membrane in a regulated and activity dependent manner. Constitutively active TRPML1 mutants localizes only in the plasma membrane, which is likely a consequence of lysosomal exocytosis owing to the elevated Ca^{2+} efflux via TRPML1 (Dong *et al.,* 2009). TRPML2 and TRPML3 function is not well known and still remain to be fully characterized. Those genes localize to intracellular vesicles and long-tubular structures and, similar to TRPML1, TRPML2 has been observed in the late endosomes/lysosomes compartment (Venkatachalam *et al.,* 2007; Song *et al.,* 2006). TRPML3 is very dynamic in the cell, and regulates multiple steps in the endolysosomal pathway and autophagy. Moreover, the three mammalian TRPMLs are capable of forming heteromultimers (Venkatachalam *et al.,* 2007; Zeevi *et al.,* 2008; Curcio-Morelli *et al.,* 2010). Once coupled into heteromultimers with either TRPML1 or TRPML2, the localization of TRPML3 is largely determined by either TRPML1 or TRPML2 indicating hierarchical control of TRPML subcellular distributions (Venkatachalam *et al.,* 2007).

Nevertheless, despite the potential for the mammalian TRPMLs to physically interact, these interactions may have limited overall biological significance since different TRPML are differentially expressed in different cell types. Interestingly, the hierarchical control of TRPML2 by TRPML1 in cells where they are co-expressed may also take the form of transcriptional regulation, indeed TRPML2 transcription correlates with the presence and/or activity of TRPML1 (Samie *et al.,* 2009).

All the TRPML channels tested so far are activated by phosphatidylinositol 3,5-bisphosphate ($PI(3,5)P_2$), a phosphoinositide enriched in endolysosomes. The findings that TRPMLs are activated by $PI(3,5)P_2$ are consistent with the notion that TRPMLs mainly function in endolysosomes (Dong *et al.,* 2008; Feng *et al.,* 2014).

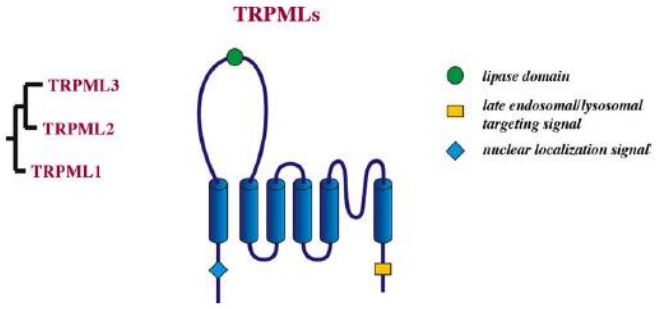


Fig. 12: *TRPML family (Pedersen, Owsianik, Nilius, 2005).*

TRPP family and PKD1, PKD2

The TRPP ("polycystin") family is structurally divided into two groups, (1) the polycystic kidney disease 1 (PKD1)-like proteins, comprising PKD1/TRPP1, PKDREJ, PKD1L1, PKD1L2, and PKD1L3, and the polycystic kidney disease 2 (PKD2)-like proteins, comprising PKD2/TRPP2, PKD2L1/TRPP3, and PKD2L2/TRPP5 (Delmas, 2004; Moran *et al.,* 2004).

PKD2 was discovered as the gene whose mutation cause the pathology of polycystic kidney (ADPKD) and, as well as *PKD1*, is widely expressed, while the other family members exhibit more restricted expression patterns (Mochizuki *et al.,* 1996; Delmas *et al.,* 2004,2005).

The PKD2 (polycystin 2)-like proteins have a predicted topology of six putative TM domains and a large extracellular loop between the first and second TM (Delmas *et al.,* 2004, 2005). They are cation permeable channels, usually with $P_{Ca}/P_{Na} > 1$, and exhibit significant homology to other TRPs, even if they are characterized by the absence of both a TRP domain or ankyrin repeats.

Several domains present in N- and C-termini of Pkd2 are responsible for Pkd2 protein–protein interactions and Ca^{2+} sensitivity. A naturally occurring truncation mutation that removes this C-terminal domain, and thus presumably abrogates all of its interactions and regulatory potential, is sufficient to cause ADPKD (Mochizuki *et al.,* 1996). At least two domains, one in each cytoplasmic tail, contribute to Pkd2 oligomerization and immediately after last transmembrane domain a functionally complex region is present that includes coiled-coil, EF-hand, and ER retention domains. Again a calcium-binding EF

hand domain begins upstream of and extends into coiled-coil region that is fundamental for Pkd1 interaction (Mochizuki *et al.,* 1996; Qian *et al.,* 1997; Celić *et al.,* 2008), and the helix-loop-helix structure of the EF-hand permits the protein to sense or to buffer changes in Ca^{2+} (Gifford *et al.,* 2007). The Pkd2 EF-hand has a single Ca^{2+}-binding site with micromolar affinity (Celić *et al.,* 2008). Then a sequence is present that is required for maintaining Pkd2 ER and Golgi localization (Cai *et al.,* 1999).

Pkd2 localizes to both motile and primary cilia, and recent evidence strongly implicates it as a mechanosensor in the nonmotile, primary cilia, but *PKD2* also appears to function as an intracellular Ca^{2+} channel (Cahalan, 2002).

Indeed, Pkd2 is a calcium-activated channel that releases calcium from intracellular stores in response to increases in cytosolic calcium concentrations (Vassilev *et al.,* 2001; Koulen *et al.,* 2002). Also *TRPP2/PKD2* homologous in yeast, *S. pombe Sppkd2,* is involved in a Ca^{2+} signaling pathway, cell wall synthesis and membrane trafficking and localizes in Golgi and plasma membrane. It seems to act as a mechanosensitive ion channel modulator regulated by calcium, even if the detail of its regulation are not clear (Aydar, Palmer, 2009).

The Pkd1 (polycystyn 1)-like proteins are structurally distinct from the channel-forming TRPP2-like TRPPs. Indeed, they are characterized by 11 putative TM domains, a very long extracellular domain and a C-terminal domain, which can interact with the C-terminus of Pkd2 (Wu *et al.,* 2000; Qian *et al.,* 1997; Tsiokas *et al.,* 1997). The large extracellular Pkd1 N-terminus contains 15 PKD repeat motifs, two complete leucine-rich repeat motifs flanked by cysteine-rich sequences, and a C-type lectin domain (Hughes *et al.,* 1995; Bycroft *et al.,* 1999). Many of these domains are crucial for Pkd1's functions and play established roles in protein–protein or protein–matrix interactions (van Adelsberg, 1999; Ibraghimov-Beskrovnaya *et al.,* 2000; Babich *et al.,* 2004; Streets *et al.,* 2009). TRPP1 has been proposed to work as a cation channel on its own (Babick *et al.,* 2004); however, more recent studies have excluded this possibility (Delmas *et al.,* 2004, 2005).

Actually, a great number of data indicates that Pkd1 and Pkd2 are physically coupled and act as a single signaling complex that may also participate in sensing fluid flow and pressure in the kidney (Patel and Honoré, 2010). This interaction is also necessary for Pkd2 plasma membrane localization (Hanoka *et al.,* 2000) even if Pkd2 has many functions that do not depend on Pkd1

interaction (Volk *et al.,* 2003). Indeed Pkd2 has been found in the same complex with a number of other TRPs, indicating that it could be involved in a great variety of functions (Tsiokas *et al.,* 1997).
The Pkd1, Pkd2 proteins modulate diverse signaling pathways, and there is a long list of proteins known to interact with them (Somlo *et al.,* 2001). However, the interaction between Pkd1 and Pkd2 is known to regulate many cellular signaling pathways such as negative growth regulation, G protein activation, Wnt pathway modulation and the regulation of differentiation, apoptosis and proliferation in a NFAT and calcineurin dependent manner (Horsley and Pavlath, 2002).
The interaction between Pkd1 and G proteins is known to activate the nuclear factor of activated T cells (NFAT) that is also connected to calcium signaling and Pkd2 localization. NFAT is activated by calcineurin, which is activated by elevation of cytosolic Ca^{2+} levels. Calcineurin, once activated, can dephosphorylate NFAT, leading to its nuclear accumulation; NFAT is then phosphorylated by glycogen synthase kinase 3β (GSK-3β) causing NFAT to move back into the cytoplasm. It is likely that Pkd1 could activate calcineurin through the action of G proteins, ending in NFAT relocation into the nucleus (Horsley and Pavlath, 2002).
Moreover, studies in *C. elegans* provided the evidence of a direct interaction between Pkd2 and calcineurin, since calcineurin-mediated dephosphorylation of Pkd2 permits its ciliary localization (Hu *et al.,* 2006).

Calcium related Spray protein in *Neurospora crassa*

Numerous studies have shown that calcium ions have an important role in polarized growth of cells. Indeed, gradients of calcium concentration have been reported to be present in a great variety of biological structures such as root hairs, pollen tubes and fungal hyphae (Ehrhardt *et al.,* 1996; Hepler, 1997; Silverman-Gavrila and Lew, 2000). The *spray* (*sp*) protein takes its name from a mutant phenotype, characterized by a slow growth, hypersensitivity to calcineurin inhibition, and a dense dichotomous branching spreading out from the colony center in a pattern resembling a spray of flowers or of water (Perkins, 1959).
Originally the *spray* gene was thought to encode an enzyme related to cell wall synthesis, because in the mutant carrying the deletion of this gene the protein

content in cell walls was only half that of a wild type strain (Wrathall and Tatum, 1974). However, addition of 50–500 mM of exogenous Ca^{2+} is sufficient to correct the mutant phenotype, thus the gene seems to have a role in regulating Ca^{2+} homeostasis in *N. crassa* (Dicker and Turian, 1990).

spray is a large gene, spanning more than 5 kb from promoter to termination signals. The gene structure has an ORF of 3452 bp and encodes for a protein of 1144 amino acids. The protein is composed of 48% nonpolar and 52% charged residues and sequence analysis revealed the presence of eight putative transmembrane domains indicating that Spray is likely a membrane protein.

A bioinformatics analysis revealed a similarity of part of the Spray sequence with uncharacterized hypothetical membrane proteins from *S. cerevisiae* (*ACS1/GCV3*, *YPL221w*, *YGL139w*, *MRF1/SEC27*, and *YOR365c*) and from *Schizosaccharomyces pombe* (SpPkd2) enforcing the hypothesis that Spray localizes in cellular membranes, although the lack of homology with Spray and plasma membrane calcium transporters (Cch1 for example) could indicate a possible intracellular localization. It is likely that it could be located in the endoplasmic reticulum, calcium-storing vesicles, vacuoles, or mitochondria. Indeed in the Spray mutant, the Ca^{2+} capacity to restore defective mutant could be due to an organellar depletion of calcium stores, also making the mutant particularly sensitive to calcineurin inhibition (Bok *et al.,* 2001).

Finally, a further evidence for a connection between the function of the Spray protein and the calcineurin-mediated regulation of Ca^{2+} transport is their similar effect on asexual development. Conidiation of the Spray mutant is not induced by conidiating medium (Delvecchio and Turian, 1968) as well as in a strain with dysfunctional calcineurin catalytic subunit (Prokisch *et al.,* 1997). Even if the role of Spray protein is not clear yet, its requirement for calcium homeostasis in *N. crassa* is likely (Bok *et al.,* 2001).

CALCIUM RELATED DISEASE

Mutations in many calcium channels and transporters result in a large number of human diseases, collectively termed channelopaties, for example Brody Myopathy, a neuromuscular disease, which clinically manifests as a cramping of skeletal muscles post activity caused by a mutation in the Pmc1 homolog ATP21p, a subunit of the calcium ATPase.

Two diseases related to mutations in genes encoding for TRPP or TRPML calcium channels in humans will be discussed in detail below. Mucolipidosis type IV (MLIV) is a lysosomal storage disorder characterized by psychomotor retardation and visual abnormalities, caused by mutations in MCOLN1/TRPML1. The polycystic kidney disease (PKD) is a genetic disorder of the kidneys, linked to calcium transport and homeostasis and characterized by the presence of a polycystic kidney, caused by mutations in a calcium channel, Pkd2, or the related protein Pkd1.

Polycystic Kidney disease (PKD)

Polycystic kidney disease (PKD) is an inherited disorder in which clusters of cysts develop primarily within kidneys. Cysts are noncancerous round sacs containing water-like fluid. The cysts vary in size and, as they accumulate more fluid, they can grow very large. Although kidneys usually are the most severely affected organs, polycystic kidney disease can cause cysts to develop in the liver and elsewhere. The disease causes a variety of serious complications such as high blood pressure and kidney failure.

There are two types of PKD: autosomal dominant polycystic kidney disease (ADPKD) and the less-common autosomal recessive polycystic kidney disease (ARPKD).

Autosomal recessive polycystic kidney disease (ARPKD) is a cause of significant renal and liver-related morbidity and mortality in children. The majority of individuals with ARPKD classically present in the neonatal period with enlarged echogenic kidneys. More than 50% of children with ARPKD who have a classic presentation progress to end-stage renal disease within the first decade of life. One of the worst consequences of ARPKD is pulmonary hypoplasia, which occurs in a number of affected infants. Approximately 30% of these infants die in the neonatal period or within the first year of life from respiratory insufficiency or pulmonary infections. The diagnosis of ARPKD is based on clinical findings in the proband and the absence of renal disease in the proband's biological parents. *PKHD1* is the only gene in which mutation is known to be associated with ARPKD (Bisceglia *et al.,* 2006).

This gene encodes for a 67-exon transcript for a large protein of 4074 amino acids termed fibrocystin, but, even if its function remains unknown, its seems to be implied in calcium homeostasis (Yang *et al.,* 2007).

Autosomal dominant polycystic kidney disease (ADPKD) is the most common of all the hereditary cystic kidney diseases, indeed the massive enlargement of the kidney and the substitution of an irregular profusion of glistening cysts for its usual striated architecture afflict approximately 1 in 1000 individuals (Torres, 1998; Calvet and Grantham, 2001; Grantham, 2001; Igarashi and Somlo, 2002; Wilson, 2004). Cysts increase in size and number over the space of decades, displacing and destroying adjacent renal parenchyma, leading ultimately to end-stage renal disease in 50% of cases (Gabow, 1993).

This pathology is caused by the mutation of *PKD1* (85% of cases) or *PKD2* (15% of cases), that localize to the primary cilium. Thus, ADPKD is the founding member of the "ciliopathies," a recently defined class of genetic disorders that result from mutations in genes encoding cilia-associated proteins. These disorders are often characterized by the presence of renal cysts as well as by additional pathologies including neural tube defects, retinal malformations, and polydactyly (Badano *et al.,* 2006).

The kidneys of an ADPKD patient who inherits one mutated copy of Pkd1 or Pkd2 will develop and function normally into adulthood. Over time, however, cysts will form in this patient's kidneys and several studies suggest that the cells that line these cysts will have lost both functional copies of a polycystin gene (Qian *et al.,* 1997; Brasier and Henske, 1997). This indicates that an additional somatic mutation may cause cysts to form. According to this model, each cyst arises because of a distinct somatic mutation event, explaining the disease's slow progression over the course of decades (Lu *et al.,* 1997; Reynolds *et al.,* 1999; Pritchard *et al.,* 2000; Lantinga-van Leeuwen *et al.,* 2004; Rossetti *et al.,* 2009).

Mucolipidosis

The mucolipidoses (ML) are a group of inherited metabolic diseases that affect the body's ability to carry out the normal turnover of various molecules within cells. In ML, abnormal amounts of carbohydrates and lipids accumulate in cells.

The mucolipidoses derived their name from the similarity in symptoms presentation to both mucopolysaccharidoses and sphingolipidoses.

A biochemical understanding of these conditions has changed how they are classified. Although four conditions (type I, II, III, and IV) have been labelled as mucolipidoses, type I (sialidosis) is now classified as a glycoproteinosis, and

type IV (Mucolipidosis type IV) is now classified as a gangliosidosis (McMillan *et al.,* 2006).

Mucolipidosis type IV (MLIV) is a lysosomal storage disorder (LSD) presenting unique features in the LSD group, either in the clinical picture, or in the nature of the basic metabolic defect. This disease is a neurodegenerative disorder characterized by severe psychomotor retardation, ophthalmological abnormalities, iron deficiency and achlorohydria. This syndrome has extremely slow clinical progression and, if plasma iron levels are balanced appropriately, patients can live for over four or five decades (Altarescu *et al.,* 2002; Bach, 2001, 2005). MLIV was defined as a mucolipidosis due to the simultaneous lysosomal accumulation of membranous lipid substances together with granulated water-soluble materials in cells of every tissue and organs of the body (Merin *et al.,* 1975; Newell *et al.,* 1975; Tellez-Nagel *et al.,* 1976; Folkerth *et al.,* 1995). Chemical analyses indicated the storage of a heterogeneous group of lipids, including gangliosides, phospholipidosis and acid mucopolysaccharides, together with various glycoproteins (Bach, 2001). By the way this storage in MLIV apparently does not stem from a defective or deficient lysosomal hydrolase activity or a related protein, which is the cause in most other LSDs, but rather from a defect probably in the biogenesis of lysosomes (Bargal *et al.,* 1997; Chen *et al.,* 1998; Pryor *et al.,* 2006).

Most of the mutations in the *MCOLN1* (Mucolipin-1) gene cause mucolipidosis type IV. Mucolipin-1 acts as a channel, allowing positively charged atoms (cations) to cross the membranes of lysosomes and endosomes. A lack of functional mucolipin-1 impairs transport of lipids and proteins, causing these substances to build up inside lysosomes, ending in MLIV (Bargal *et al.,* 2000; Bassi *et al.,* 2000; Sun *et al.,* 2000).

MCOLN1 shares a great sequence homology with both *MCOLN2* and *MCOLN3*, whose function as cation channels is not already clear. Although only mutation in *MCOLN1* gene is known to cause human disease (Bargal *et al.,* 2000).

MATERIALS AND METHODS

Strains

Escherichia coli

DH5α: *deoR, AndA1, gyrA96, hsdR17 ($rk^- mk^+$), recA1, relA1, supE44, thi-1, Δ (lacZYA-argFV169), ϕ80lacZΔM15, F^-.*

Saccharomyces cerevisiae

Strain	Main genotype	Reference
K601 (W303-1A)	*MATα ade 2-1 can 1-11 his 3-11,15 leu 2-3,112 trp 1- 1 ura 3-1*	Locke *et al.*, 2000
ELY117	K601 *cch1::TRP1*	Locke *et al.*, 2000
ELY138	K601 *mid1::LEU2*	Locke *et al.*, 2000
ELY151	K601 *mid1::LEU2 cch1::TRP1*	Locke *et al.*, 2000
RT960	K601 *yvc1::Sphis5*	Groppi *et al.*, 2011
RT973	K601 *mid1::LEU2 cch1::TRP1 fig1::Sphis5*	Groppi *et al.*, 2011
RT974	K601 *mid1::LEU2 cch1::TRP1 fig1::Sphis5 yvc1::KanMX4*	Groppi *et al.*, 2011
RT1170	K601 *cnb1::Sphis5*	Groppi *et al.*, 2011
RT1172	K601 *mid1::LEU2 cch1::TRP1 cnb1:: Sphis5*	This work
RT1251	K601 *ecm7::Sphis5*	This work
RT1252	K601 *cls2::Sphis5 yvc1::KanMX4*	This work
RT1253	K601 *cls2::Sphis5*	This work
RT1255	K601 *yor365c::Sphis5*	This work
RT1256	K601 *mid1::LEU2 cch::TRP1 fig1::Sphis5*	This work

	yor365c::KanMX4	
RT1257	K601 *flc2::Sphis5*	This work
RT1258	K601 *mid1::LEU2 cch::TRP1 fig1::Sphis5 flc2::KanMX4*	This work
RT1259	K601 *yor365c::Sphis5 flc2::KanMX4*	This work
RT1330	K601 *crz1::Sphis5*	This work
RT1300	K601 *flc1::Sphis5*	This work
RT1310	K601 *flc3::Sphis5*	This work
RT1311	K601 *flc1::Sphis5 flc3::KanMX4*	This work
RT1312	K601 *flc2::Sphis5 flc3::KanMX4*	This work
RT1290	K601 *yor365c::YOR365-eGFP-Sphis5*	This work
RT1313	K601 *mid1::LEU2 flc2::KanMX4*	This work
RT1314	K601 *cls2::Sphis5 flc2::KanMX4*	This work
RT1315	K601 *mid1::TRP1 cch1::LEU2 fig1::Sphis5 flc1::KANMX4*	This work
RT1316	K601 *mid1::TRP1 cch1::LEU2 fig1::Sphis5 flc3::KANMX4*	This work

Plasmids

pVTU-AEQ: multicopy plasmid used in luminescence assays, carrying apo-equorin sequence (Trópia *et al*., 2006).
pAMS366: multicopy plasmid containing *4xCDRE::lacZ* reporter (Stathopoulos and Cyert, 1997).
p1366: multicopy plasmid carrying Mpk1 responsive *PRM5::LacZ* reporter
pMCZ-Y: multicopy plasmid carrying *UPRE::LacZ* reporter

GROWTH CONDITION AND CULTURAL MEDIUM COMPOSITION

Escherichia coli

E. coli strains can be conserved for many years at -80°C in cultural medium or in an aqueous solution containing 50% glycerol, and for shorter periods (weeks) in liquid culture or in plates of LB medium (0.1% glucose, 1% NaCl, 1% Bacto-

peptone and 0.5% yeast extract by Biolife, USA. For solid medium 1.5% agar was added) at 4°C. Cells were grown with shaking, at 37°C.
For AmpR selection, ampicillin was added to a final concentration of 50 mg/L.

Saccharomyces cerevisiae

S. cerevisiae strains can be conserved for many years at -80°C in an aqueous solution containing 15% glycerol, and for shorter periods they are maintained on plates containing the appropriate cultural medium at 4°C. Culture strains conserved in that way are refreshed every 2 weeks, inoculating them from the previous cultures.
Yeast strains were grown in YPD medium (2% glucose, 2% Bacto-tryptone, 1% yeast extract and 2% agar for solid medium by Biolife, USA, supplemented with 50 mg/L adenine) or in minimal medium (2% glucose, 0.67% Yeast nitrogen base w/o aminoacids YNB by Difco, USA, and supplemented with 50 mg/L adenine, histidine, leucine and tryptophan) with shaking at 30°C.
Yeast strains carrying a plasmid were grown in selective medium, prepared with complete supplemented synthetical medium, containing 2% glucose, 0.67% YNB, the appropriate CSM drop-out (BIO101, USA) at 0.62 g/L, 2.5% agar for solid media (Biolife, USA) supplemented with 50 mg/L tryptophane.

Growth curves

Cell density was determined by measuring optical density at 600 nm (OD600) or by Coulter Counter (Coulter Electronics Z2).

MUTANT STRAIN CREATION

CRZ1, ECM7, CLS2, *YOR365C*, *FLC2*, *FLC1* and *FLC3* genes were deleted in wild-type or mutant strains indicated in Table 1 using a disruption cassette generated by PCR using pFA6a-His3MX4 plasmid, containing an expression cassette for the heterologous marker *his5*$^{+}$ (Watch *et al.,* 1997) from *Schizosaccharomyces pombe*, as a template. The primers are listed in the primer table. 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, described in Wach *et al*., 1997, 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 strains 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 (Reinke *et al.,* 2004) was a kind gift from B. Glick (University of Chicago, USA). The YIplac204T/CHmg1DsRED plasmid was previously described (Belotti *et al.,* 2012).

For luminescence assay yeast cells were transformed by lithium acetate method with the multicopy pVTU-AEQ plasmid (Tropia *et al.,* 2006). For β-galactosidase assay yeast cells were transformed either with multicopy pAMS366 plasmid (containing *4xCDRE::LacZ* reporter) (Stathopoulos, Cyert, 1997), a kind gift from M. Cyert (Stanford University, CA), or with p1366 (carrying Mpk1 responsive *PRM5::LacZ* reporter) (Jung *et al.,* 2002), kindly provided by D. Levin (John Hopkins University, MD), or with pMCZ-Y (carrying *UPRE::LacZ* reporter) (Mori *et al.,* 1996), 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.

Gene	Primer	Sequence
CNB1	Cnb1pFA6aFOR	TTAAAAATCACTAGTTTCTTTTTTAGCGGAA TGCAATAAACGGATCCCCGGGTTAATTAA
CNB1	Cnb1pFA6aREV	CGTATTATTCTTCTTTTCTTAAAAATATTGG CATACCATATGGATCTGATATCATCGATG
ECM7	ECM7pFA6aFOR	TTAGTTTCATTTACACCATTTCTTTGTGTATC AGTCATTGCGGATCCCCGGGTTAATTAA
ECM7	ECM7pFA6aREV	TTTTCTCTTCTTCGTTGCTTAATGATGTTTAG TTCTGAAATGGATCTGATATCATCGATG
CLS2	CLS2pFA6aFOR	TGAAAGAAATGTTTGTAGGCCATTTTCTTCC AGAACAGATCGGATCCCCGGGTTAATTAA
CLS2	CLS2pFA6aREV	GAAGAACATCCTAGTAATTGTCTTGAAAAT AATCAAGATATGGATCTGATATCATCGATG
YOR365C	YOR365CpFA6aFOR	ATATTTTGGAAGGATGATGAAGCAAAGTCG TAATGCTATTCGGATCCCCGGGTTAATTAA
YOR365C	YOR365CpFA6aREV	ATTTAGTTCCGAGATGGTCTTACAACCTTTC AAATTCCAATGGATCTGATATCATCGATG
YOR365C	YOR365CeGFP-FOR	CCTTTGTAAACAAAATTCGAACTGTGAATAT CTGTATATGGGAGCAGGTGCTGGTGCT
YOR365C	YOR365CeGFP-REV	TCCAAAATCAGAAGAAATTTTGCAGGAAGG CAAATCCTACTCTGGGCAGATGATGTCGAG
FLC2	YAL053WpFA6aFOR	CATTACGATTATATTGACGTGATAAAAAGA TTATATAGCCCGGATCCCCGGGTTAATTAA
FLC2	YAL053WpFA6aREV	TATTTATATAATAAGTTGTTACATGTGAGTA TATATTGGATGGATCTGATATCATCGATG
FLC2	FLC2GAP-REP For	GCTCTAGAGCGTTCATACTCATGTTCCTTCT TCTGCTGCGCAGTGGCGGCGCGGATCCGCG CGGGATCCCG
FLC2	FLC2GAP-REP Rev	GCGAATTCGCGATATCCGTACATTTAAGGA AGCAGAGCCAAAGCGAATTTCACATTCGGG ATCCCGCGCGGATCCGC
FLC2	FLC2GenomeFor	ACTCATGTTCCTTCTTCTGCTGCGC
FLC2	FLC2GenomeRev	CATTTAAGGAAGCAGAGCCAAAGCG
CRZ1	CRZ1pFA6aFOR	TTAGTCTCGATTGGAAGTTTCGTCAGACAG TACAAGGAAGCGGATCCCCGGGTTAATTAA

CRZ1	CRZ1pFA6aREV	AAAAAAAATTCCTATTCAAAGCTTAAAAAA ACAAAAATAATGGATCTGATATCATCGATG
FLC1	FLC1pFA6aFOR	CATTATTGACACACATACCCGACCAAAAAC GGCGTTAAGACGGGATCACTCTCGGCAT
FLC1	FLC1pFA6aREV	AATTACGTTGTTCTTTTTACCTTTATCCCATC GACAAAGCAGCGAGGAAGCGGAAGAG
FLC3	FLC3pFA6aFOR	AACAAATAGTGGAGACTAGAAAAGTATACC CCTCACAGGCCGGGATCACTCTCGGCAT
FLC3	FLC3pFA6aREV	TTTTTATGATAGGAGCAGAACTTTTGTATAT CGTAAAGACAGCGAGGAAGCGGAAGAG

CELLULAR AND MOLECULAR BIOLOGY TECHNIQUES

"MIDI-prep" extraction of plasmid DNA from E. coli

1. Inoculate 50 ml of *E. coli* culture at 37°C overnight.
2. Centrifuge cells at 4000 rpm for 30 min.
3. Resuspend the bacterial pellet in 4 ml of ice-cold Lysis buffer and incubate the tube on ice for 10 min. (Lysis buffer glucose 50 mM, EDTA 10 mM ,Tris-HCl (pH 8) 25 mM)
4. Add 8 ml of freshly prepared 0.2 N NaOH/ 1% SDS solution, mix and store the tube 10 min. at room temperature.
5. Add 6 ml of ice-cold 5 M HSS and mix the content by vortexing. Store the tube on ice for 10 min.
6. Centrifuge at 4000 rpm at 4°C for 10 min.
7. Pour the supernatant into a clean tube through a small two-ply square of cheesecloth placed in the centre of a funnel.
8. Precipitate the nucleic acids by adding equal volume of isopropanol to the tube and store it at -20°C for 15-20 min. Centrifuge at 4000 rpm at 4°C for 30 min.
9. Discard supernatant and dry the pellet as well as possible.
10. Dissolve the pellet in 1 ml of TE buffer, add 1 ml of 5 M LiCl and mix the content by inverting the tube.
11. Incubate the tube 30 min. on ice.
12. Centrifuge at 13000 rpm at 4°C for 10-15 min.

13. Pour off the supernatant containing plasmid DNA into a clean tube. Add 0.1 volume of 3 M NaAc pH 5.2 and a volume of isopropanol.
14. Precipitate the nucleic acids at -20°C for 10-20 min.
15. Centrifuge at 13000 rpm at 4°C for 20 min. and dry the pellet.
16. Resuspend the pellet in 500 µl of TE containing DNAse-free pancreatic RNAse (40 µg/ml). Vortex briefly. Store at 37°C for 20 min.
17. Extract proteins from the plasmid DNA adding an equal volume of phenol/chloroform/isoamyl alcohol. Vortex vigorously for 30 seconds. Centrifuge at full speed for 5 min. at room temperature.
18. Remove upper aqueous layer containing the plasmid DNA carefully and repeat passage 17.
19. Add 5 M KAc (final 0.3 M) and 2 volumes of absolute ethanol to precipitate the plasmid DNA.
20. Store at -80°C for 15-30 min. Centrifuge at full speed for 15 min. at 4°C. Dry the pellet.
21. Dissolve the pellet in 50-100 µl of TE or H2O.
22. Measure the concentration of the plasmid DNA by gel electrophoresis.

DNA gel electrophoresis

Gel electrophoresis allows to separate a mixture of molecules through a stationary material (gel) in an electric field. Agarose gel is usually used as a support for separation of the DNA fragments.

Agarose powder was dissolved in TAE buffer (pH 8): Tris-HCl 242 g, glacial acetic acid 57.1 ml, EDTA 18.61 g water to 1 l.

Gels were run in the same buffer at 4-6 Volt/cm for 1-2 hours.

DNA samples were prepared by mixing with a “loading buffer” containing 50% glycerol and 0.25% bromophenol blue.

Molecular weight markers, a mixture of DNA fragments with known molecular weights, are used to estimate the sizes of DNA fragments in the sample.

DNAs were visualized with UV light by staining the gel with ethidium bromide 5 µg/ml.

Rapid extraction of total DNA from *S. cerevisiae*

1. Inoculate 4 ml of *S. cerevisiae* culture in YPD medium and incubate culture at 30°C overnight, until the late exponential phase (2-8 x 107 cells /ml).
2. Centrifuge cells at 4000 rpm for 15 min.
3. Discard the supernatant and resuspend cells in 200 µl of SZB buffer (sorbitol 1 M, Na citrate 0.1 M, EDTA 0.06 M, pH 8, 2-mercaptoethanol 115 mM, zymoliase 100000 U/g)
4. Incubate at 37 °C for 40 min., with shaking.
5. Centrifuge at 2000 rpm for 10 min.
6. Discard the supernatant and resuspend the pellet in 100 µl SDS/TE (SDS 2%
1. Tris-HCl 100 mM, pH 9, EDTA 10 mM) and mix by inverting.
7. Incubate at 60-65°C for 15 min.
8. Add 100 µl 6 M KAc and mix by inverting.
9. Keep cells on ice for 30-60 min.
10. Centrifuge at 13000 rpm for 10 min. and transfer the supernatant in a clean tube. Repeat this passage.
11. Add 160 µl of 5 M ammonium acetate and 650 µl of ice-cold isopropanol, mixing after each addition.
12. Incubate at -80°C for 15-30 min., better overnight.
13. Centrifuge at 13000 rpm for 10 min.
14. Discard the supernatant and wash the pellet with 70% ethanol. Centrifuge again at 13000 rpm for 10 min.
15. Discard the supernatant and dry carefully the pellet.
16. Resuspend the pellet in 20 µl of dH2O.

Transformation of *S. cerevisiae* with LiAc method

1. Inoculate 50 ml of *S. cerevisiae* culture in YPD medium and incubate culture at 30°C overnight until OD600 of 0.4-1.5.
2. Harvest the cells by centrifugation at 4000 rpm for 10 min. (10 OD cells/treatment needed).
3. Wash the cells with 25 ml of sterile H2O. Centrifuge again at 4000 rpm for 10 min.

4. Resuspend the pellet in 1 ml of 0.1 M LiAc and transfer the cells into a 1.5 ml microcentrifuge tube.
5. Centrifuge at 13000 rpm for 15 sec.
6. Resuspend the pellet in 100 mM LiAc at 2 x 109 cells/ml.
7. Transfer 50 µl of the cell suspension in new 1.5 ml tubes.
8. Add to each sample the transformation mix containing the adequate volume of plasmid DNA (0.1-10 µg), 240 µl of polyethylene glycol (PEG) 4000 (50% w/v), 31 µl of 1 M LiAc, 5 µl of salmon sperm DNA (Sigma) and sterile H2O to volume. Mix by vortexing.
9. Incubate samples at 30°C for 30 min.
10. Heat shock cells at 42°C for 15 min.
11. Centrifuge at 6000 rpm for 2 min., discard the supernatant and wash cells with 1 ml of sterile H2O.
12. Resuspend the pellet in 50 µl of sterile H2O, spread the cells on the appropriate selective medium plates and incubate for 2-3 days at 30°C.

In vivo bioluminescence assay to determine intracellular Ca^{2+} level

All the yeast strains analyzed were transformed with pVTU-AEQ plasmid, carrying the sequence of the non-functional apoequorin. In the presence of Ca^{2+} ions and the synthetic cofactor coelenterazine, the functional bioluminescent aequorin protein is reconstituted: Ca^{2+} binding to aequorin generates a transient bioluminescence with the emission of light at 466 nm, caused by coelenterazine oxydation to coelentheramide. The light emission, proportional to the cytosolic Ca^{2+} level, can be monitored through a luminometer (Berthold Lumat LB 9507).

Monitoring intracellular Ca^{2+} level in response to hypotonic shock

1. Inoculate yeast strains in YPD medium (6 OD/treatment) from a culture grown overnight in SD (-URA) medium and grown overnight at 30°C.
2. Harvest exponentially growing cells (5-6 x 106 cells/ml) by centrifugation at 4000 rpm for 10 min.
3. Resuspend the cells in YPD medium at a density of about 108 cells/ml. Transfer the cellular suspension into a microcentrifuge tube.
4. Centrifuge at 7000 rpm for 2 min. Discard the supernatant.

5. Resuspend the cells in 20 µl of YPD medium for each treatment.
6. Add 50 µM coelenterazine (stock solution 1 µg/µl dissolved in 99.5% methanol, conserved in the dark at -20°C, Molecular Probes).
7. Incubate at room temperature in the dark for 20 min.
8. Centrifuge at 7000 for 2 min. Discard the supernartant in order to eliminate the excess coelenterazine.
9. Wash three times with YPD medium (200 µl/wash) and by centrifugation at 7000 rpm for 1-2 min.
10. Resuspend the cells in the adequate volume of YPD medium, (200 µl/treatment).
11. Transfer the cellular suspension into the luminometer tubes (200 µl each), in the presence of 1 mM of the calcium chelator ethylene glycol tetraacetic acid (EGTA) (stock solutions prepared in H2O) and of solution of $CaCl_2$ at the final concentrations indicated in the legends of the figures.
12. Monitor light emission with the luminometer at intervals of 5 s for 1 min. before (until the signal is stable) and for at least 3 min. after the addition of 4 volumes of H2O. Light emission is reported in relative luminescence units (RLU/s).

Free calcium concentration in the medium was estimated by Maxchelator (http://www.stanford.edu/~cpatton/maxc.html), considering ion concentrations evaluated by Loukin and Kung, 1995. Light emission was recorded with the luminometer at intervals of 5 s for at least 1 min. before (until the signal is stable) and for at least 3 min. after the addition of 4 volumes of water, and reported in calcium concentration according to Blinks *et al.*

At the end of each experiment aequorin expression and activity was tested by lysing cells with 0.5% Triton X-100 in the presence of 10 mM $CaCl_2$ (stock solution 100 mM $CaCl_2$), 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 just in order to avoid overlay of the lines.

Parameters estimation of the calcium transport was performed as previously described with the difference that in the experiments utilized for these estimations the 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 investigated by polynomial mixed effect model.
To transform RLU in $[Ca^{2+}]$ we took advantage of the calibration curve reported in Blinks et al., (1978), which could be approximated by the relation:

$$Log_{10}[Ca^{2+}](M) = 0,4612*Log_{10}(L/L_{max}) – 4,1935$$

with a linear correlation coefficient R^2 of $R^2 = 0,9948$.

β-galactosidase reporter assays

Exponentially growing cells (5-6 x 10^6 cells/ml) in YPD were harvested by filtration on nitrocellulose filters (Millipore, pore sizes 0.22µm), washed twice with cold water (1 volume/wash) and then resuspended in fresh medium at a cell density of 1.25 x 10^7 cells/ml. To measure the β-galactosidase activity in exponentially growing cells, the method described by Kiechle *et al.* was used. Briefly, 200 µl of the cell suspension ('exp', for exponentially growing treatment) were 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 separated and collected and processed as described above.
For detection, 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 and after incubation for suitable time (t_{inc}) at 37°C

the reaction was stopped with 200 µl of 1M Na_2CO_3. Then samples were centrifuged at 13000 rpm for 15 min and the supernatant was read at 574 nm. β-galactosidase activity was calculated as follows and expressed in Miller Units (MU):

$$MU = [(OD_{574} - OD_{574}\ ctrl) \times 1000] / (OD_{600} \times t_{inc})$$

Each value is the average of at least three independent experiments of the same strain, each with at least double dosage. Student t test was used to assess significance of the observed differences compared to the respective control.

Epifluorescence microscopy

Cells were grown at 30°C in the proper selective SD medium 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).

RESULTS

Hypotonic shock induced calcium signalling mainly involves calcium efflux from intracellular stores

Hypotonic shock (HTS) was reported to induce a transient calcium peak in yeast cells growing on a synthetic complete medium. This cytoplasmic calcium raise was reported to derive initially from intracellular stores, while influx of calcium from extracellular environment was thought to be fundamental for the maintainance of the transient peak (Batiza *et al.,* 1996).

Calcium availability is different in synthetic or rich media; since this condition is known to regulate calcium transporters functionality in yeast (REF) we tested hypotonic shock induced calcium signal in cells grown on a rich medium (YPD) (Muller *et al.,* 2001; Groppi *et al.,* 2001; Trópia *et al.,* 2006).

In these conditions, it is possible to observe the presence of two different components that contribute to the HTS-driven increase in cytosolic calcium concentration (Fig. 13A e B). The first component drives a fast increase in intracellular calcium, reaching the maximum peak only after 5-10 s after water addition. Moreover, this fast calcium response is inhibited by high concentrations of extracellular calcium but cannot be observed if external calcium is not available, indicating that cytoplasmatic calcium raise in these condition is due to an influx of calcium from extracellular environment.

The second component drives a slower $[Ca^{2+}]_i$ increase, reaching its maximum 20-30 s after HTS, which is present only when cells are exposed to EGTA, an extracellular calcium chelator, in order to reduce free calcium concentration in the medium to the submicromolar range. The same results were obtained when BAPTA or EDTA were used instead of EGTA, indicating that while the fast response is probably due to a calcium influx from external medium, the slowest peak should be rather related to a calcium release from intracellular stores, which is peculiarly inhibited by extracellular calcium concentration in a micromolar range.

In particular, as it is evident in Fig13 B, the contributes of both fast and slow components are not mutually exclusive in a large range of extracellular calcium concentrations, while it is also evident that the calcium release from internal stores progressively disappears at extracellular calcium concentration exceeding 1 μM.

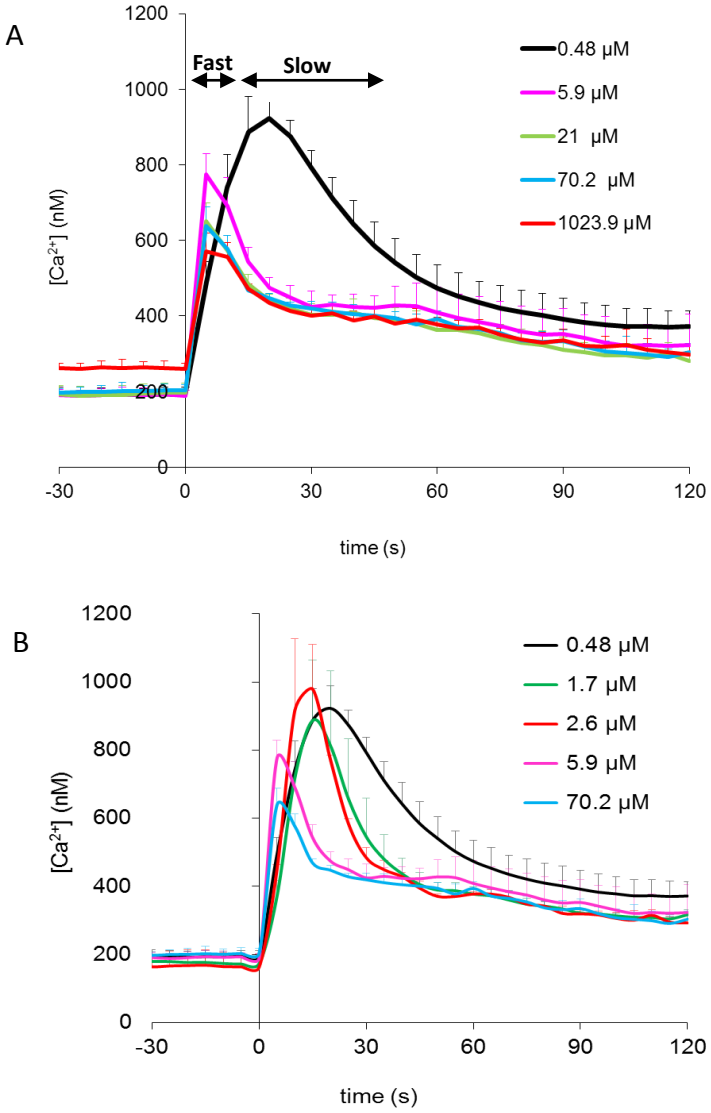


Fig. 13 A, B: *hypotonic shock triggered calcium peak was observed as described in Materials and Methods section in K601 strain cells exponentially growing in YPD medium additioned with 1 mM EGTA and different concentrations of $CaCl_2$ giving the indicated free calcium ion concentrations (A,B). In Fig. 13B $[Ca^{2+}]i$ was estimated using calibration curve described in material and methods.*

In order to evaluate the contribution of extracellular calcium to the whole response we recorded the raise of intracellular calcium concentration with 1-second time points instead of 5 seconds. Then we evaluated the initial rate of calcium concentration increase as the maximal value of the first derivative of the calcium concentration increase, usually registered between 3 and 6 seconds after the HTS stimulus.

Calcium release from intracellular compartments is slower than calcium influx from extracellular environment (Fig. 13A and B) so, these experimental conditions will give a good estimation of the calcium influx, while the contribution of calcium release will be surely underestimated.

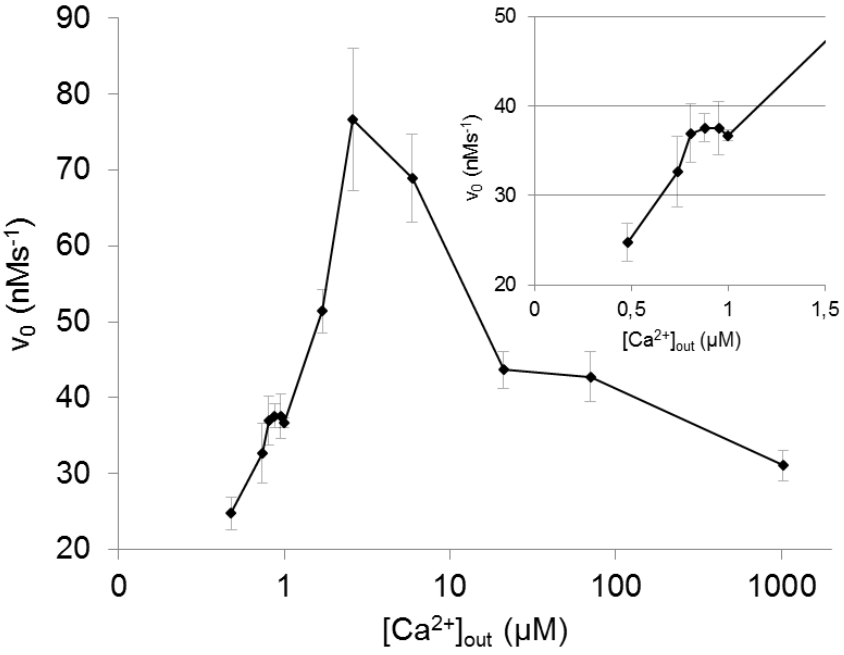


Fig. 14: *The initial rate in cytosolic calcium increase in wild type cells was plotted against free calcium concentration in the extracellular medium. In the upright corner a detail of initial rate at submicromolar extracellular calcium concentration.*

As shown in figure 14 the initial rate of calcium increase plotted against extracellular free calcium concentration draws a very complex graph. Considering also the data registered at submicromolar extracellular free calcium concentration, it is not possible to fit the curve by any Hill function (Fig. 14), suggesting a more complex scenario than a simple influx from extracellular environment. This is consistent with the simultaneous contribute in $[Ca^{2+}]_i$ raise after HTS of both slow and fast component, as observed in figure 13.
On the contrary, the initial rate registered in the micromolar external calcium concentration range can be almost perfectly fitted by a Hill function, with a K_M value of 1.1 ± 0.2 μM, suggesting the existence of a transporter on the plasma membrane. Further rising of external concentration induces an almost complete inhibition of calcium influx, with an IC_{50} of 20.6 ± 0.4 μM (Fig. 14). This could explain why this transport has not been previously identified, since usual growing media used for budding yeast contains free calcium concentrations slightly lower than millimolar, dramatically inhibiting both of these transports.

Involvement of HACS and LACS in hypotonic shock induced calcium signal

Stretch-activated channels (SACs) have been suggested as transducers in response to a variety of mechanical perturbations, such as hypotonic shock (Bargmann, 1994; Christensen, 1987; Gustin *et al.,* 1988; Sukharev *et al.,* 1997). Evidence has suggested that Mid1 might be a stretch response activator of calcium influx (Yoshimura *et al.,* 2004). Furthermore, Mid1 is sensitive to gadolinium, which was reported to inhibit calcium raise after hypotonic shock (Batiza *et al.,* 1996). Thus, in order to characterize the channels involved in this response we challenged mutants defective in HACS subunits, Mid1, Cch1 or Ecm7, with hypotonic shocks.
Surprisingly, cytoplasmic calcium raise after hypotonic shock in *mid1Δ* strain is comparable to a wild type strain (Fig. 15 A), indicating that Mid1, even if it was previously proposed as a mechanosensitive channel, is not involved in HTS response. Interestingly, *MID1* gene deletion only slightly affects the calcium response signature by delaying the calcium release from intracellular compartments at low extracellular calcium concentrations, but not the calcium influx (Fig. 15 A), probably because of the function of Mid1 ER fraction.

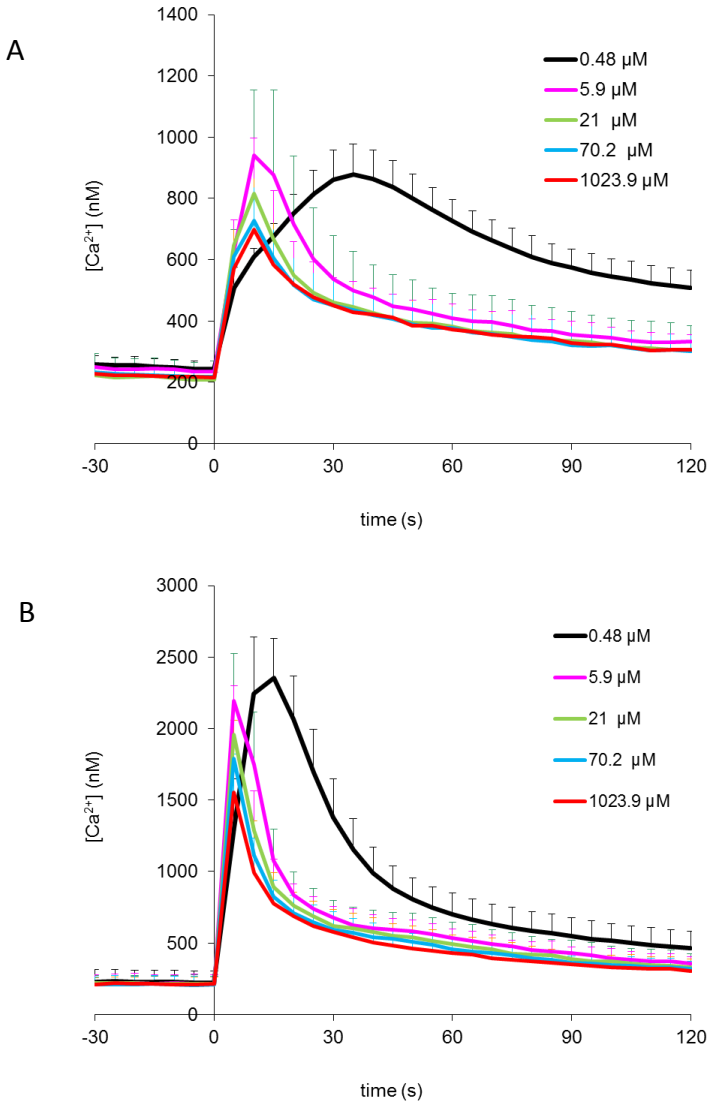


Fig. 15 A, B: *hypotonic shock triggered calcium peak was observed as described in Materials and Methods section in mid1Δ (A) and cch1Δ (B) strain cells exponentially growing in YPD medium additioned with 1 mM EGTA and different concentrations of $CaCl_2$ giving the indicated free calcium ion concentrations.*

n YPD growing cells, hypotonic shock revealed a surprising pattern in strains carrying the deletion of Cch1 HACS component, the voltage-gated channel pore forming α subunit homolog. In *cch1Δ* mutant, both the intensity of calcium release from internal compartments and of calcium influx are even higher than in the wild type strain (Fig. 15 B).
Anyway, in cells disrupted in both *CCH1* and *MID1* genes together the delay of intracellular calcium release observed in *mid1Δ* mutant strain is no longer observed and *mid1Δ cch1Δ* cells behave as the single *cch1Δ* mutant strain (data not shown), suggesting that Cch1 is the only HACS subunit involved in regulation of the HTS response.
We also tested the other known component of HACS, Ecm7, but since calcium raise in the *ecm7Δ* mutant after hypotonic shock is comparable to the wild type (Fig. 16), we can exclude a role of this protein in HTS induced response.

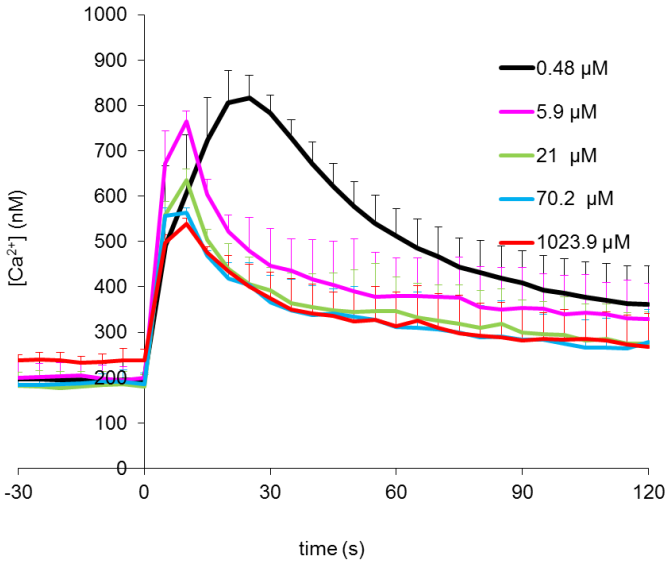


Fig. 16: *hypotonic shock triggered calcium peak was observed as described in Materials and Methods section in ecm7Δ mutant strain cells exponentially growing in YPD medium additioned with 1 mM EGTA and different concentrations of $CaCl_2$ giving the indicated free calcium ion concentrations.*

Fig1 is the only protein identified up to now involved in LACS calcium transport, likely with the role of a regulator. Fig1 regulate cytoplasmic calcium increase in response to mating factor exposure (Muller *et al.,* 2003) and seems to also have a role in the regulation of calcium transporters involved in calcium influx upon glucose addition to starved cells. Indeed, a genetic interaction between Fig1 and GIC transporter have been reported (Groppi *et al.,* 2011).
FIG1 gene was deleted together with *MID1* and *CCH1* genes, in order to get rid of HACS contribution. The calcium response observed in the triple mutant, *mid1Δ cch1Δ fig1Δ* after hypotonic shock has no difference if compared to the double mutant *mid1Δ cch1Δ* (data not shown) or to *cch1Δ* (compare Fig. 17 and Fig. 15 B) mutant strain, that share the same behavior with the double mutant as observed before. Therefore, LACS system seems not to be involved in HTS response, or at least not to require Fig1 to be activated by HTS.

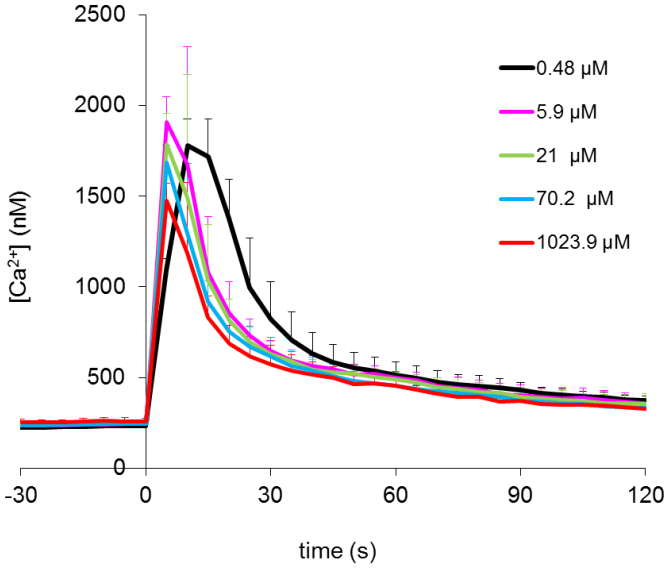


Fig. 17: *hypotonic shock triggered calcium peak was observed as described in Materials and Methods section in mid1Δ cch1Δ fig1Δ mutant strain cells exponentially growing in YPD medium additioned with 1 mM EGTA and different concentrations of $CaCl_2$ giving the indicated free calcium ion concentrations.*

Involvement of known intracellular calcium transporter in hypotonic shock induced calcium signal

Vacuole is the most important intracellular calcium storage for *S. cerevisiae* for the purposes of both detoxification and signalling. Calcium can be released from this organel by the action of Yvc1, a homolog of the constitutively active inwardly rectifying calcium channels in mammals known as TRP (Transient Receptor Potential) channels. Yvc1 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 (Zhou *et al.,* 2003). To investigate a possible role of Yvc1 in HTS-induced calcium response we observed the raise in cytosolic calcium in *yvc1Δ* mutant strain exposed to HTS (Fig. 18). Both the calcium peak driven by the slow and the fast component are completely comparable to the peak observed in the wild type strain. Moreover, synthetic deletion of HACS and LACS components in *mid1Δ cch1Δ fig1Δ yvc1Δ* strain produces a response similar to *mid1Δ cch1Δ fig1Δ* strain (data not shown), suggesting that the HTS-induced calcium release do not involve Yvc1-driven calcium release from the vacuole.

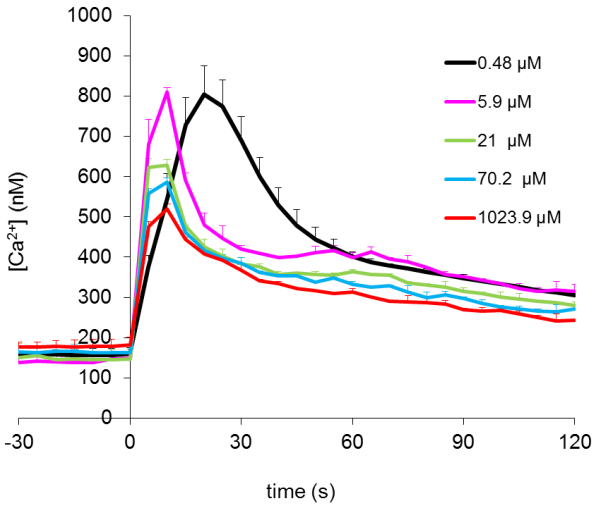


Fig. 18: *hypotonic shock triggered calcium peak was observed as described in Materials and Methods section in yvc1Δ mutant strain cells exponentially growing in YPD medium additioned with 1 mM EGTA and different concentrations of $CaCl_2$ giving the indicated free calcium ion concentrations.*

Cls2 is a hydrophobic ER protein characterised by the presence of an EF-hand type domain, in the cytoplasmic loop, that is able to bind Ca^{2+}.
Even if a role was proposed for Cls2 as a calcium channel (Tanida *et al.,* 1996), recent data demonstrated that it is required for mannosylation of inositolphosphorylceramide (Tkach JM, et al. 2012; Stock SD, et al. 2000). Although the role of Cls2 in calcium homeostasis is still to be cleared, we decided to observe HTS-induced calcium raise in *cls2Δ* mutant strain despite the marginal role of ER as a calcium store in yeast. Surprisingly, the deletion of *CLS2/CSG2* stimulates HTS-induced calcium release (Fig. 19), in particular at very low extracellular calcium concentration (Slow component), pointing to the hypothesis of ER or Golgi involvement in intracellular calcium release after hypotonic shock.

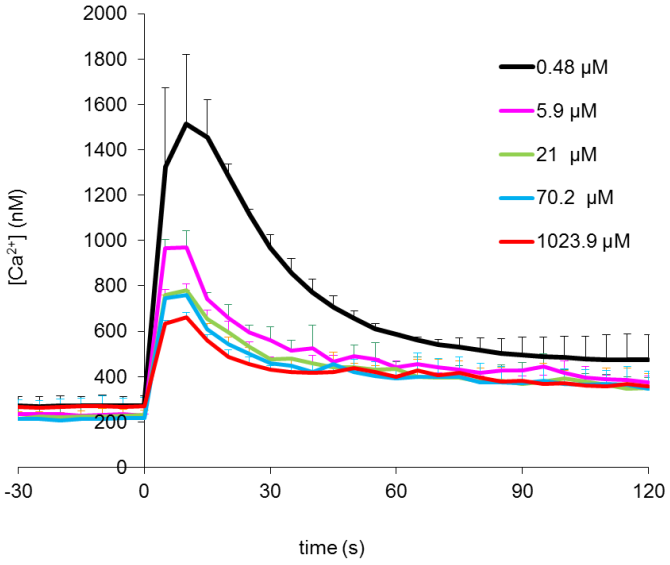
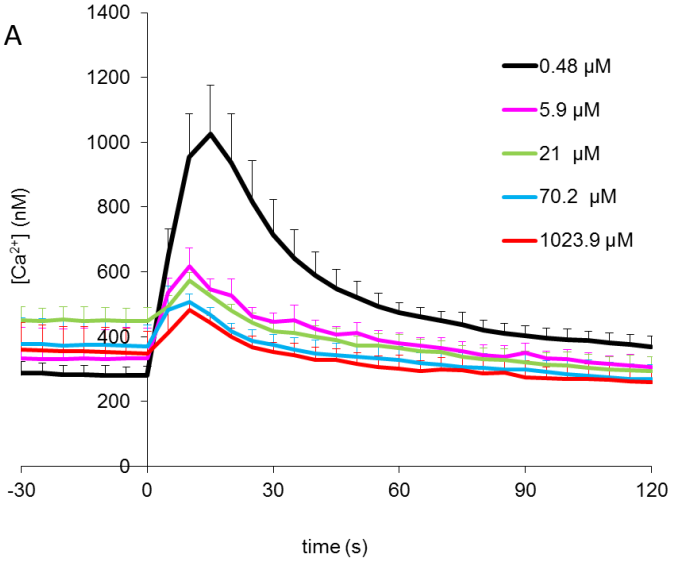


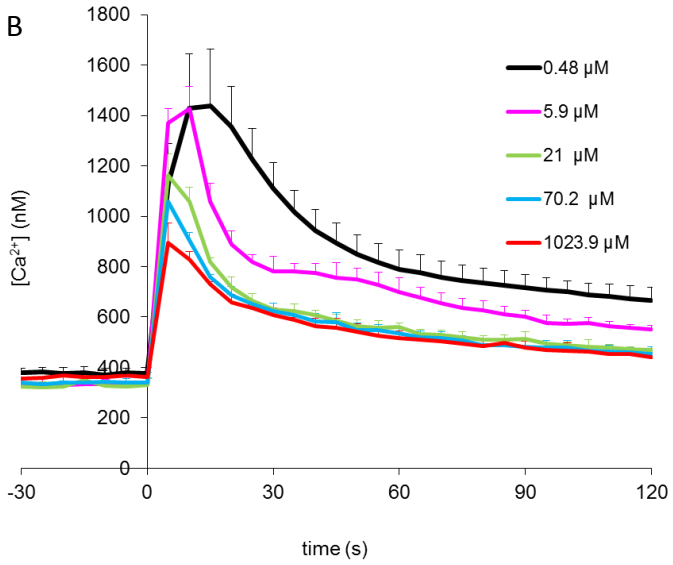
Fig. 19: *hypotonic shock triggered calcium peak was observed as described in Materials and Methods section in cls2Δ mutant strain cells exponentially growing in YPD medium additioned with 1 mM EGTA and different concentrations of $CaCl_2$ giving the indicated free calcium ion concentrations.*

Calcineurin role in HTS-induced response

The major player in Ca^{2+} dependent signal transduction pathways and the main effector of cytosolic calcium level and regulator of calcium homeostasis is the Ca/calmodulin/calcineurin phosphatase (Kraus *et al.,* 2003). Consequently, the effect of calcineurin inactivation was investigated, in order to assess if extracellular calcium availability could regulate calcium response by activating calcineurin after hypotonic shock.

Calcineurin inactivation in *cnb1Δ* mutant led to a slight increase in the calcium release from intracellular stores in presence of EGTA, but also a dramatic reduction in the response when calcium chloride is added in the extracellular environment (Fig. 20 A). This phenotype 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 (Muller *et al.,* 2001); 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 *mid1Δ cch1Δ cnb1Δ* shows a far less dramatic calcium inhibition of HTS-induced calcium influx (Fig. 20 B), confirming that Cch1 mediated calcium influx is responsible for the inhibition of the HTS response, rather than being a significant component of the response itself.


A
1400
1200
1000
800
600
400
200
0
[Ca2+] (nM)
-30
0
30
60
90
120
time (s)
0.48 µM
5.9 µM
21 µM
70.2 µM
1023.9 µM


B
1800
1600
1400
1200
1000
800
600
400
200
0
[Ca2+] (nM)
-30
0
30
60
90
120
time (s)
0.48 µM
5.9 µM
21 µM
70.2 µM
1023.9 µM

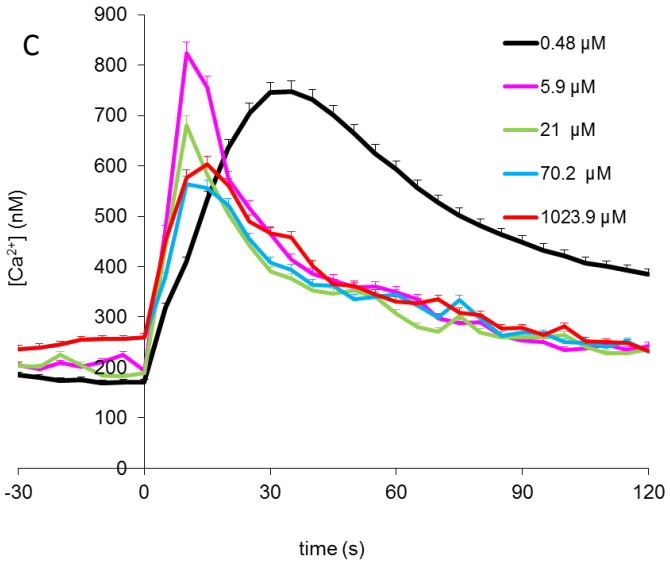


Fig. 20 A, B, C: *hypotonic shock triggered calcium peak was observed as described in Materials and Methods section in cnb1Δ (A), midΔ cch1Δ cnb1Δ (B) and crz1Δ (C) mutant strain cells exponentially growing in YPD medium additioned with 1 mM EGTA and different concentrations of* $CaCl_2$ *giving the indicated free calcium ion concentrations.*

Consistent with this hypothesis, deletion of calcineurin-dependent transactivation factor encoding gene, *CRZ1* (Cyert, 2003), do not cause any effect on the HTS-induced calcium signal, indeed the calcium response in *crz1Δ* mutant strain is comparable to a wild type response (Fig. 20 C). This confirms that the effect of calcineurin deletion is due to a lack of post-transcriptional regulation, likely on Cch1 transporter.

Involvement in HTS response of novel uncharacterized proteins

YOR365C gene product was previously identified as a 703 amino acids putative ionic transporter on the basis of a phylogenetic classification of different yeast unknown membrane proteins (De Hertogh *et al.,* 2002).

Yor365c displays some similarity to the mammalian TRP Ca^{2+} transporters superfamily, indeed Yor365c sequence is characterized by the presence of a conserved TRP-like domain as well as numerous transmembrane domains (Fig. 21) (NCBI http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). Yor365c

sequence conservation of TRP-like domains was evident when aligned to a consensus obtained with other TRP family members from different species, indicating a possible role as ion transporter.
YOR365C has a paralog, *FLC2/YAL053W*, that arose from the whole genome duplication (Byrne *et al.,* 2005). Flc2 was recently proposed as a member of a fungal specific family, named FLC, which is required for transport of FAD into endoplasmic reticulum (Protchenko *et al.,* 2006). However also *FLC2* encoded protein, as observed in *YOR365C* gene product, shows numerous TM domains and a conserved TRP-like domain in its sequence (Fig. 21). Indeed, Flc2 was previously proposed as the homolog of the fission yeast TRP-like calcium channel *PKD2* (Palmer *et al.,* 2005).

Fig. 21: *Schematic representation of Yor365c and Flc2 structure, with TRP-like and TM domain in evidence (www.yeastgenome.org)*

In order to better understand the role of those two proteins we performed numerous alignments among calcium transporters in fungi. This alignments lead to the identification of a fungal specific family of calcium transporters that comprehend yeast proteins previously proposed as flavin transporters, homolog to *FLC* genes from *S. cerevisiae*, and fungal calcium-related spray proteins, homologous to spray protein from *Neurospora crassa*. It is not possible to find significative differences based on sequence similarity among

the members of this family, indicating that all of them belong to a unique family, likely of calcium transporters.

All these protein are distantly related to TRP subfamilies typical of higher eukaryotes, and the TRP-like domain is situated in the middle-C-terminal part of the sequence, which is the most conserved region. The C-terminal tail is on the contrary quite divergent, or even really short, as in the case of Yor365c. Nonetheless, this region is 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 (*PKD2*) calcium channel, we decided to perform an alignment between middle-C-term region of budding yeast Flc2 and *N. crassa* spray proteins with TRP P and TRPML (the most closely related to TRPP among TRP subfamilies) protein family members.

All the fungal proteins observed share a great similarity among the sequence spanning transmembrane domains TM4 to TM6 and the large loop between TM5 and TM6, which is likely the pore-forming region of the channel (Fig. 22).

Actually, Flc2 shows some features typical of PKD channels, with an aminoacidic identity above 20% between Flc2 and human Pkd2 in the pore-forming region, but, together with *spray* protein, results to be more similar to TRPML branch of TRP family when conservative amino acids substitutions are considered (Fig. 23).

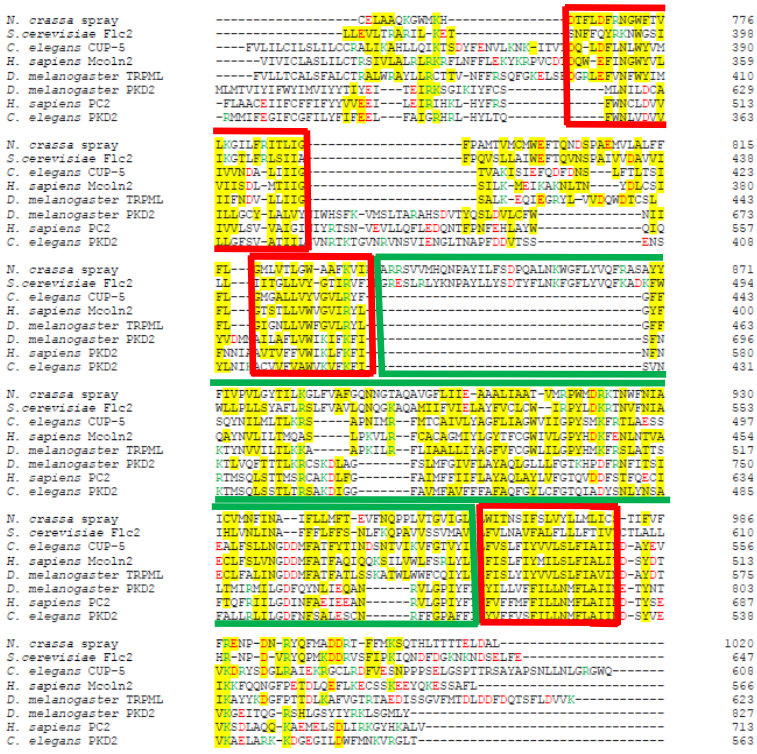


Fig. 22: *Alignment of sequence, from TM domain 4 to TM domain 6, of Neurospora crassa calcium-related spray (Accession number AAG03077.1), S. cerevisiae Flc2 (P39719.2), C. elegans CUP-5 (AF338583_1), H. sapiens MCOLN2 (NP_694991.2), D. melanogaster TRPML (NP_649145.1), C. elegans PC2 (Q9U1S7.3), D. melanogaster PC2 (AAQ18122.1), H. sapiens PC2 (Q13563.3). Red boxes, Transmembrane (TM) domains. Green Box, pore-forming region. Yellow marks, conserved amino acids.*

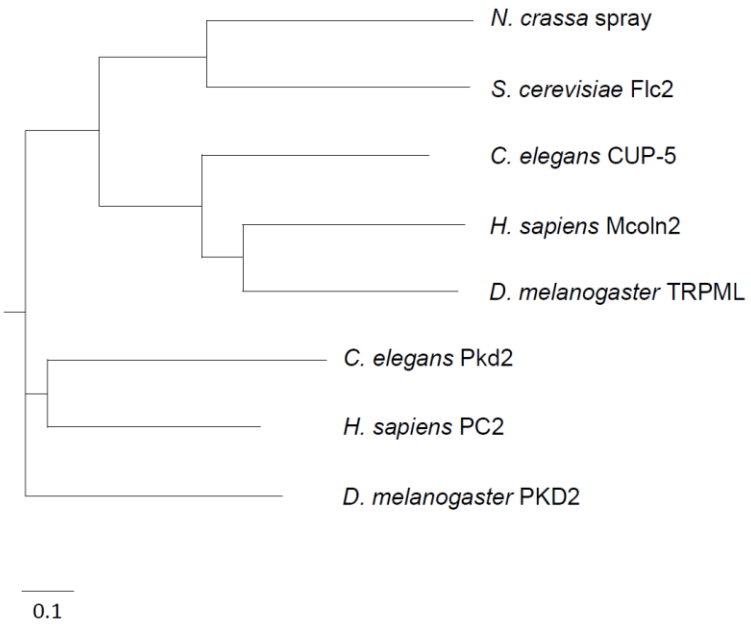


Fig. 23: *This phylogenetic tree has been constructed by the alignment shown in Fig. 22. The branch length is proportional to the number of substitutions per site calculated with ClustalOmega as indicated by the bar.*

Indeed, according to the phylogenetic tree (Fig. 23) Flc2 is more similar to TRPML than to Pkd2 as previously reported.

Flc2, but not Yor365c, is mainly involved in HTS induced-calcium response

In order to confirm our theory of a role of Flc2 and Yor365c as calcium channels, we tested hypotonic shock induced calcium signal both in *flc2Δ* and *yor365cΔ* mutant strains.

Deletion of *YOR365C* only slightly affects calcium release from intracellular compartments at low extracellular calcium concentration (Fig 24 A). Indeed, calcium raise in extracellular calcium shortage shows only a marginal decrease compared to a wild type strain (Fig. 13 B).

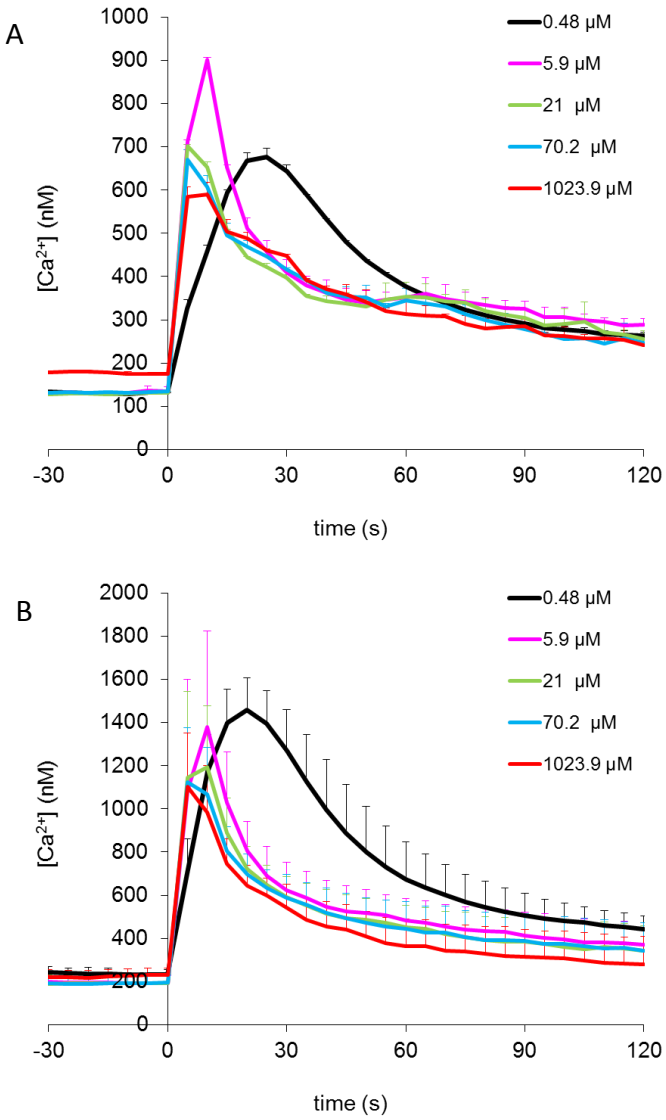
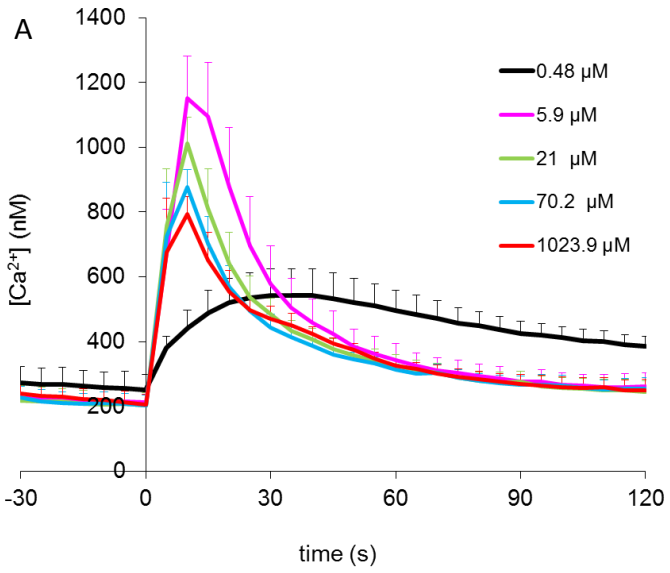


Fig. 24 A, B,: *hypotonic shock triggered calcium peak was observed as described in Materials and Methods section in yor365cΔ (A), midΔ cch1Δ fig1Δ yor365cΔ (B) mutant strain cells exponentially growing in YPD medium additioned with 1 mM EGTA and different concentrations of $CaCl_2$ giving the indicated free calcium ion concentrations.*

The slower peak is still only slightly inhibited in the *mid1Δ cch1Δ fig1Δ yor365cΔ* (Fig. 24 B) strain compared to the *mid1Δ cch1Δ fig1Δ* strain (Fig. 17), indicating that this hypothetic transporter could have only a marginal role, if any, in response to hypotonic shock.
Conversely, the deletion of *FLC2* dramatically affects the signal at low external calcium concentration, suggesting that this protein is involved in HTS-induced calcium release from internal stores. Thus, Flc2 may not be itself the transporter involved in calcium release from internal stores, but at least it seems to be involved in its regulation.
In contrast, the fast response caused by an influx of calcium from extracellular environment seems not to be impaired, but rather increased, in *flc2Δ* mutant strain (Fig. 25 A).
The phenotype observed in cells carrying the deletion of *FLC2* is not reverted in *mid1Δ cch1Δ fig1Δ flc2Δ* mutant strain (Fig. 25 B), indicating that lack of slow calcium response after HTS is not due to an indirect role of Flc2 on HACS.



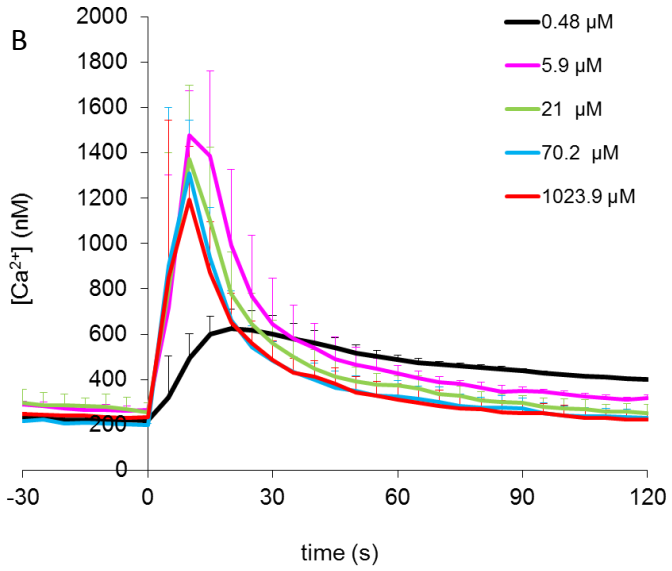


Fig. 25 A, B: *hypotonic shock triggered calcium peak was observed as described in Materials and Methods section in flc2Δ (A), mid1Δcch1Δfig1Δflc2Δ (B) mutant strain cells exponentially growing in YPD medium additioned with 1 mM EGTA and different concentrations of $CaCl_2$ giving the indicated free calcium ion concentrations.*

To study Yor365c localization we created a strain carrying a version of this putative TRP channel fused with eGFP. The resulting protein localized as a mainly mitochondrial protein in YPD exponentially growing cells (Fig. 26), as consistently predicted by WoLF PSORT software (Horton *et al.,* 2007) too.
Conversely, 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) (Huh *et al.,* 2003) and to the ER and probably cell membrane by membrane fractionation (Protchenko *et al.,* 2006). Thus, the localization of the two proteins is not identical, suggesting that the two homologs could have diverged in function, too.

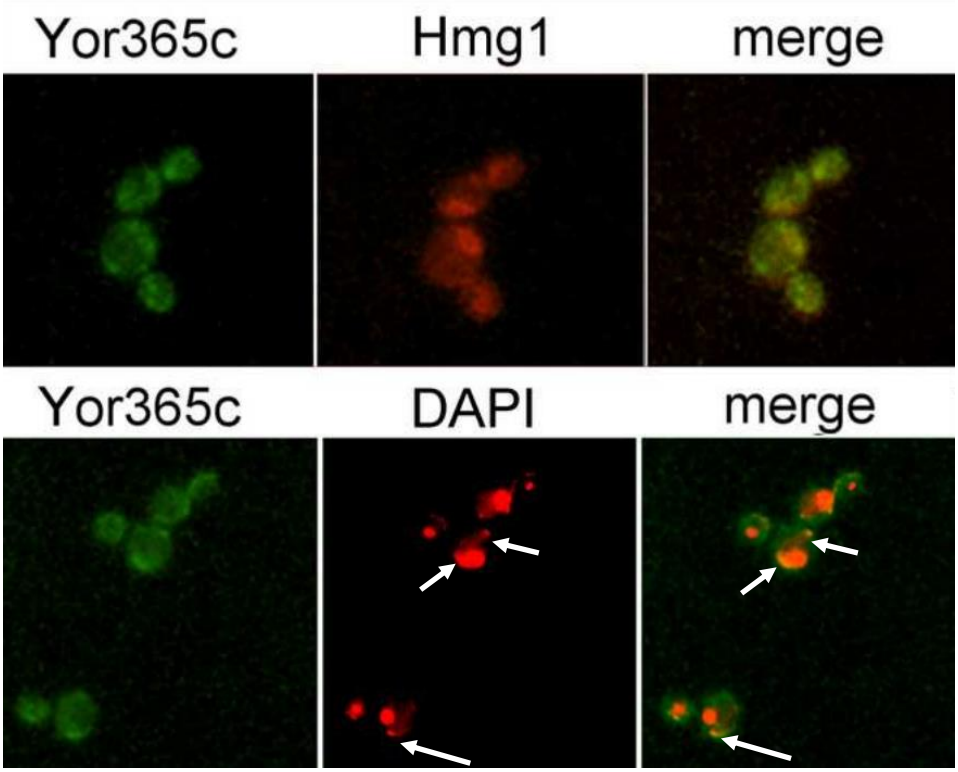


Fig. 26: *Cells expressing a Yor365c-eGFP protein fusion, transformed with the YIplac204T/CHmg1DsRED plasmid, driving expression of an Hmg1-DsRed fusion protein, localizing in ER, were grown in synthetic complete medium, dyed with DAPI and observed with an epifluorescence microscope. Co-localization of Yor365c-eGFP (Yor365c) fluorescence and either Hmg1-DsRed (Hmg1) fluorescence or DAPI fluorescence are shown in the upper and lower lanes, respectively.*

Since Flc2 was reported to localize on ER and Golgi we decided to challenge with hypotonic shock cells lacking both *FLC2* and *CLS2*, in order to assess if the impairment of calcium release after HTS could be due to an indirect effect on ER calcium homeostasis. The effect of Flc2 deprivation is not reverted in *flc2Δ cls2Δ* mutant strain (Fig. 27), indicating that Flc2 likely have a direct role on calcium release from ER after hypotonic shock and not an indirect effect on ER calcium homeostasis.

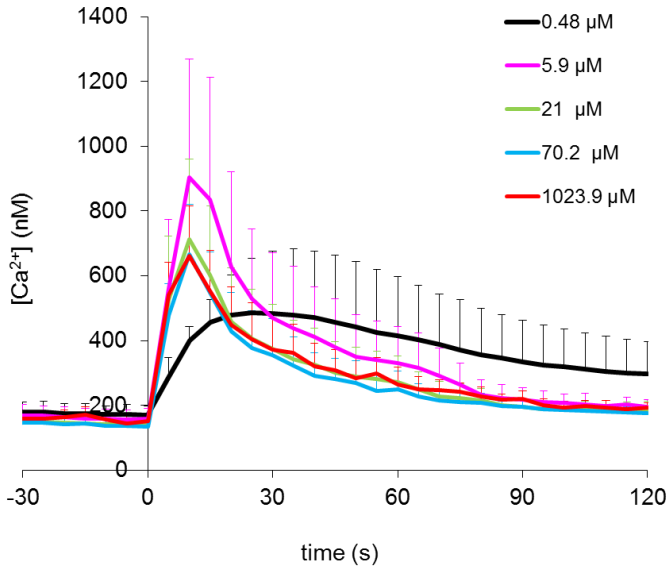


Fig. 27: *hypotonic shock triggered calcium peak was observed as described in Materials and Methods section in flc2Δcls2Δ mutant strain cells exponentially growing in YPD medium additioned with 1 mM EGTA and different concentrations of $CaCl_2$ giving the indicated free calcium ion concentrations.*

Furthermore, again supporting the hypothesis of a direct role of Flc2 in calcium release from ER after HTS, the presence of *FLC2* gene on a multicopy plasmid dramatically perturbs both the calcium level in exponentially growing cells and the response to HTS (Fig. 28). Indeed, in K601 cells transformed with YEplac112-*FLC2* both calcium release from intracellular stores and calcium influx from extracellular environment are significantly higher if compared to a wild type strain, as well as the $[Ca^{2+}]_i$ before the shock.

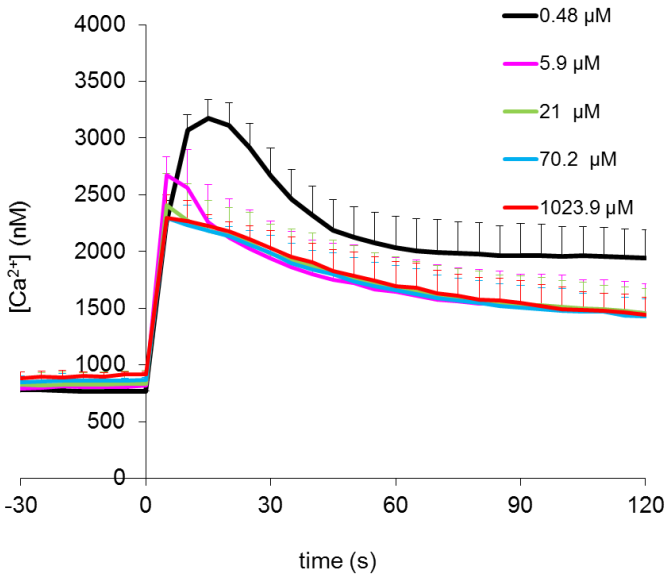
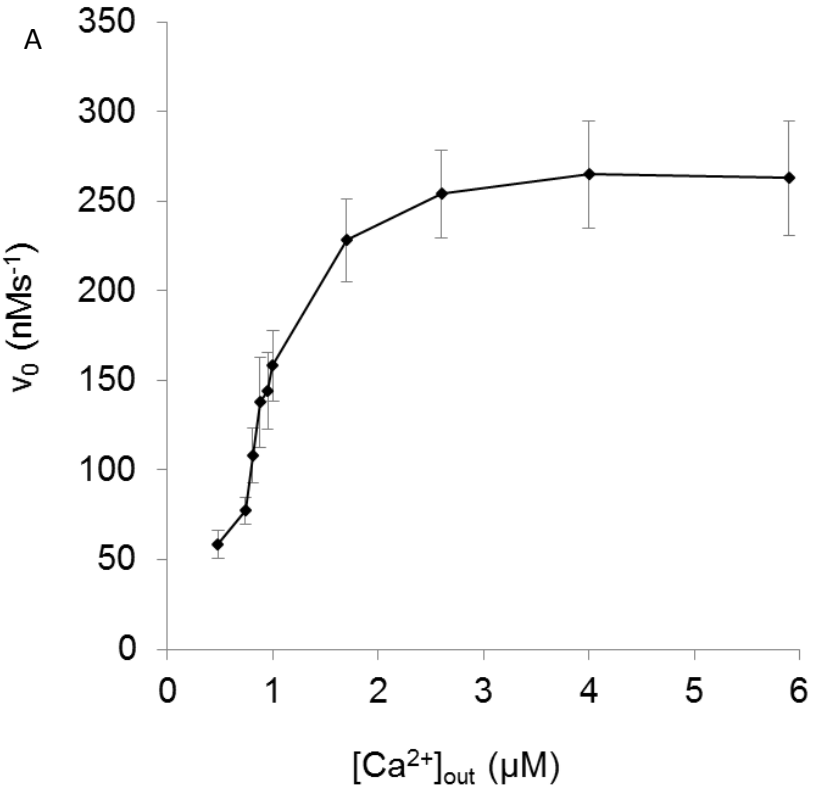


Fig. 28: *hypotonic shock triggered calcium peak was observed as described in Materials and Methods section in K601-YEplac112-FLC2 cells exponentially growing in YPD medium additioned with 1 mM EGTA and different concentrations of $CaCl_2$ giving the indicated free calcium ion concentrations.*

In order to better analyze the role of *FLC2* in HTS-driven calcium raise, we assayed the initial rate of cytosolic calcium rise in the presence of different external calcium concentrations in *flc2Δ* mutant strain. The behavior of the HTS-triggered calcium influx from extracellular environment in the absence of Flc2 activity was completely different if compared to the wild-type strain (Fig. 14): the curve in the *flc2Δ* 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 (Fig. 29 A). Even if cytosolic calcium raise of the *flc2*Δ mutant is lower in presence of only EGTA if compared to the wild type, this does not correlate with any defects in calcium influx rate, which in contrast is higher also in scarcity of external calcium.

Surprisingly, also the dramatic inhibition occurring in wild type cells above 5 µM free external calcium concentration disappeared (Fig. 29 B), leading to a maximal inhibition of less than half of the maximal initial rate, when free extracellular calcium concentration is 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.
Furthermore, Flc2 also seems to be involved in the high sensitivity to calcium of the HTS-responsive plasma membrane calcium transporter; indeed, in the absence of Flc2 protein, the calcium influx rate of this transporter is 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 *mid1Δ cch1Δ fig1Δ flc2Δ* when compared to the *flc2Δ* strain (Fig. 25 A, B): the relief of Cch1-mediated inhibition requires the presence of Flc2.



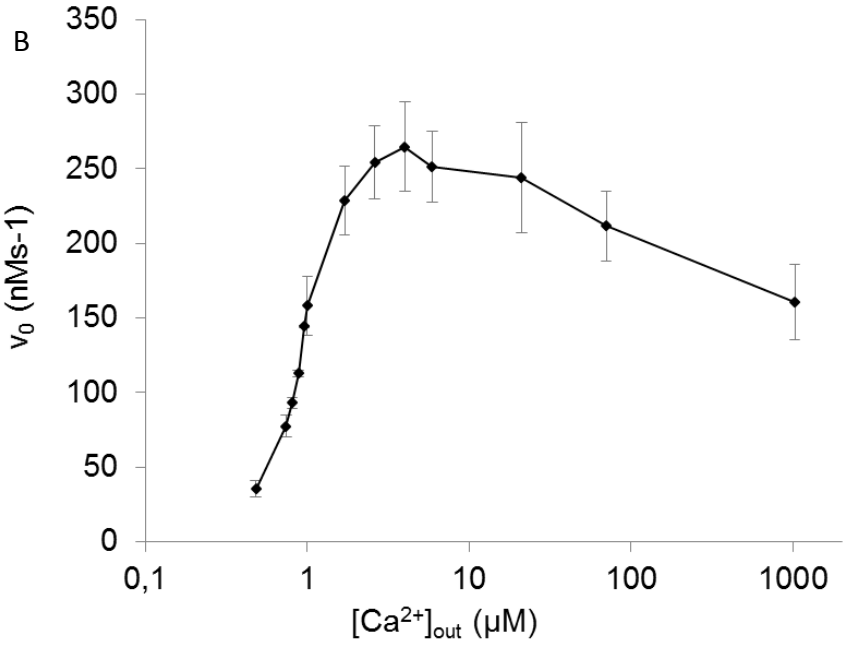
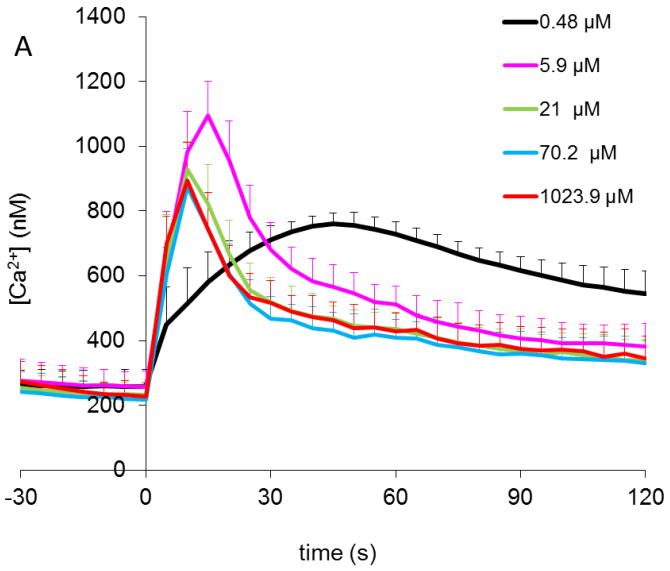


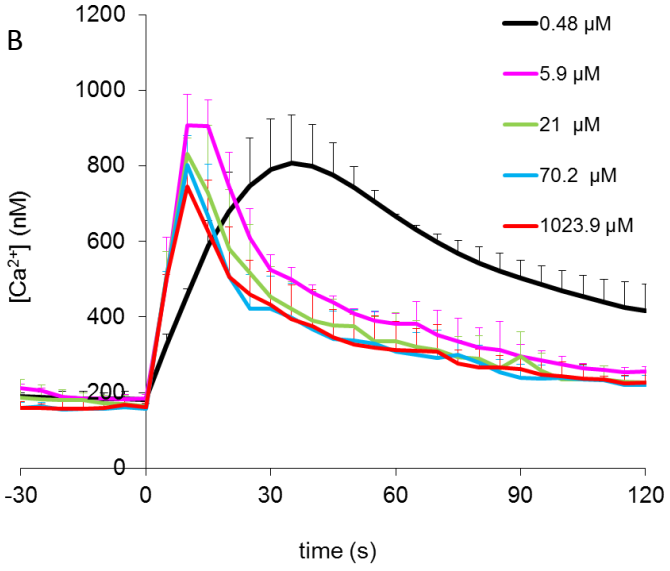
Fig. 29 A, B: *The initial rate in cytosolic calcium increase in flc2Δ cells was plotted against free calcium concentration in the extracellular medium.*

The other member of FLC family, Flc1 and Flc3, are not required in HTS-induced $[Ca^{2+}]_i$ raise

FLC2 belongs to *FLC* family of genes together with its homologs *FLC1* and *FLC3*, although *FLC2* is more similar to *YOR365C* than to other *FLC* genes.

Protchenko and coworkers (2006) proposed a redundant role for Flc2 and Flc1, but not Flc3, since double deletion of *FLC1* and *FLC2* genes is lethal. In contrast with this hypothesis, we have not noticed any redundant effect on HTS-induced calcium release from ER. Indeed we observed almost the same defect in both *flc1Δ* and *flc3Δ* (Fig. 30 A, B) strains, which is additive in the *flc1Δ flc3Δ* (Fig. 30 C) double mutant, with a slower calcium mobilization but no decrease in response intensity.


A
1400
1200
1000
800
600
400
200
0
[Ca2+] (nM)
-30
0
30
60
90
120
time (s)
0.48 µM
5.9 µM
21 µM
70.2 µM
1023.9 µM


B
1200
1000
800
600
400
200
0
[Ca2+] (nM)
-30
0
30
60
90
120
time (s)
0.48 µM
5.9 µM
21 µM
70.2 µM
1023.9 µM

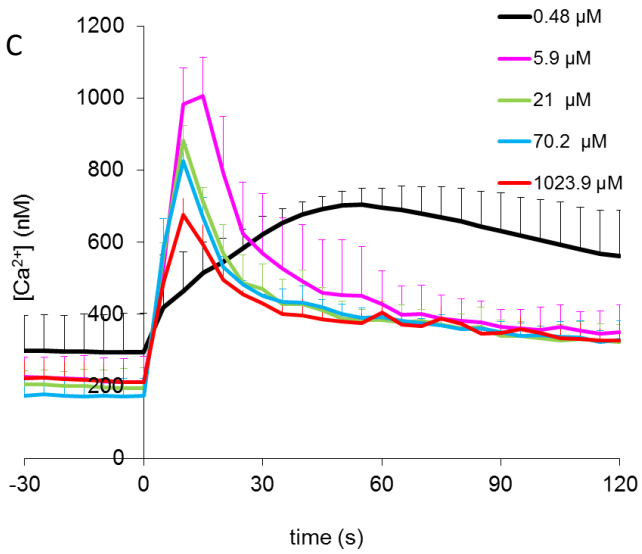
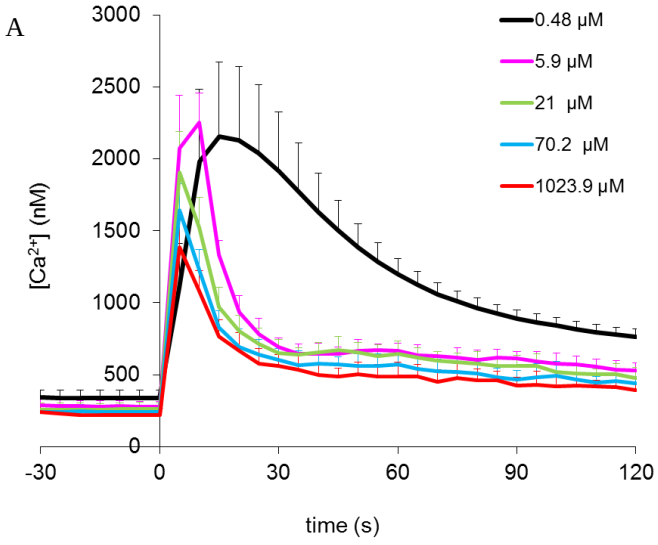


Fig. 30 A, B, C: *hypotonic shock triggered calcium peak was observed as described in Materials and Methods section in flc1Δ (A), flc3Δ (B) and flc1Δ flc3Δ (C) mutant strain cells exponentially growing in YPD medium additioned with 1 mM EGTA and different concentrations of* $CaCl_2$ *giving the indicated free calcium ion concentrations.*



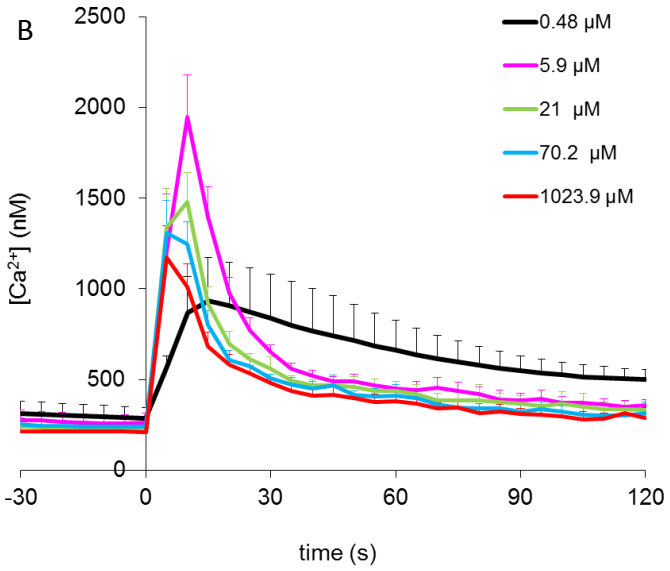


Fig. 31 A, B: *hypotonic shock triggered calcium peak was observed as described in Materials and Methods section in mid1Δ cch1Δ fig1Δ flc1Δ (A) and mid1Δ cch1Δ fig1Δ flc3Δ (B) mutant strain cells exponentially growing in YPD medium additioned with 1 mM EGTA and different concentrations of $CaCl_2$ giving the indicated free calcium ion concentrations.*

This effect is very similar to the response delay observed in *mid1Δ* mutant strain (Fig. 15 A), and it probably indicates an indirect effect of *MID1*, *FLC1* or *FLC3* disruption on calcium homeostasis in endoplasmic reticulum.
Furthermore, the deletion of *FLC1* in a background lacking HACS and LACS (*mid1Δ cch1Δ fig1Δ*) generate a response similar (Fig. 31 A) to the one observed in *mid1Δ cch1Δ fig1Δ* (Fig. 17) mutant strain, while deletion of *FLC3* in the same background (Fig. 31 B) shows a HTS-induced calcium raise similar to *flc3Δ* single mutant (Fig. 30 B). This could indicate a possible role of Flc3 in regulation of HTS-driven calcium release from ER. As in the *flc2Δ* strain, in *flc1Δ* and *flc3Δ* strains the HTS-triggered calcium influx from extracellular environment is not defective (see Fig. 25 A, 30 A, B), but inhibition of the influx at higher extracellular calcium concentration was less evident than in the wild-type strain.
A mathematical model was developed in collaboration with Dott. R. Ambrosini (University of Milano-Bicocca) to quantitatively investigate the different

behavior of the *FLC* genes defective mutants 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, reduces to the minimum the contribution of external calcium influx (Fig. 14).
This mathematical model confirmed 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 does not take into account the small contribution of the calcium influx, which is almost negligible at these external calcium concentrations.

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

Several strains carrying defects in calcium channels are highly sensitive to cell wall perturbing agent, due to a misregulation of Cell Wall Integrity (CWI) pathway, probably caused by lack of calcium signaling crosstalk with CWI pathway. Thus, defects in calcium signalling can also involve a certain fragility in the response to cell wall damage (Nobel *et al.,* 2000; De Groot *et al.,* 2001). One of the most common agents used to observe cell wall stress is Calcofluor white (CW), a fluorescent molecule used to stain bud scar in yeast and cell wall in plants, which is able to interfere with cell wall synthesis by blocking the crosslinking of chitin and glucan.
Cells carrying the deletion of *FLC2* have been reported to be sensitive to Calcofluor white, further confirming a role in calcium transport (Protchenko *et al.,* 2006). Therefore, in order to study possible genetic interactions between *FLC2* and other proteins involved in calcium transport and homeostasis, we tested sensitivity to Calcofluor white on a YPD plate for mutants lacking this putative transporter, alone or together with other known calcium transporters (Fig. 32 A). We also decided to add sorbitol to the plates at a final concentration of 0,5 M as an osmoprotector.
As expected, the deletion of *FLC2* cause a great sensitivity to CW cell wall perturbing agent in a dominant manner. Indeed, the single deletion of *FLC2* is sufficient to stop growth on CW, either alone or in*flc2Δ mid1Δ*, *flc2Δ yvc1Δ*, *flc2Δ cls2Δ* (Fig. 32 A) and *flc2Δ flc3Δ* (Fig. 32 B) double mutants.

In contrast, deletion of *YOR365C* gene did not cause an enhanced sensitivity to Calcofluor white if compared to wild type strain (data not shown), confirming that these two genes, although paralog, have clearly diverged in function.
We also tested the sensitivity to Calcofluor white of the other members of *FLC* family, *FLC1* and *FLC3*. Even in this case, the deletion of *FLC1* and *FLC3*, alone or together, do not imply a different sensitivity to Calcofluor white than the wild type strain (Fig. 32 B), confirming that *FLC* proteins, albeit homologues, play different roles within the cells.

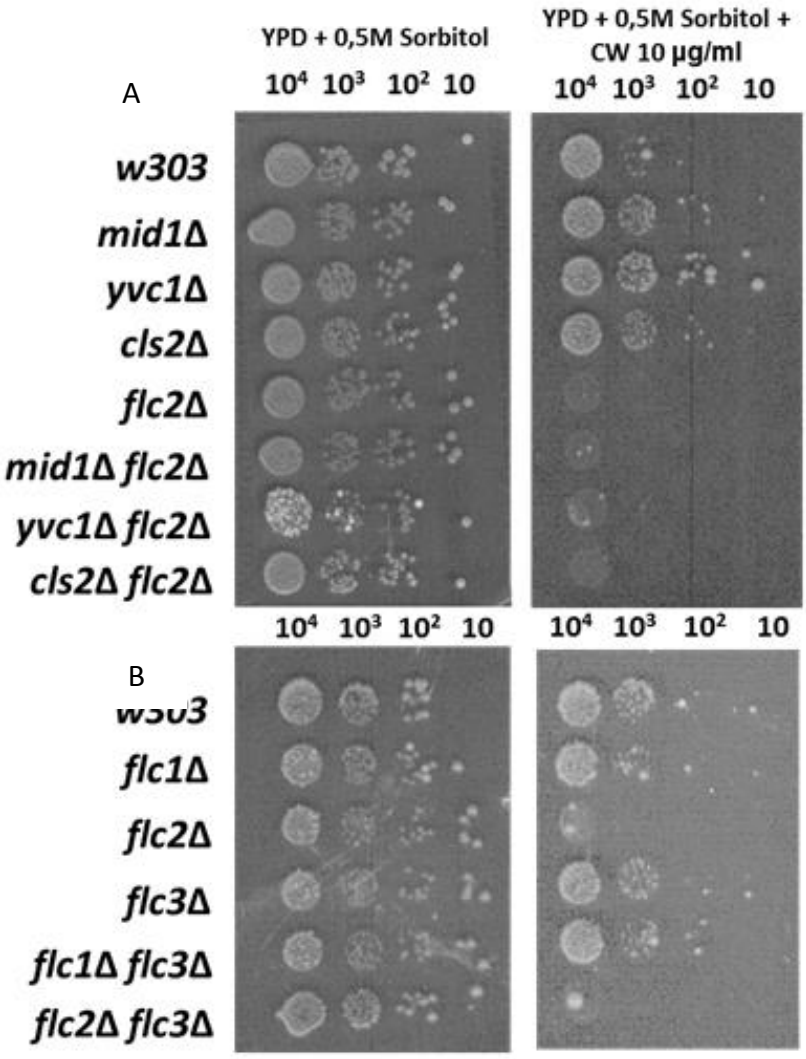


Fig. 32 A, B: *Indicated strains were grown in YPD medium until exponential phase and then diluted to appropriate cell density to spot 10^4, 10^3, 10^2 and 10 cells. Spots were laid on YPD medium additioned with DMSO (left) or YPD additioned with 0.5 M sorbitol and 10 µg/ml Calcofluor white (right).*

Flc2 is reported to localize on ER and late Golgi and its deletion seems to cause severe ER stress (Protchenko *et al.,* 2006). To test this severe ER stress we tested the sensitivity of *flc2Δ* mutant strain, as well as strain mutated in known calcium channels and protein related to calcium homeostasis, to tunicamycin. Tunicamycin is a mixture of homologous nucleoside antibiotics that blocks N-linked glycosylation (N-glycans) and causes cell cycle arrest in G1 phase and activation of unfolded protein response due to ER stress.
Surprisingly, single deletion in *FLC2* is not sufficient to confer higher sensitivity than the wild type strain to 0.25 µg/ml tunicamycin in YPD, but interestingly the *mid1Δ flc2Δ* double deletion mutant is extremely sensitive to tunicamycin (Fig. 33), suggesting a genetic interaction between these two genes impinging on ER integrity.

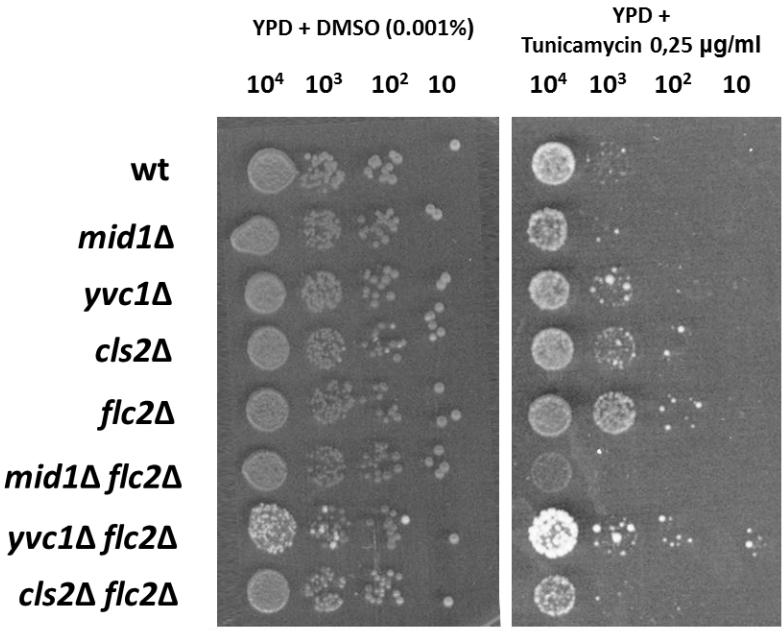


Fig. 33: *Indicated strains were grown in YPD medium until exponential phase and then diluted to appropriate cell density to spot 10^4, 10^3, 10^2 and 10 cells. Spots were laid on YPD medium additioned with DMSO (left) or YPD additioned with 0.25 µg/ml tunicamycin (right).*

Finally, we tested ER stress level in our strains by evaluating the basal activation of UPR pathway. The plasmid pMCZ-Y, which encodes a fusion between the unfolded protein responsive element of the *KAR2* gene, minimal *CYC1* promoter elements and the coding sequence for the *Escherichia coli* β-galactosidase enzyme, was used to observe UPR activation in strains indicated in figure 34.

UPR-dependent transcription was not higher in any of the mutants tested (Fig. 34), and was even lower in the *flc2*Δ strain, confirming that the defect in calcium release after hypotonic shock is not due to a general stress in the ER but likely to a consistent function of Flc2 as calcium transporter.

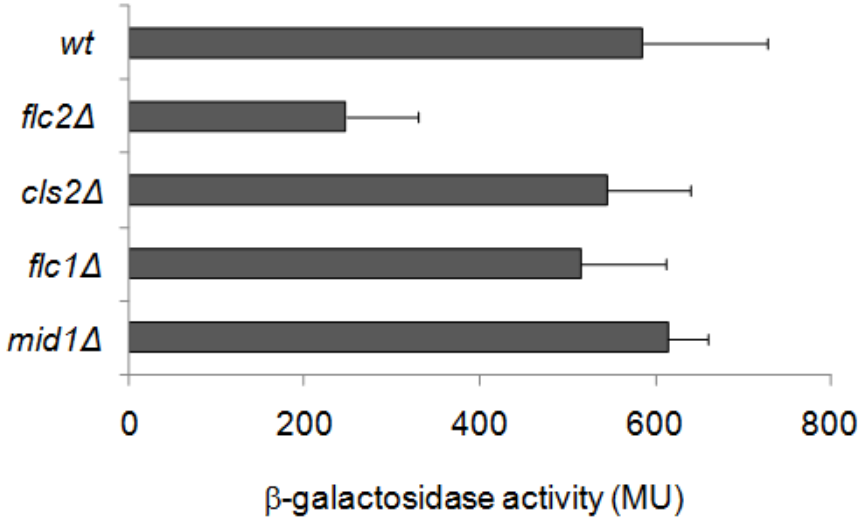


Fig. 34: *Exponentially growing cells of the indicated strains transformed with pMCZ-Y plasmid (UPR-responsive reporter) were assayed for β-galactosidase activity as described in Materials and methods section. *, P=0.001 vs. wt.*

The deletion of *FLC2* induces the activation of both calcineurin and Mpk1 kinase pathway.

The most important regulator of calcium homeostasis and calcium transporters activity in yeast is the calmodulin/calcineurin pathway. Beside direct and indirect post-translational regulation, calcineurin controls transcription of many genes involved in calcium signaling through the action of Crz1 transcription factor. To investigate calcineurin activity before and after hypotonic shock we took advantage of a plasmid carrying the *Escherichia coli* β-galactosidase enzyme under the control of a promoter containing four CDRE (Calcineurin Dependent Responsive Elements) elements, recognized by Crz1.

Calcineurin activity was tested in cells transformed with pAMS366 grown on YPD and challenged with EGTA and four volumes of water or EGTA alone.

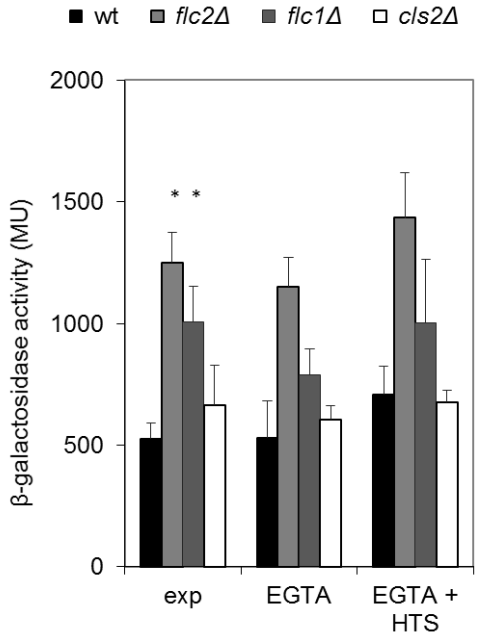


Fig. 35: *Wild-type (closed rectangles), flc2Δ (light grey rectangles), flc1Δ (dark grey rectangles) or cls2Δ (open rectangles) strains cells were transformed with pAMS366 (Crz1-responsive reporter) plasmid. Exponentially growing cells in YPD medium (exp) were exposed for 1.5 h to 1 mM EGTA (EGTA) or to 1 mM EGTA and HTS (EGTA + HTS), 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).*

In the *flc1*Δ and *flc2*Δ strains not exposed to HTS, a hyper-activation of calcineurin is evident (Fig. 35) when compared with the wild-type strain. This is consistent with the higher basal calcium concentration in the cytosol observed in *flc1*Δ (Fig. 30 A) and *flc2*Δ (Fig. 25 A) strains. The activation level of calcineurin do not significantly raise after hypotonic shock in any of the strains tested, probably because the cytosolic calcium increase is not sufficient to activate calcineurin pathway (Fig. 35). No hyperactivation was noticed in *cls2Δ* mutant strain when compared to wild type cells (Fig. 35).

Cell wall integrity pathway is known be activated in yeast cells in response to hypotonic shock. The kinase cascade of CWI signaling involved the activation of Mpk1 kinase that controls the localization of Rlm1 transcription factor (Watanabe *et al.,* 1995; Kamada *et al.,* 1995).

We used the p1366 plasmid, carrying the LacZ gene under control of Rlm1 responsive elements, to monitor Mpk1 and consequently CWI pathway activation in our strains.

As expected, we observed a 3-fold increase in β-galactosidase activity in wild type cells challenged with HTS (Fig. 36). Furthermore, *flc2Δ* strain revealed a hyperactivity of the Rlm1-dependent reporter already 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 (Fig. 36). HTS treatment was able to induce further β-galactosidase production, indicating that *flc2Δ* strain has no defects in the activation of Mpk1 pathway by HTS (Fig. 36). Deletion of *FLC1* do not cause any hyperactivity of CWI in exponentially growing cells, further indicating that Flc1 and Flc2 do not have a redundant function (Fig. 36).

Surprisingly, *cls2Δ* mutant cells showed a great reduction in Mpk1 activation in untreated cells, as well as a minor capacity of activating CWI response after HTS (Fig. 36). This suggests that cytosolic calcium increase is not correlated with Mpk1 activation, but that ER calcium homeostasis could have an important effect on CWI pathway, which is also confirmed by the similar effect observed in *flc1*Δ strain (Fig. 36).

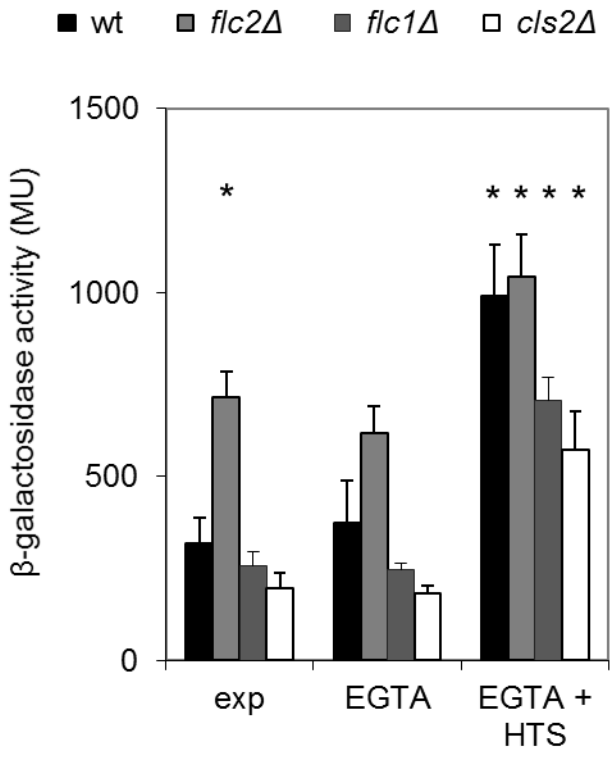


Fig. 36: *Wild-type (closed rectangles), flc2Δ (light grey rectangles), flc1Δ (dark grey rectangles) or cls2Δ (open rectangles) strains cells were transformed with p1366 (Rlm1-responsive reporter) plasmid. Exponentially growing cells in YPD medium (exp) were exposed for 1.5 h to 1 mM EGTA (EGTA) or to 1 mM EGTA and HTS (EGTA + HTS), 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).*

DISCUSSION

Calcium plays a very important role as a second messenger being involved in the regulation of many fundamental cellular processes, most of them activated in presence of environmental stresses. Indeed, *S. cerevisiae* cells respond to hypertonic and hypotonic shock, as well as nutrients, with a cytoplasmic calcium pulse (Batiza *et al.,* 1996; Groppi *et al.,* 2011; Denis *et al.,* 2002).

Hypotonic shock imposes a high mechanical stretch on the plasma membrane, thus probably activating a transport system that has mechanosensitive properties (Kung *et al.,* 2010).

S. cerevisiae genome lacks any mechanosensitive (MscS) homologues, which are present in the genomes of prokaryotes and cell-walled eukaryotes, for example, plants, algae and even other fungi (Nakayama *et al.,* 2012; Kung *et al.,* 2005; Chang *et al.,* 2010). Recently, in yeast and in higher eukaryotes numerous TRP channels have been identified with mechanosensitive properties (Kung *et al.,* 2005; Chang *et al.,* 2010), that likely could compensate for the lack of MscS in *S. cerevisiae*.

In budding yeast, hypotonic shock stimulates phospholipase C activity (Perera *et al.,* 2010), although this is not required to generate the calcium signal (Tisi *et al.,* 2002 and data not shown), which was previously supposed to be directly controlled by mechanosensitive calcium channels on cell surface. In electrophysiological studies of ion channels in the plasma membrane of the yeast *Saccharomyces cerevisiae*, in fact, channels were observed that were activated by, and adapted to, stretching of the membrane, passing both cations and anions, showing voltage-dependent adaptation to mechanical stimuli (Gustin *et al.,* 1988). Furthermore, Mid1 channel was found to show mechanosensitive features in a heterologous system (Kanzaki *et al.,* 1999).

In YPD growing cells challenged with hypotonic shock the presence of two kinetically distinct components that control $[Ca^{2+}]_i$ raise is evident (Fig. 13 A, B), suggesting that calcium is transported by two different systems. The fast response is dependent on extracellular calcium, but it is also severely inhibited by free calcium concentration above the micromolar range in the wild-type strain.

Low affinity for calcium do not make of LACS a good candidate for HTS-driven calcium influx from extracellular environment. Indeed, deletion of the only

identified LACS subunit, *FIG1*, does not cause any particular phenotype for what concern calcium flux after hypotonic shock (Fig. 17 and data not shown).
A good hypothetical model fitting our data could involve a stretch-activated channel with high affinity for calcium, whose opening then promotes calcium release from internal calcium stores, and which is inhibited at high calcium concentrations, suggesting HACS as a good candidate.
However the deletion of *CCH1*, *MID1* and *ECM7* alone or together, did not reveal any severe defect in response to HTS (Fig. 15 A, B), indicating that HACS do not solve any decisive role in calcium influx almost in this case. Conversely Cch1 seems to have an inhibitory role on HTS response, since its deletion causes a large increase in calcium signal (Fig. 15 B), while the only observed phenotype in *mid1Δ* mutant strain is a slightly delay in calcium release from intracellular stores (Fig 15 A), probably due to a marginal regulating function of this putative strech channel in HTS-induced calcium signal only in extracellular calcium shortage condition. This is particularly interesting since the budding yeast *Saccharomyces cerevisiae* employs a CCE-like mechanism to refill Ca^{2+} stores within the secretory pathway involving HACS system (Locke *et al.,* 2000), suggesting the existence of a regulatory loop between the ER and Golgi intracellular calcium compartments and HACS system.
In *N. crassa*, treatment with the antibiotic staurosporine causes a raise in cytoplasmic calcium concentration both due to a calcium influx from extracellular environment and to a calcium release from organelles. Calcium influx seems to be regulated by a novel fungal system that resembles a TRP channel, which is also upregulated in the absence of HACS, while calcium efflux from intracellular stores is mediated by IP_3 generation (Goncalves *et al.,* 2014). Moreover, IP_3 is known to regulate hyphal growth in *N. crassa* by creating a tip-high $[Ca^{2+}]_i$ gradient that is derived by intracellular Ca^{2+} channels (Silverman-Gavrila *et al.,* 2001; 2002; 2003), even if IP_3 receptor seems not to be present in fungi (Borkovich *et al.,* 2002; Zelter *et al.,* 2002).
IP_3 is generated by a stretch-activated tip-localized phospholipase C that senses tension due to hyphal expansion (Silverman-Gavrila *et al.,* 2002; 2003), promoting the release of Ca^{2+} through the activity of two channels. The first is a large conductance channel that localizes on the vacuolar membrane, while the second is associated with endoplasmic reticulum (ER) and Golgi-derived vesicles and is characterized by small conductance capacity.

Conversely, in budding yeast, deletion of phospholipase C *PLC1* gene causes a great increase in calcium influx after hypotonic shock if compared to a wild type strain, contrasting with the model proposed for *N. crassa*, at least for IP_3 regulation (Tisi *et al.,* 2002 and data not shown).

When budding yeast is exposed to extracellular calcium shortage conditions, the HTS-driven calcium response completely changes its kinetical characteristics. In these conditions $[Ca^{2+}]_i$ raise is likely achieved through an intracellular transporter driven efflux of calcium from intracellular compartments. Moreover, this component of HTS-induced calcium response is very sensitive to high concentration of extracellular calcium, since it is active only when extracellular calcium is very low (submicromolar free calcium concentration). The presence of two different components with such particular characteristic could be a very useful trick to economize calcium storage, but to assure the response in nature, when the shock usually comes from rain, which is very poor in ions.

Yvc1 was the first intracellular calcium channel identified in yeast, since it controls efflux of calcium from vacuole, which is the biggest calcium store in *S. cerevisiae*.

Deletion of *YVC1* does not cause any change in HTS-induced calcium raise, either we consider slow or fast component of the response (Fig. 18), suggesting that the system that controls calcium efflux does not involve vacuole or Yvc1 function.

Cls2 is a protein present on ER membrane required for mannosylation of inositolphosphorylceramide (Tkach JM, *et al.,* 2012; Stock SD, *et al.,* 2000) which also has a role in calcium homeostasis. Albeit not a calcium channel, we studied HTS-induced calcium response in *cls2Δ* mutant strain in order to observe the effect of ER calcium homeostasis defects on calcium release after hypotonic shock.

Surprisingly, deletion of *CLS2* causes a great increase in calcium release involved in the slow component observed at low extracellular calcium concentration, while influx of calcium was not modified in this mutant strain (Fig. 19). These data suggest a clear role of ER calcium in HTS response, although ER solves a less significant function for what concerns calcium storage in budding yeast.

A bioinformatics analysis lead to the identification of two putative calcium channels: Yor365c and its paralogue Flc2 which is a member of ER FLC family of

putative flavin carriers (Protchenko *et al.,* 2006), but it was also previously proposed as a homolog of *PKD2* calcium channel of *S. pombe* (Palmer *et al.,* 2005).

The *FLC* genes are part of a conserved fungal gene family of integral membrane proteins, characterized by the presence of a TRP-like domain. These genes have been identified in *S. cerevisiae* thanks to the homology with *C. albicans FLC* genes (Protchenko *et al.,* 2006). Budding yeast 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 named *FLC1*, *FLC2*, and *FLC3*, respectively (Protchenko *et al.,* 2006). Conversely, *YOR365C* exhibits minor homology to *CaFLC1* (28% identity) but higher similarity to *FLC2* than to *FLC1* or *FLC3*, suggesting that those gene, albeit similar, have diverged within evolution.

YOR365c and *FLC* genes represent a category of genes recently identified as "fungal specific" since they are present in all fungal species but not in prokaryotes or higher eukaryotes (Hsaing and Baillie 2005).

As mentioned above, *FLC2* was reported to be the homolog of *S. pombe PKD2* gene, which encodes for a calcium channel that belongs to the TRP-like ion channel family. S. pombe *PKD2* has low similarity to polycystic-kidney-disease (PKD)-related ion channel genes, in particular to *PKD2* (PC2 or TRPP2), a member of *PKD* genes located in tubular epithelial cells, which encodes for a Ca^{2+} permeable ion channel. Pkd2 is responsive to a mechanical stimulus triggered by ciliary action, which increases the intracellular Ca^{2+} levels causing cell cycle arrest (Nauli *et al.,* 2003; Nilson 2004).

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 (Aydar and Palmer, 2009). The fission yeast Pkd2 protein is localized mainly to Golgi and to plasma membrane, similarly to *FLC* proteins localization in budding yeast (Protchenko *et al.,* 2006). This family of genes is also present in several fungal species, where it has been formerly identified as a calcium-related spray proteins family. The most studied member of this family is the *Neurospora crassa* spray protein (Bok *et al.,* 2001), an ER-localized calcium channel that regulates the ability of *N. crassa* cells to respond to a topographical stimulus by altering the axis of growth of the hyphae (thigmotropism) (Stephenson *et al.,* 2014).

By performing partial sequences alignment, we revealed that fungal *spray* proteins are more similar to the TRPML (mucolipin) branch of TRP channels than to TRPP, although TRPML is the most closely related subfamily to TRPP class within TRP calcium transporters. Mucolipins mostly reside in membranes of organelles of the endolysosomal system such as early and late endosomes, recycling endosomes, lysosomes, or lysosome-related organelles and function as ion transporters. Indeed, TRPML1 is a calcium and iron permeable intracellular channel in lysosomes, and thus the loss of function impairs endosomal/lysosomal function and autophagy (Colletti and Kiselyov, 2011).

Phylogenetic tree observed in figure 22 derive from the alignment of *FLC2* and *N. crassa spray protein* sequence spanning from TM4 to TM6 (the pore forming region of TRP-ion channels), with members of TRPML *(H. sapiens MCOLN2* and *D. melanogaster TRPML*) and TRPP (*C. elegans PC2*, *D. melanogaster PC2* and *H. sapiens PC2*) branch of TRP family. It is evident that all these proteins are closely related, but *FLC2* is more similar to *spray protein* and TRPML than to TRPP, as previously suggested.

Based on these sequence alignments and on previous works, both FLC genes and spray proteins seem to belong to a new fungal specific family of calcium transporters similar to TRPML and TRPP branch of TRP family of cation channels. Thus, renaming the *FLC1*, *FLC2*, *FLC3* and *YOR365C S. cerevisiae* genes as *TPR* (for TRP Related) *1*, *2*, *3* and *4* would seem more appropriate.

The phenotype observed in cells carrying the deletion of *FLC2* strongly suggest a role of this protein as a calcium channel involved in HTS-induced release of calcium from ER.

Indeed, in addition to sequence alignments results, in *flc2Δ* mutant strain hypotonic shock driven calcium raise is completely inhibited in extracellular calcium shortage condition (Fig. 25 A). This phenotype is not reverted by HACS impairment (Fig. 25 B), nor by perturbing ER calcium homeostasis (Fig. 27), suggesting a direct role of Flc2 in calcium transport.

The initial rate of calcium concentration increase plotted against extracellular calcium concentration in wild type cells draws a very complex graph (Fig. 14), consistent with our model of two different components working in HTS response. At submicromolar external calcium concentration an interference by an additional transport is clearly observable, probably due to release of calcium from ER fully active in these conditions. Conversely, when extracellular calcium concentration is above 5 μM the initial rate of calcium raise starts to be

seriously inhibited (Fig. 14). Surprisingly, both these two phenomena completely disappeared when we evaluated initial rate of calcium concentration in *flc2Δ* mutant cells, which draws a graph perfectly fitted with a Hill function (Fig. 29). Thus, this indicates a double role of Flc2 in HTS-induced calcium signalling: first, Flc2 is the intracellular calcium transporter that drives calcium release, likely from ER, after hypotonic shock. Second, Flc2 is also part of the plasma membrane system that controls calcium influx in these conditions, and the disappearance of initial rate inhibition suggests that it is the calcium responsive subunit.

Another validation to our hypothesis comes from the observation of the massive increase of calcium release from intracellular storage after hypotonic shock when *FLC2* is overexpressed (Fig. 28). Overexpression of *FLC2* is characterized not only by a higher calcium peak, but also by a persisting signal, likely determined by a dramatic misregulation of ER calcium homeostasis.

The evaluation of the initial rate of calcium raise is a reliable method to study the plasma membrane component involved in HTS response. Indeed, calcium release from intracellular compartments is slower (30 sec.) than calcium influx from extracellular environment (5-10 sec.) (Fig. 13A, B) so, this will give a good estimation of the calcium influx, while the contribution of calcium release will be surely underestimated.

The K_M evaluated in wild type cells, taking consideration of the initial rate calculated in the micromolar external calcium concentration range, is 1.1 ± 0.2 μM, very similar to the k observed in Hill function that characterizes initial rate in *flc2Δ* mutant strain (0.92 ± 0.04 μM). Thus, the K_M of plasma membrane calcium transporter is around 1 μM either Flc2 component is present or not.

Moreover, the IC_{50} for extracellular calcium, caused by Flc2 presence on plasma membrane, is 20.6 ± 0.4 μM. Therefore, extracellular calcium concentration in yeast growing media used for research purpose is higher enough to completely inhibit HTS responsive components.

Finally, the cooperation number of 2,9 ± 0,5 in the Hill function fitting the initial rate of calcium raise in *flc2Δ* mutant strain indicates that plasma membrane transporter is formed by more transporting subunits, and the presence of Flc2 in this transporter suggests that it could be an heterotetrameric complex, as it was often observed for TRP channels (REF).

According to our mathematical model, all *FLC* genes are involved in calcium release from ER caused by hypotonic shock. However, we could not observe in

any of the mutant tested such dramatic phenotype if compared to *flc2Δ* mutant strain.

Indeed, in *flc1Δ*, *flc3Δ*, *flc1Δ flc3Δ* and *yor365cΔ* mutant strain, calcium efflux from intracellular stores is only slightly reduced (Fig. 24 A, 30 A, B, C) but always observable in presence of *FLC2* gene. *FLC1* and *YOR365C* seems to have a marginal role in HTS response, but we cannot exclude that the reduction in calcium efflux observed in strains lacking these two genes could be due to an indirect effect on ER stability or on calcium homeostasis in general. Conversely, the effect of *FLC3* disruption seems to bring to a more severe phenotype if compared with *FLC1* deletion. Indeed, whether calcium release from ER is not more severely affected in *flc3Δ* mutant cells than in *flc1Δ* mutant cells, if we consider *mid1Δ cch1Δ fig1Δ flc3Δ* mutant strain we can appreciate a much more severe defect than in the *mid1Δ cch1Δ fig1Δ flc1Δ* mutant strain. This suggests a more direct role of *FLC3*, than *FLC1* or *YOR365C*, in response to hypotonic shock, likely as a regulatory protein of Flc2 calcium channel on endoplasmic reticulum.

As mentioned above, *S. cerevisiae FLC* genes belongs to a specific fungal family of calcium channels similar to TRPML and TRPP. Our alignments strongly suggest a role of calcium transporter not only for *FLC2* but also for *FLC1*, *FLC3* and *YOR365C* (Data not shown).

However, analysis of calcium flux after hypotonic shock demonstrates that only Flc2 has a role as a calcium channel in these conditions, while other *FLC* genes likely solves regulatory roles. This suggests that genes of this family have greatly diverged in functions during evolution, consistent with the high difference in the C terminus aminoacidic sequence observed within *FLC* genes.

This difference in function is also evident if we consider Calcofluor white (CW) sensitivity assays. Only the deletion of *FLC2* confers a major sensitivity to cell wall perturbing agent CW, while cells lacking *FLC1*, *FLC3* or *YOR365C* show a survival rate similar to a wild type strain (Fig. 32 A, B).

Consistently with the observed hypersensitivity to Calcofluor white, Flc2 was also previously reported to be regulated by the cell wall biogenesis and maintenance pathway and to be regulated by Rlm1, the cell wall integrity responsive transcription factor.

Indeed, lack of *FLC2* causes a significative increase in β-galactosidase gene transcription when under the control of Rlm1-responsive promoter in exponentially growing cells. This is consistent with a role of Flc2 in the response

to cell wall integrity pathway (CWI), whose activation is nonetheless not impaired by *FLC2* deletion.

Indeed, hypotonic shock induces the Pkc1 signal transduction cascade, which activates the Cell Wall Integrity (CWI) MAP kinase cascade (Davenport *et al.,* 1995), even in *flc2Δ* mutant strains, suggesting that Flc2 is important for cell wall stability but not for the activation of CWI pathway (Fig. 36).

Surprisingly, HTS do not activate calcineurin, even if it is hyperactivated in both *flc1Δ* and *flc2Δ* exponentially growing cells (Fig. 35), neither in a direct manner nor via Crz1 transcription factor (Fig. 20 C). Our data on HTS-driven calcium flux suggest that defect in calcium influx from extracellular environment, observed in *cnb1Δ* mutant strain (Fig. 20 A), is due to a hyperactivation of Cch1, as confirmed by the reverted phenotype of *mid1Δ cch1Δ cnb1Δ* mutant strain (Fig. 20 B). Calcineurin is indeed known to inhibit Cch1 in rich media (Muller *et al.,* 2001) and the lack of HACS regulation causes a major influx of calcium in resting cells, inhibiting HTS response.

However, hyperactivation of calcineurin dependent transcription observed in *flc1Δ* and *flc2Δ* exponentially growing cells could be explained by the higher level of basal cytosolic calcium observed in those mutant strains, further confirming a role in calcium homeostasis for both these FLC proteins.

Protchenko and coworkers performed their assays to test FAD transport on a strain lacking both *FLC1* and *FLC2* genes, whose viability was kept by the expression of *FLC1* on a repressible plasmid. In those condition, they also observed a hyperactivation of unfolded protein response (UPR), caused by an extreme ER stress, after repression of *FLC1* expression.

Conversely, the single *flc2*Δ mutant, as the other mutants tested, does not show any induction of UPR dependent transcription (Fig. 34), suggesting that ER stress is not an issue in the single mutant. Moreover, deletion of *FLC2* do not cause an increased sensitivity to ER stressing agent tunicamycin (Fig. 33), confirming that lack of *FLC2* alone does not impinge on ER function.

Surprisingly, we found a genetic interaction between *MID1* and *FLC2* as far as ER stress is concerned, since *mid1Δ flc2Δ* mutant strain is not viable on 0,25 µg/ml tunicamycin. Anyway, this higher sensitivity to tunicamycin could be merely due to the additive effect of *MID1* and *FLC2* deletion on ER calcium homeostasis, but are nonetheless worthy further investigation.

The similarity of FLC proteins with channels permeable to calcium and iron could be involved in the previously reported involvement of *S. cerevisiae* FLC

proteins overexpression in yeast survival on hemin (Protchenko *et al.,* 2006). 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.

Thus, even if Flc2 was reported to be a flavin carrier, it is evident from our data that the previously described role of Flc1 and Flc2 on FAD transport could be due to an indirect effect of ER stress caused by *FLC1* and *FLC2* simultaneous deletion (Protchenko *et al.,* 2006). Indeed, FLC proteins are more likely involved in calcium transport, as demonstrated by sequence alignments with known calcium transporters and by our data on HTS-induced calcium fluxes.

RIASSUNTO

Nelle cellule eucariotiche il calcio è una delle molecole segnale maggiormente versatili, poiché, oltre ad essere un cofattore fondamentale per numerose funzioni proteiche, rappresenta un secondo messaggero intracellulare ubiquitario.

Durante la crescita, il lievito *S. cerevisiae* mantiene la concentrazione citosolica di calcio ($[Ca^{2+}]_i$) in un *range* compreso tra 50 e 200 nM, indipendentemente dalla concentrazione extracellulare di tale ione. La $[Ca^{2+}]_i$ è controllata principalmente da pompe, scambiatori e canali localizzati sia sulla membrana plasmatica che sulle membrane intracellulari. Sulla membrana plasmatica sono noti agire Mid1 e Cch1, che formano il complesso HACS (*High Affinity Calcium System*), Fig1, unica proteina identificata fino ad ora del complesso LACS (*Low Affinity Calcium System*) e un trasportatore ancora non identificato denominato GIC. Sulle membrane interne troviamo invece Yvc1, un canale TRP vacuolare, meccanosensibile e attivato dal Ca^{2+} stesso, e Mid1, localizzato sulle membrane dell'ER.

Nelle cellule di lievito, così come in quelle di mammifero, lo shock ipotonico causa uno stress a livello della membrana, in risposta al quale le cellule attivano dei flussi ionici al fine di regolare il volume e la pressione cellulare. Studi presenti in letteratura hanno dimostrato che nel lievito *S. cerevisiae* lo stress generato dallo shock ipotonico induce un aumento transiente della concentrazione di Ca^{2+} citosolica. Dai nostri studi risulta che lo shock ipotonico è mediato, in cellule cresciute in terreno ricco, da due componenti cineticamente distinte, una molto rapida (circa 5 secondi), leggermente inibita da calcio e dipendente dal calcio extracellulare, e l'altra più lenta (10-20 sec.), fortemente sensibile alla presenza di calcio nell'ambiente esterno, al punto da non essere rilevabile anche a concentrazioni extracellulari di 1µM. Mentre la prima risposta sembra essere mediata da un trasportatore localizzato sulla membrana plasmatica la seconda è chiaramente dipendente da un rilascio dagli *store* intracellulari.

Alcuni dati presenti in letteratura sui geni regolati dal calcio ci hanno suggerito di studiare il ruolo nella risposta a shock ipotonico di due geni omologhi, di cui il primo è stata proposto come codificante per un putativo canale per ioni simile ai canali TRP di mammifero: *YOR365C* e *FLC2*.

Flc2 è localizzata sulle membrane plasmatica, di Golgi ed ER ed è stato proposto come putativo trasportatore di flavine assieme ai suoi omologhi Flc1 e Flc3. TuttaviaFlc2 era già stato precedentemente identificato come un omologo del canale del calcio pkd2 di *S. pombe.* Effettivamente in base a nostri allineamenti è risultato evidente come Flc2 appartenga a una sottofamiglia di canali TRP-*like* tipica dei funghi, a cui appartengono anche le mucolipine umane e le proteine spray fungine, ma non le proteine Pkd2. Mentre nel caso dei ceppi privi di *YOR365C*, *FLC1* o *FLC3*non è stata rilevata nessuna differenza nei flussi di calcio rispetto al selvatico, la mancanza di *FLC2* abbatteva completamente il rilascio di calcio dagli *store* intracellulari in carenza di calcio extracellulare, indicando che questi geni, pur appartenenti alla stessa famiglia, svolgono funzioni molto diverse.

Per caratterizzare in modo efficace i flussi di calcio dovuti allo shock ipotonico abbiamo calcolato la velocità iniziale di incremento della $[Ca^{2+}]_i$ (v_0) sia nel ceppo selvatico che nel ceppo *flc2Δ* al variare della concentrazione di calcio extracellulare.

Nel ceppo selvatico i dati in nostro possesso non potevano essere interpolati da nessuna equazione di Hill, a causa di una componente additiva presente a concentrazioni submicromolari di calcio extracellulare, mentre prendendo in considerazione i dati rilevati a concentrazioni di calcio superiori a1µM possiamo osservare una perfetta Michaelis-Menten caratterizzata da una K_M di 1 µM. A concentrazioni di calcio superiori a 1 µM invece la $v_0$subisce una forte inibizione dovuta al calcio stesso, con una IC_{50} di 20 µM.

Al contrario i dati delle $v_0$calcolati nel ceppo *flc2*Δ sono interpolati da una curva di Hill anche a concentrazioni submicromolari di calcio, in quanto non si rileva alcuna componente additiva (dovuta probabilmente al rilascio di calcio dall'ER controllato da Flc2) a basse concentrazioni di calcio, né d'altra parte si osserva l'inibizione ad alte concentrazioni. Anche in questo caso la *k* è di circa 1 µM e la curva mostra un numero di cooperatività di 3.

Questi dati ci portano ad affermare che il flusso di calcio a seguito di uno shock ipotonico è caratterizzato dall'attività di due diversi complessi: uno localizzato sull'ER, attivo solo quando non è disponibile calcio extracellulare e regolato da Flc2, che ha probabilmente funzione di trasporto o di attivazione del complesso; l'altro sulla membrana plasmatica, attivo solo quando è presente calcio nell'ambiente extracellulare a concentrazioni micromolari. La peculiarità di quest'ultimo complesso è che, alla luce della scomparsa

dell'inibizione ad alte concentrazioni di calcio osservata nel ceppo *flc2Δ*, anch'esso sembra coinvolgere l'attività della proteina Flc2, che è probabilmente la subunità regolatoria sensibile al calcio del trasportatore in questione.

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Cell Calcium xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Cell Calcium

journal homepage: www.elsevier.com/locate/ceca

Hypotonic stress-induced calcium signaling in *Saccharomyces cerevisiae* involves TRP-like transporters on the endoplasmic reticulum membrane

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ARTICLE INFO

Article history:
Received 13 August 2014
Received in revised form 5 December 2014
Accepted 6 December 2014
Available online xxx

Keywords:
Budding yeast
Mucolipin like calcium channels
Polycystin 2 like calcium channels
PKD2 like calcium channels
Osmotic shock
TRPML
TRPP

ABSTRACT

Saccharomyces 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 *Schizosaccharomyces pombe pkd2* and *Neurospora 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.

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 and 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

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.

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http://dx.doi.org/10.1016/j.ceca.2014.12.003
0143-4160/

technique allows 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 (OD_{600}) or by Coulter Counter (Coulter Electronics Z2).

Hypotonic shock was applied by diluting the cells suspension with 4 vol. 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 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. [24], and the same primers listed in 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 Wisconsin-Madison, WI).

In order to overexpress *FLC2* gene, a PCR was performed using FLC2GAP-REP For and FLC2GAP-REP Rev primers (see Table S2) 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 × 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 × 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

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(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 [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 vol. 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 s 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 biological 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:

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

Finally, patterns of variation in $[Ca^{2+}]_i$ of different cellular strains were compared with those of the wild type by pairwise 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 × 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 × 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 13,000 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 vol. 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 1 M Na_2CO_3. Samples were then centrifuged at 13,000 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 40× or a 60× 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 (McMaster 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

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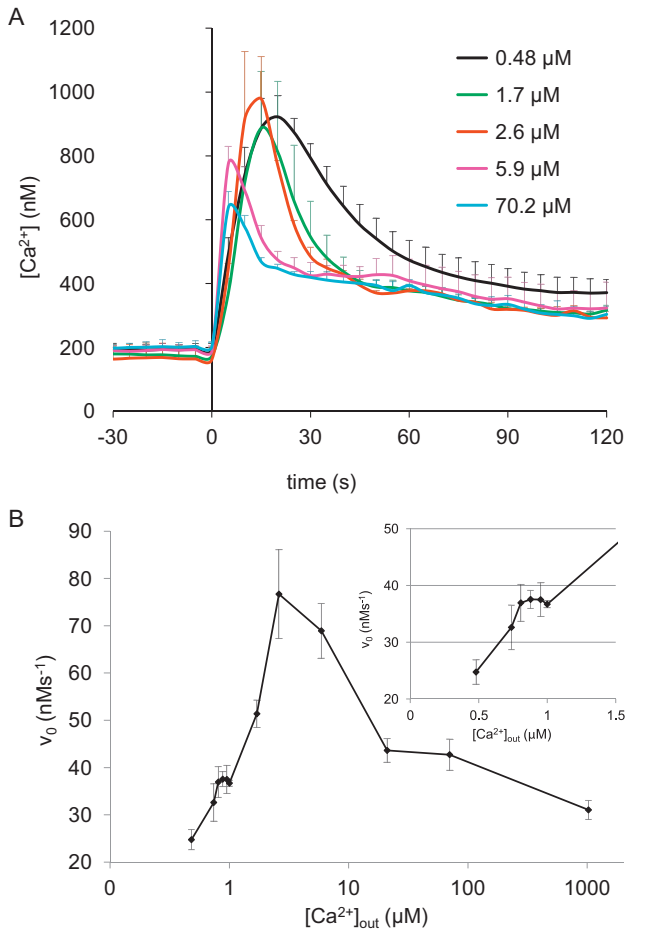

Fig. 1. Hypotonic shock-induced calcium response is regulated by calcium availability in extracellular medium. (Panel A) Hypotonic shock-triggered calcium peak was observed as described in Section 2 in K601 strain cells exponentially growing in YPD medium added with 0.2 mM EGTA and different concentrations 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.

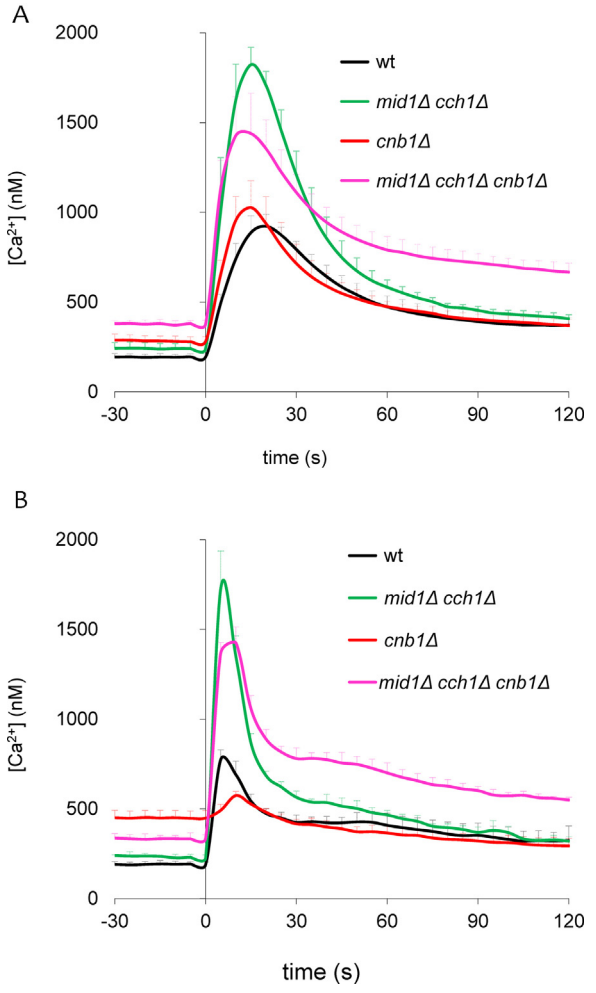

Fig. 2. HACS components are involved in inhibition of calcium response to HTS by calcineurin. Hypotonic shock triggered calcium peak was observed as described in Section 2 in *mid1*Δ *cch1*Δ strain, *cnb1*Δ strain and *mid1*Δ *cch1*Δ *cnb1*Δ strain exponentially growing in YPD medium, at the free calcium 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.

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

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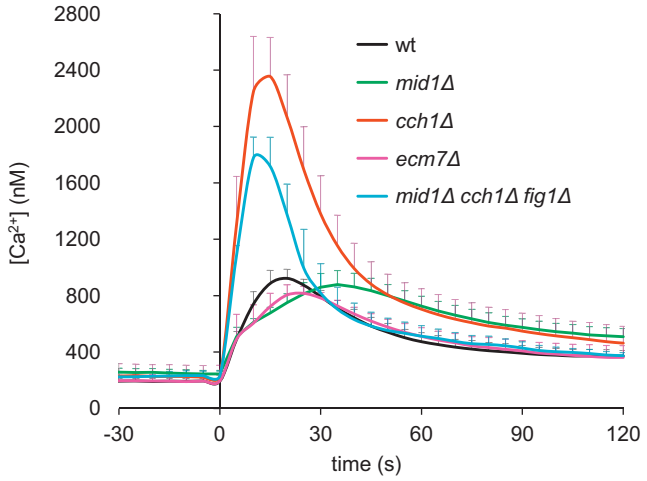

Fig. 3. HACS or LACS calcium transport systems components are not required for calcium release induced by HTS. Hypotonic shock triggered calcium release was observed as described in Section 2 in *mid1*Δ strain, *cch1*Δ strain, *ecm7*Δ strain and *mid1*Δ *cch1*Δ *fig1*Δ strain cells exponentially growing 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.

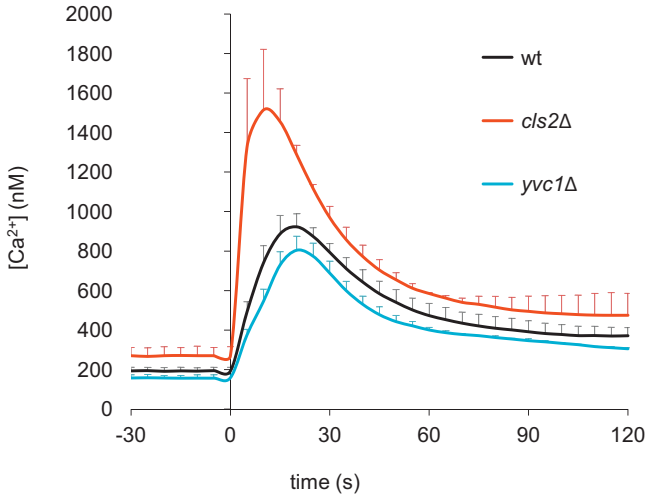

Fig. 4. Involvement of intracellular calcium stores in HTS response. Hypotonic shock-triggered calcium release was observed as described in Section 2 in *yvc1*Δ strain and *cls2*Δ strain cells exponentially growing in YPD medium at the presence of 0.48 μM free extracellular calcium concentration. Bars represent standard deviations and are reported on the upper side of each curve only, to avoid overlap.

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 Fig. 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 *ecm7*Δ 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 [34,35]. 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

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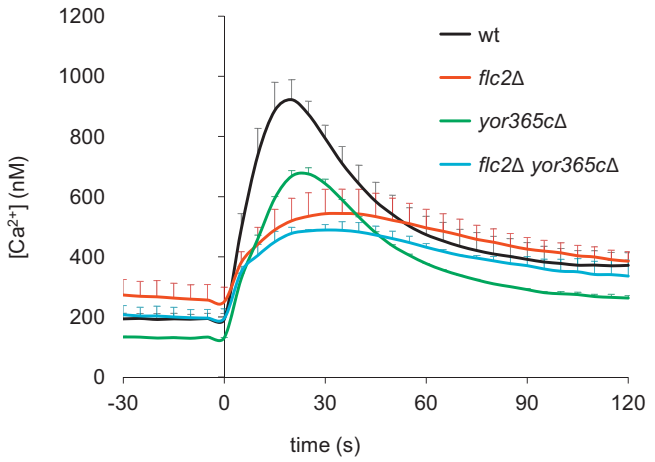

Fig. 5. Involvement of Flc2 and Yor365c paralog proteins in HTS-induced calcium release. Hypotonic shock-triggered calcium peak was observed as described in Section 2 in *flc2*Δ strain, *yor365c*Δ strain and *flc2*Δ *yor365c*Δ strain cells exponentially growing in YPD medium at the presence of 0.48 μM free extracellular calcium concentration. Bars represent standard deviations and are reported on the upper side of each curve only, to avoid overlap.

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,

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Table 1
Results from the polynomial mixed model of variation in $[Ca^{2+}]_i$ between zero and 20 s 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 errors 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 (b_0)	8.20	5	0.146	wt	192.63	31.18	52	6.178	<0.001
				*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 × Time (b_1)	84.49	5	<0.001	wt	72.12	3.74	52	19.301	<0.001
				*flc1*Δ	−36.53	5.71	52	−6.400	<0.001
				*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 × Time^2 (b_2)	35.20	5	<0.001	wt	−1.78	0.17	52	−10.559	<0.001
				*flc1*Δ	0.93	0.26	52	3.627	0.011
				*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.

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).

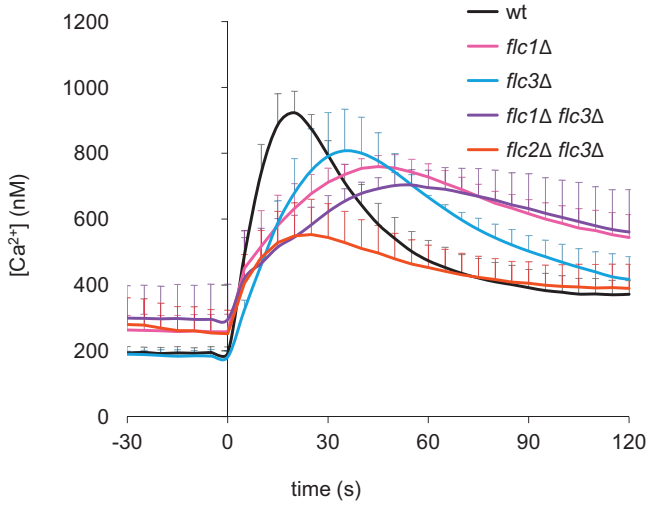

Fig. 6. Involvement of Flc2 homologous proteins in HTS-induced calcium release. Hypotonic shock-triggered calcium peak was observed as described in Section 2 in the indicated strains exponentially growing in YPD medium at the presence of 0.48 μM free extracellular calcium concentration. Bars represent standard deviations and are reported on the upper side of each curve only, to avoid overlap.

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 contribution to the observed variation in $[Ca^{2+}]_i$ is by far lower than that of calcium

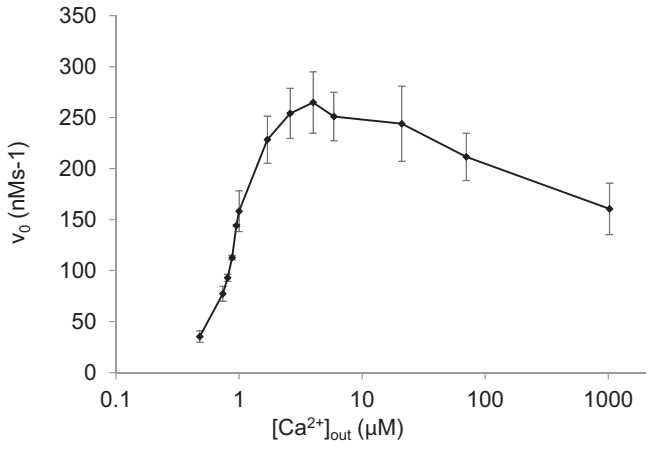

Fig. 7. Flc2 is the calcium responsive protein in HTS induced calcium response. Initial rate in cytosolic calcium increase in the *flc2*Δ strain was plotted against free calcium concentration in the extracellular medium.

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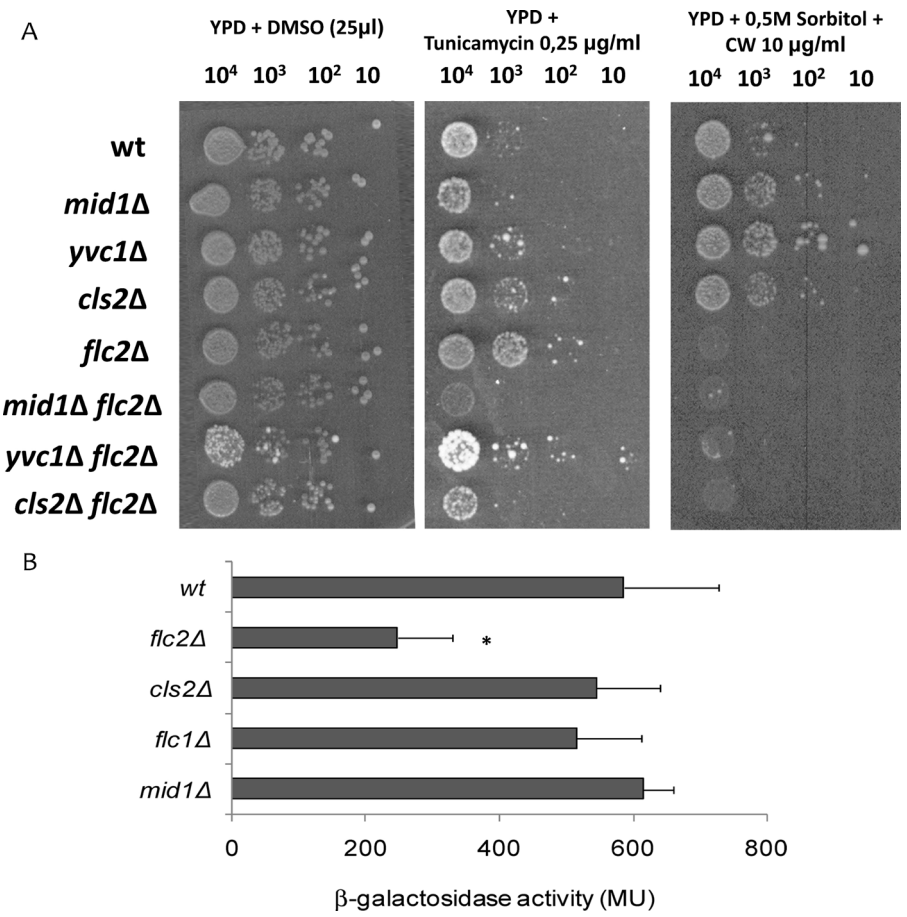

Fig. 8. (Panel A) Disruption of *FLC2* gene confers Calcofluor white hyper-sensitivity but has not any effect on sensitivity to tunicamycin. Indicated strains were grown in YPD medium until exponential phase and then diluted to appropriate cell density to spot 10^4, 10^3, 10^2 and 10 cells. Spots were laid on YPD medium either added with DMSO (left) or with 0.25 μg/ml tunicamycin (middle) or with 0.5 M 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 Section 2. *, $P = 0.001$ vs. wt.

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

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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 Fig. 6). 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.

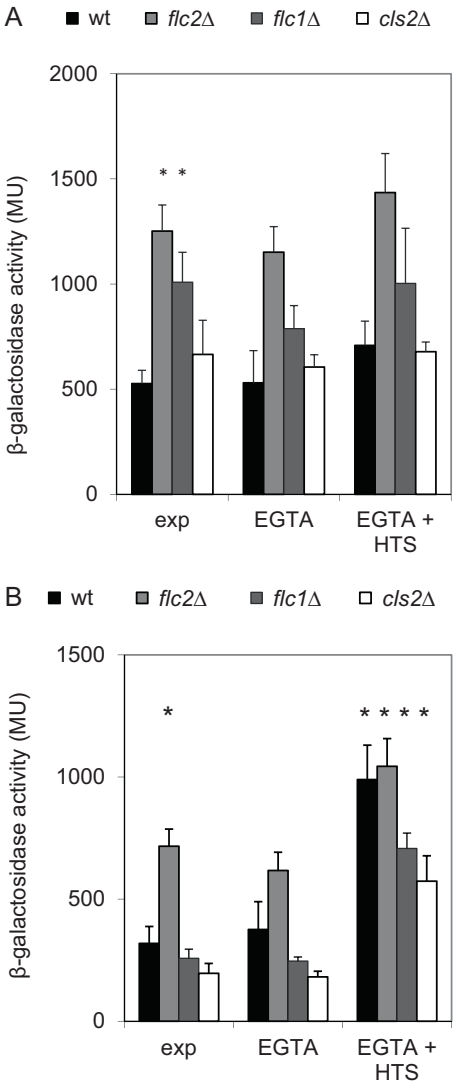

Fig. 9. Disruption of *FLC2* gene impinges on calcineurin and Mpk1-dependent transcription. Wild-type (closed rectangles), *flc2*Δ (light gray rectangles), *flc1*Δ (dark gray rectangles) or *cls2*Δ (open rectangles) strains cells transformed either with pAMS366 (Crz1-responsive reporter) plasmid (panel 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 Section 2. *, $P < 0.01$ vs. wt (for exp) or EGTA (for EGTA + HTS).

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 *S. 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 *S. 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

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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*. Hsiang 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 [74]. 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.

Acknowledgements

We thank B. Glick (University of Chicago, USA), M. Cyert (Stanford University, USA), D. Levin (John Hopkins University, USA), D. Eide (University of Wisconsin-Madison, USA) for kindly providing materials and yeast strains. This work was partially supported by University of Milano-Bicocca funding to EM and RT.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ceca.2014.12.003.

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Biochimica et Biophysica Acta 1823 (2012) 1208–1216

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Localization of Ras signaling complex in budding yeast

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ARTICLE INFO

Article history:
Received 13 September 2011
Received in revised form 26 April 2012
Accepted 30 April 2012
Available online 7 May 2012

Keywords:
Saccharomyces cerevisiae
Cdc25
Ira
Adenylate cyclase
Membrane fractionation
Mitochondria

ABSTRACT

In *Saccharomyces cerevisiae*, cAMP/pKA pathway plays a major role in metabolism, stress resistance and proliferation control. cAMP is produced by adenylate cyclase, which is activated both by Gpr1/Gpa2 system and Ras proteins, regulated by Cdc25/Sdc25 guanine exchange factors and Ira GTPase activator proteins.
Recently, both Ras2 and Cdc25 RasGEF were reported to localize not only in plasma membrane but also in internal membranes. Here, the subcellular localization of Ras signaling complex proteins was investigated both by fluorescent tagging and by biochemical cell membrane fractionation on sucrose gradients. Although a consistent minor fraction of Ras signaling complex components was found in plasma membrane during exponential growth on glucose, Cdc25 appears to localize mainly on ER membranes, while Ira2 and Cyr1 are also significantly present on mitochondria. Moreover, PKA Tpk1 catalytic subunit overexpression induces Ira2 protein to move from mitochondria to ER membranes. These data confirm the hypothesis that different branches of Ras signaling pathways could involve different subcellular compartments, and that relocalization of Ras signaling complex components is subject to PKA control.

1. Introduction

In *Saccharomyces cerevisiae*, cAMP regulates PKA activity, which is involved in post-translational regulation of a variety of proteins, for example key enzymes for gluconeogenesis or glycolysis [1], is required for G1 to S phase progression in the cell cycle and modulates the critical cell size required for budding and for entry into mitosis [2]. Cyclic AMP is synthesized by Cyr1 adenylate cyclase, which is controlled by two different systems: the G-protein-coupled receptor system (GPCR), acting through Gpa2 G-protein [3,4], and Ras small G protein system [5]. Ras protein activity is controlled by regulatory proteins: Cdc25 and Sdc25 guanine nucleotide exchange factors (GEFs) [6], which stimulate the GDP/GTP exchange on Ras, and Ira1 and Ira2 GTPase activating proteins (GAPs), which promote the intrinsically low Ras GTPase activity [7].

Literature data suggest that upstream components of cAMP/PKA pathway interact to constitute a large multiprotein complex: after detergent extraction, adenylate cyclase behaves as a large complex on gel filtration and sucrose density centrifugation [8–11]; consistently, adenylate cyclase interacts with Ras proteins [12,13], and with Cdc25 SH3 motif [14]; moreover, Ira1 was shown to be involved in Cyr1 association to membranes [8,15].

Cdc25 and Ira proteins were shown to be tightly attached to membrane fraction [16,17], adenylate cyclase is only loosely bound to the membranes, migrates in lower density internal membrane fractions on a sucrose gradient [18] and seems to rely on Ira1 for its membrane targeting [8,15].

Recent data have shown that mammalian Ras isoforms are differentially distributed within cell surface nanoclusters and on endomembranous compartments [19]. The three abundant mammalian isoforms – H-Ras, N-Ras and K-Ras4B – have a high degree of sequence identity in their G-domain, the structural element required for the switch function and for effector binding; the differences among the isoform localization and function can be attributed to different post-translational lipid modifications that are encoded within their divergent C termini. In yeast, Ras2 localization on internal membranes was also investigated: Ras proteins are synthesized as cytosolic precursors and then they are anchored into membranes *via* post-translational modifications; most of the Ras modification steps occur on the cytoplasmatic surface of the endoplasmic reticulum, Ras2 is engaged to the ER membrane by Eri1 protein [20] and the subsequent Ras translocation from ER to plasma membrane doesn't require the classical secretory pathway in yeast [21,22]. Besides, recent works point to a possible role for mitochondria in Ras localization [23]. Moreover, we have reported that Cdc25 and Ira1 are found not only associated with membranes, but also in the nucleus [24,25], and active Ras was also detected in the nuclear compartment, even if the meaning of this result is still under investigation [26]. Finally, we recently reported that PKA activity is not only able to negatively control Cdc25 localization in the plasma membrane, but

Abbreviations: cAMP, 3′-5′-cyclic adenosine monophosphate; GEF, guanine exchange factor; GAP, GTPase activating protein; ER, endoplasmic reticulum; PKA, protein kinase A; GPCR, G-protein coupled receptor

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doi:10.1016/j.bbamcr.2012.04.016

also in the nuclear compartment [25]. Ras localization was also recently found to be affected by PKA activity [25,27].

Consequently, even if the complex has been initially thought to function at the plasma membrane level, only Ras2 showed a clear, but partial, plasma membrane localization, while no clear data are available for the localization of the proteins involved in this pathway. Here we demonstrate that, during exponential growth on glucose, Ras2 signaling complex components are associated mostly to internal membranes, in particular to ER membranes, suggesting the presence of a large signaling complex working inside the cell rather than on its surface, at least during exponential growth on glucose. In addition a possible role of mitochondria in Ras signaling is reinforced by our observation on the partial localization of both Cyr1 and even more of Ira2 on these organelles.

2. Materials and methods

2.1. Strains and cultures

Top10 *E. coli* strain (Invitrogen) was grown in LB medium (1% NaCl, 1% Bacto-peptone, 0.5% yeast extract, 0.1% glucose) at 37 °C. For Amp^R selection, ampicillin was added to a final concentration of 50 mg/L. For solid media, 1.5% agar was added.

Yeast strains utilized in this work are described in Table 1. Yeast cells were grown in YP (1% yeast extract, 2% Bacto-peptone) supplemented with 2% w/v glucose (YPD). Yeast strains carrying a plasmid were grown in synthetic medium (SC) containing 0.67% w/v yeast nitrogen base w/o amino acids, CSM synthetic amino acid mixture (BIO101, USA) required and the appropriate sugar (2% glucose or 2% galactose with 0.1% glucose). Solid medium contained 2–2.5% agar. In all conditions cells were grown at 30 °C. Cell density was determined by measuring optical density (OD_{600}) or by Coulter Counter (Coulter Electronics mod. Z2). Budding index (BI) was determined by direct microscopic count of at least 300 cells fixed in 4% formalin and mildly sonicated. Cell volume distributions were determined with a Coulter Counter Channelyzer 256 [2].

Cells starved for nutrients or for carbon source were grown in YPD or in SC until exponential phase, then collected by filtration, washed with water, resuspended in 0.25 M MES pH 6.5 and incubated for 4 h.

2.2. Strains and plasmid construction

Strains carrying 4-HA tagged Ira1 and Ira2 were constructed according to the method first described by Wach et al. [28], by transforming the W303-1A strain with a PCR generated cassette amplified from pDHA plasmid [29] (kind gift from P. Coccetti, University of Milan-Bicocca) with Pfu Turbo enzyme (Promega) using as primers: for *IRA1* cassette, IRA1HA FOR and IRA1HA REV; for *IRA2* cassette, IRA2HA FOR and IRA2HA REV (Table 2).

YIplac204T/CSec7-7xDsRed, YIplac204T/CHmg1CFP [30] and YIplac204/TKC-DsRED-HDEL [31] were kind gifts from B. Glick (University of Chicago, USA).

The YIplac204T/CHmg1dsRED plasmid was obtained by substituting the *NcoI–PvuII* fragment in YIplac204T/CHmg1CFP with the *NcoI–HincII* fragment from pDsREDMonomer1 (Clontech).

The pYX-TPI-IRA2 plasmid was created by inserting the 506 bp *EcoRV–SalI* fragment from pd46 plasmid [7] (kind gift from JF. Cannon, University of Missouri, USA) in *XhoI–SmaI* digested pYX012 (Ingenius). The plasmid was linearized with *BglII* and inserted at *IRA2 locus* in YOL081W strain (Invitrogen) giving RT1200 strain.

Diploid RT1220 and RT1210 strains were obtained by conjugation of W303-1B transformed with YIplac204T/CSec7-7xDsRed respectively with YJL005W or RT1200, diploid RT1230 and RT1240 strains were obtained by conjugation of W303-1B transformed with YIplac204T/CHmg1xDsRed respectively with YJL005W or RT1200. RT1211 and RT1221 aploid strains were obtained by sporulation respectively of RT1210 and RT1220 strains, and isolated by random spore analysis.

pYX212-EGFP-RBD-3 and YEpTPK1 plasmids were previously described [26,32].

2.3. Cellular membrane fractionation

The method was first described by Sidoux-Walter et al. [33], with modifications. Cells were grown until mid-log phase (OD_{600} 0.6–1.0) at 30 °C in YPD medium, harvested by centrifugation at 4 °C for 10–15 min, washed with 20 ml of Cell Wash Buffer (CWB) (10 mM Tris–HCl pH 7.5, 0.5 M sucrose, and either 2.5 mM EDTA or 2 mM magnesium chloride, as indicated) and collected by centrifugation. Cell pellet was weighed and frozen at −80 °C.

Table 1
Strains used in this study.

Strain	Relevant genotype	Source
W303-1A	*MAT a ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	[58]
W303-1B	*MAT α ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*	[58]
OL568-1C	W303-1A *3xHA-CDC25*	[59]
RT1170	W303-1A *IRA1-4xHA::KanMX6*	This work
RT1180	W303-1A *IRA2-4xHA::KanMX6*	This work
YLR378C	*MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SEC61-GFP::Sp his5*$^+$	Invitrogen
YEL036C	*MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ANP1-GFP:: Sp his5*$^+$	Invitrogen
YJL005W	*MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 CYR1-GFP:: Sp his5*$^+$	Invitrogen
YOL081W	*MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 IRA2-GFP::Sp his5*$^+$	Invitrogen
W303-$CDC25^{NES}$-eGFP	W303-1A [YEp$CDC25^{NES}$-eGFP]	[24]
RT1200	*MAT a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ira2::URA3-(TPI prom)IRA2-GFP::Sp his5*$^+$	This work
RT1210	*MAT a/α CAN1/can1-100 ADE2/ade2-1 his3Δ1/his3-11,15 leu2Δ0/leu2-3,112 met15Δ0/MET15 ura3Δ0/ura3-1 ira2::URA3-(TPI prom)IRA2-GFP::Sp his5*$^+$*/IRA2 trp1-1::YIplac204T/C SEC7-7xDsRed/TRP1*	This work
RT1220	*MAT a/α CAN1/can1-100 ADE2/ade2-1 his3Δ1/his3-11,15 leu2Δ0/leu2-3,112 met15Δ0/MET15 ura3Δ0/ura3-1 CYR1-GFP::Sp his5*$^+$*/CYR1 trp1-1::YIplac204T/C SEC7-7xDsRed/TRP1*	This work
RT1230	*MAT a/α CAN1/can1-100 ADE2/ade2-1 his3Δ1/his3-11,15 leu2Δ0/leu2-3,112 met15Δ0/MET15 ura3Δ0/ura3-1 ira2::URA3-(TPI prom)IRA2-GFP::Sp his5*$^+$*/IRA2 trp1-1::YIplac204T/C HMG1-DsRed/TRP1*	This work
RT1240	*MAT a/α CAN1/can1-100 ADE2/ade2-1 his3Δ1/his3-11,15 leu2Δ0/leu2-3,112 met15Δ0/MET15 ura3Δ0/ura3-1 CYR1-GFP::Sp his5*$^+$*/CYR1 trp1-1::YIplac204T/C HMG1-DsRed/TRP1*	This work
RT1211	*MAT a ade2-1 his3 leu2 met15 ura3 ira2::URA3-(TPI prom)IRA2-GFP::Sp his5*$^+$ *trp1-1*	This work
RT1221	*MAT a ade2-1 his3 leu2 met15 ura3 cyr1::CYR1-GFP::Sp his5*$^+$ *trp1-1*	This work
W303-$CDC25^{NES}$	OL568-1C with *3xHA::CDC25::NES::Sphis5*$^+$	[24]
*cdc25*Δ YEpTPK1	W303-1A *cdc25::HIS3* [YEpTPK1]	[32]

Frozen cells were thawed at 30 °C, washed with 20 ml of Homogenization Buffer (HB) (50 mM Tris–HCl pH 7.5, 0.3 M sucrose, 2.5 mg/ml BSA, and either 5 mM EDTA and 1 mM EGTA or 2 mM magnesium chloride, as indicated), harvested by centrifugation, resuspended in HB (supplemented with 2 mM DTT, 1 mM PMSF, 2 μM pepstatin, Roche EDTA-free protease inhibitor cocktail) with approximately 2 ml of buffer per gram of cells wet-weight. Cells were disrupted with Fast-prep (Savant) in the presence of glass beads.

Extracts were clarified at 13,000 rpm for 10 min, then centrifuged at 100,000 ×*g* for 60 min and the supernatant was stored at −80 °C with 20% glycerol. Pellet (Microsomal Membrane pellet or MMP) was washed with Membrane Wash Buffer (MWB, 10 mM Tris–HCl pH 7, 1 mM DTT, 20%, glycerol and either 1 mM EGTA or 2 mM magnesium chloride, as indicated) and then centrifuged for 60 min at 100,000 ×*g*. Washed MMP was resuspended with 0.5 ml of MWB and overlayed on a 11 ml-sucrose gradient, prepared in sucrose gradient buffer (10 mM Tris–HCl pH 7, 1 mM DTT, and either 1 mM EDTA or 2 mM magnesium chloride, as indicated), with the following steps: 1 ml 46%, 2 ml 50%, 2 ml 54%, 2 ml 56%, 2 ml 58%, 1.7 ml 70% sucrose. Gradients were then centrifuged at 80,000 ×*g* for 17 h, and 1 ml-fractions were collected and stored at −20 °C.

Protein concentration was determined with Bio-Rad protein assay (Bio-Rad) and proteins were separated by SDS-PAGE and visualized by Western blot.

Several protein markers for the intracellular membranes were used, and each fraction was tested for the presence of: Gas1, which is post-translationally modified in ER and Golgi compartments and then is transported to the plasma membrane level [34], although it is present in traces also in mitochondria and nuclear envelope [35,36]; Anp1 as a marker for Golgi apparatus [37], Tom40 for mitochondria [38], Pho8 for vacuolar membranes [39], nucleolar protein Nop1 for nuclear membranes [40] and mature Pma1 for plasma membrane [41], although it also marginally localizes to vacuoles [36,42]. As ER marker Sec61–GFP fusion protein was used and expressed in YLR378C strain (Invitrogen). Protein profile was analyzed by Western blot using the following antibodies: goat α-Ras2 (Santa Cruz), mouse α-HA (Roche), goat α-Cyr1 (Santa Cruz), rabbit BD Living Colors antibody against eGFP protein (BD Biosciences, Clontech), rabbit α-Gas1 (kind gift from M. Vai, University of Milano-Bicocca), rabbit α-Tom40 (kind gift from T. Endo, Nagoya University, Japan), mouse α-Pma1 (Abcam), rabbit α-Anp1 (kind gift from S. Munro, MRC Lab of Molecular Biology, Cambridge, UK), mouse α-Pho8 (kind gift from J. Winderickx, K.U. Leuven, Belgium), mouse α-Nop1 (EnCor Biotechnologies). Secondary horseradish peroxidase-conjugated antibodies were from Jackson Immunoresearch.

2.4. GDP/GTP loading on Ras2

Gradient fractions 1–5 were collected (80–100 μg) and diluted 1:2 with Shalloway buffer 2× (50 mM Hepes pH 7.5, 300 mM NaCl, 20% glycerol, 50 mM NaF, 20 mM $MgCl_2$, 2 mM EDTA, 2 mM Na-vanadate, Roche EDTA-free protease inhibitor cocktail). They were incubated with 1 mM GDP or GTP (Sigma Aldrich) at room temperature for 15 min and then Ras2-GTP/total Ras2 ratio was measured as described by Colombo et al. [32]; briefly, fractions were incubated for 1 h at 4 °C with GST–RBD pre-bound to glutathione–Sepharose and Ras2-GTP bound to GST–RBD protein was eluted with SDS sample buffer (50 mM Tris–HCl pH 6.8, 2.3% SDS, 5% β-mercaptoethanol). Ras2 was detected by Western blot using anti Ras2 antibodies (Santa Cruz Biotechnology).

2.5. Epifluorescent microscopy

Cells were grown at 30 °C in the proper selective SC medium supplemented with 2% glucose to early exponential phase and images were collected and processed as previously described [24].

Cells carrying constructs expressing GFP or DsRed fusion proteins, eventually stained with DAPI, were observed with a Nikon Eclipse C600 fluorescence microscope using standard UV, FITC and Rhodamine filters. Images were acquired with a Leica DG350F CCD camera and elaborated with MacBiophotonics ImageJ software. Panels were mounted with Adobe Photoshop™.

2.6. Confocal microscopy

Confocal microscopy images were collected using the Leica TCS SP2 confocal microscope equipped with an inverted Leica DMIRE2 microscope and a PL APO 63× oil immersion objective (numerical aperture: 1.4). An average of 10 optical sections was acquired for every single cell, and a representative single optical section is shown. Mitochondria staining was realized with 100 nM Rhodamine B, hexyl ester, perchlorate (Molecular Probes). Images were elaborated with MacBiophotonics ImageJ software.

3. Results

Even if the cAMP/PKA pathway has been extensively studied, the physiological and functional localization of Ras signaling complex components is still in debate. No evident signals are detected by different prediction softwares (such as BaCelLo [43] or TargetP [44]) in these proteins for localization in any subcellular compartment such as ER, Golgi apparatus or mitochondria. To investigate this topic in more details, tagging with eGFP or DsRED fluorescent proteins was performed.

Since Cdc25 was previously shown to be efficiently imported and retained in the nuclear compartment [24], fluorescence tagging with eGFP was performed in the W303-1A-CDC25^{NES}-eGFP strain, carrying the Cdc25^{NES} protein, a version of Cdc25 which was modified by the insertion of a strong Nuclear Export Signal, and is therefore excluded from the nucleus. This allowed to better observe the localization of the protein within the cytosolic compartment, without the interference of the intense fluorescence in the nucleus observed for the wild-type Cdc25 protein. This mutant version confirmed that, as it was already observed for the wild-type version [24], Cdc25 is not homogeneously distributed in the cytosol: the co-localization with Hmg1DsRed (a protein localizing to ER when overexpressed) (Fig. 1A) reveals a partial co-localization with this compartment, while a co-localization with Sec7DsRed (a protein partially soluble and partially associated to the Golgi vesicles [36,45]) (Fig. 1B) followed by observation with a confocal microscope, revealed no co-localization in any of the observed cells. The co-staining of mitochondria with Rhodamine B (Fig. 1C) revealed only occasional co-localization with Cdc25eGFP, suggesting that Cdc25 cannot be observed in these compartments.

The localization of other Ras signaling complex components, Cyr1 and Ira2, was then considered. Again, these proteins were too lowly expressed to allow direct immunofluorescence observation. Both the proteins were modified by tagging with GFP, and Ira2-GFP fusion was slightly overexpressed (as revealed by the mild increase in the associated fluorescent signal) by inserting constitutive TPI promoter immediately upstream the Ira2 encoding sequence. These modifications allowed to observe that Ira2 showed significant and reproducible co-localization mainly with mitochondria, stained with DAPI (detectable in all the observed cells), and endoplasmic reticulum (ER), visualized by Hmg1 protein (detectable in 72% of the observed cells), but never with the Golgi marker Sec7 (Fig. 2B). Cyr1 was also partially observed in mitochondria (co-localization with DAPI stained mitochondria was observed in 69% of the observed cells), but it was clearly present in an internal compartment identified as ER (Fig. 2A) (co-localization with Hmg1DsRed fluorescence was observed in 88% of the cells observed). As well as for Cdc25, no co-localization was observed for Cyr1-GFP fusion with the Golgi marker Sec7 (Fig. 2B).

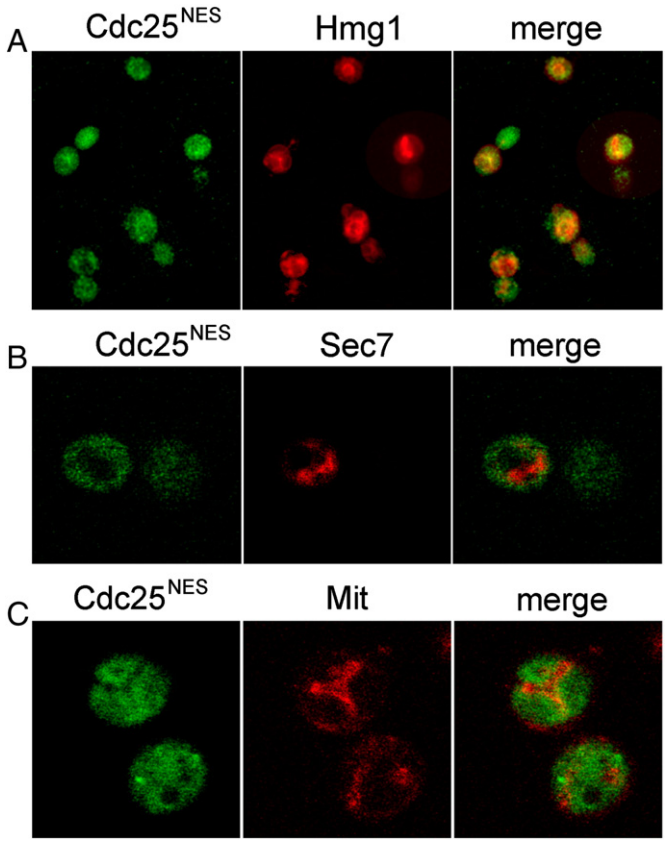

Fig. 1. Cdc25 protein is present on ER internal membranes. W303-CDC25^{NES}eGFP cells transformed either with YIplac204T/CHmg1-DsRed (expressing Hmg1-DsRED, localizing to the ER) (panel A) or with YIplac204T/CSec7-7xDsRed (expressing Sec7-DsRed, localizing to the Golgi apparatus) (panel B), or stained with the Rhodamine B dye for mitochondria (panel C) were observed using a fluorescence (A) or a confocal (B and C) microscope.

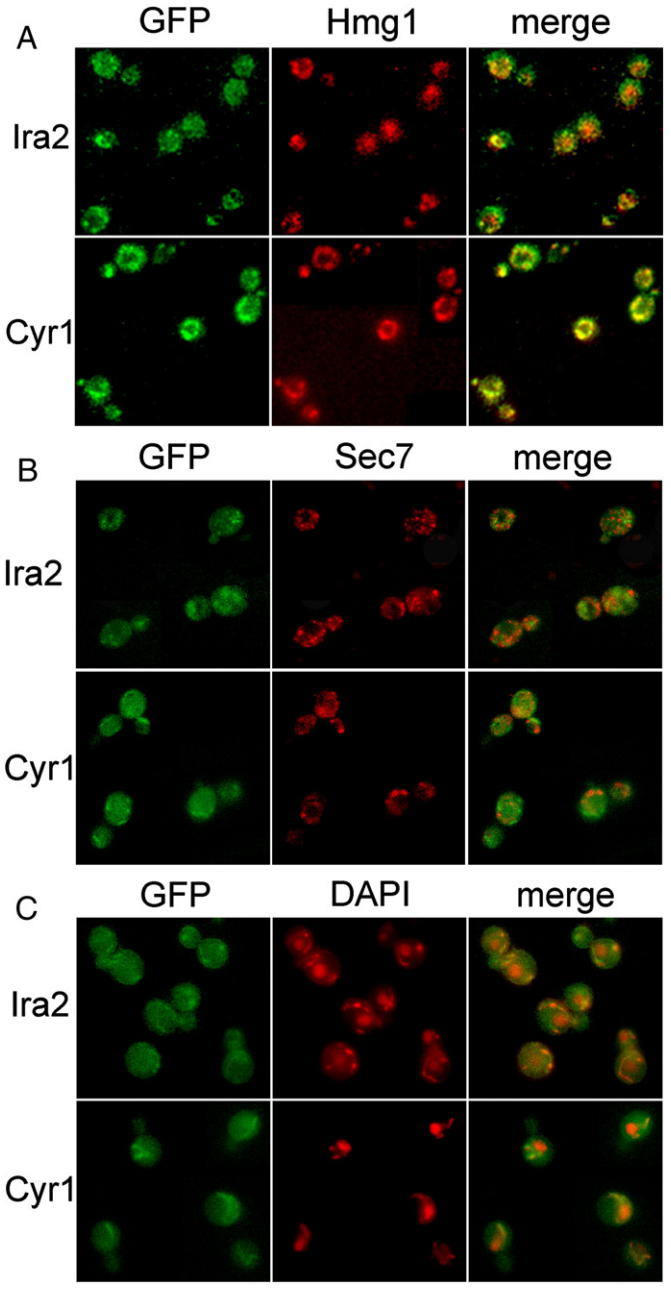

Fig. 2. Ira2 and Cyr1 proteins are present on mitochondrial membranes. RT1230 or RT1210 strains (for Ira2GFP) and RT1240 or RT1220 strains (for Cyr1GFP) were observed using a fluorescence microscope: panel A, colocalization with ER marker Hmg1 is shown; panel B, colocalization with Golgi marker Sec7; panel C, co-localization with DAPI-stained nuclei and mitochondria is shown.

Protein localization was confirmed by performing a cell extract fractionation assay on discontinuous sucrose gradients, followed by the analysis of the distribution of endogenously expressed proteins, in order to reveal any unphysiological effect on protein localization. In fact, mislocalization of Ras was already reported, due to its over-expression and/or fluorescent tagging [23].

Briefly, crude cellular membranes were layered on a 6 steps sucrose gradient (46%, 50%, 54%, 56%, 58%, 70%) and centrifuged for 17 h at 80,000 ×*g*. Eleven fractions were collected from the top of the gradient and analyzed by Western blot. As shown in Fig. 3A, internal membranes were found in fractions with lower density, while plasma membrane localized in the last fractions, consistently with previously reported data [46].

Results obtained from cellular fractionation showed that Ras2 and Cdc25 were mainly localized on internal membranes, while a consistent but minor fraction (~10%) of these proteins was localized at the plasma membrane level (Fig. 3B). Moreover, Cyr1 and Ira proteins showed the same pattern in fractionation experiments, supporting the hypothesis that the entire complex resides mostly on internal membranes. Only Ras2 and Ira proteins, but not adenylate cyclase and Cdc25, seem to reveal a concentration peak also in the lighter fractions, likely corresponding to mitochondria, according to fluorescence data, and consistently with what was already reported for Ras2 [23].

Table 2
Oligonucleotides used in this work.

Oligonucleotide	Sequence
IRA1HA FOR	AAGATTAGCAACAATGATACTGGCAAGAATGTCGTGCTCCCGGCCGCATGGATCCTATCC
IRA1HA REV	GGAAAAACGTAATAATCACTGCAATACTCTAATTTAAAAGGATGGCGGCGTTAGTATCG
IRA2HA FOR	AATAATGACAATG ATTTTATCAAGGATGTCATGTTCTGCTCGGCCGCATGGATCCTATCC
IRA2HA REV	ATAGATATTGATATTTCTTTCATTAGTTTATGTAACACCTGGATGGCGGCGTTAGTATCG

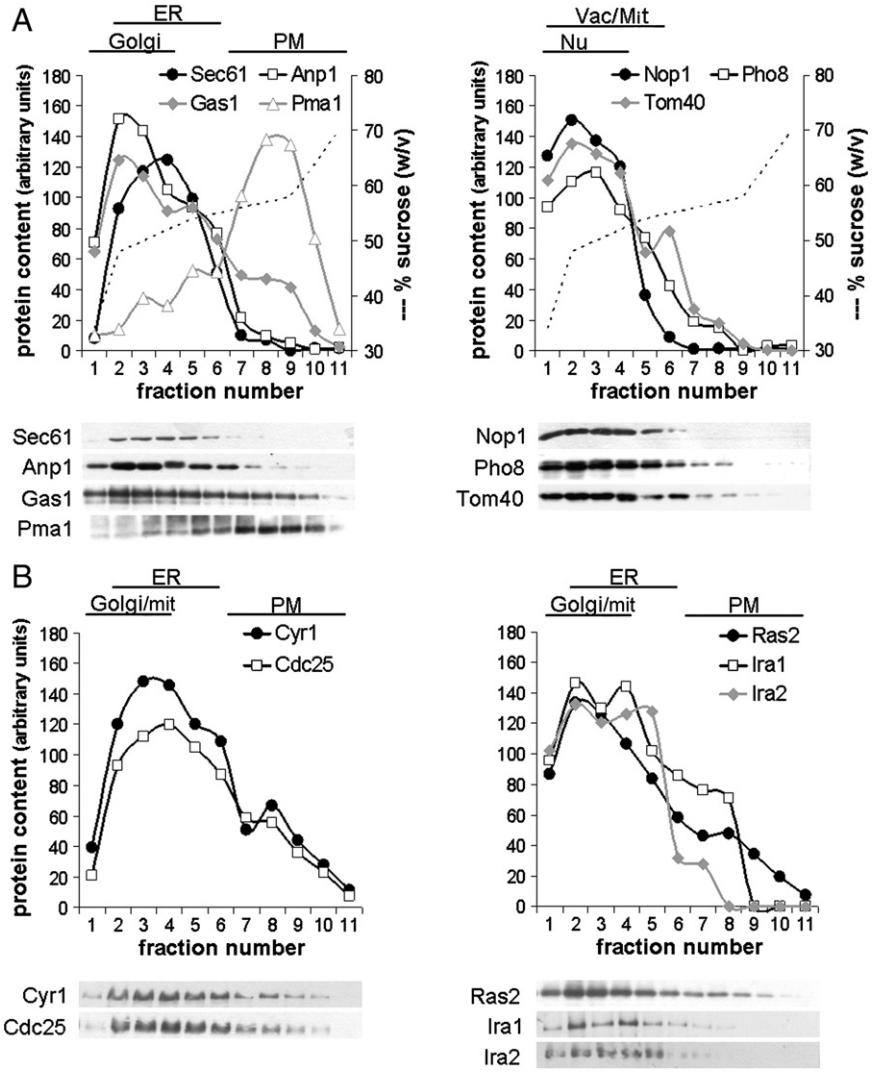

Fig. 3. Ras signaling complex mainly resides on internal membranes. Membranes were prepared from OL568-1C or YLR378C strain, then they were layered on sucrose gradient and centrifuged at 80,000 ×*g* for 17 h. 1-ml fractions were collected from the top of the gradient. Proteins were detected by Western blot. (A) Membrane marker distribution: Sec61 (ER), Anp1 (Golgi), Gas1 (ER, Golgi and plasma membrane), Pma1 (plasma membrane), Nop1 (nucleus), Pho8 (vacuolar membrane), Tom40 (mitochondria); (B) signaling transduction complex component localization. A typical distribution of the indicated proteins is shown out of at least four replicates. No differences were observed in marker distributions in different background strains.

The plasma membrane containing fractions were identified by visualizing mature Pma1. The analysis of the amount of protein present in the plasma membrane fractions revealed that the components of the Ras/adenylate cyclase complex are nonetheless significantly represented in the plasma membrane in exponentially growing cells, showing a ratio plasma membrane/internal membranes comparable to what was revealed for Gas1 protein, which has a complex internal and plasma membrane distribution [36], suggesting that in this physiological condition they could exert their essential function both on internal membranes and/or on the plasma membrane.

Since Cdc25 was previously shown to be efficiently imported and retained in the nuclear compartment [24], cellular fractionation was also performed on the W303-CDC25^{NES} strain, where Cdc25 was modified by the insertion of a strong Nuclear Export Signal, and is therefore excluded from the nucleus. This mutant Cdc25 protein has a distribution in internal membrane not different from the wild type protein (data not shown); besides, the nuclear marker, Nop1, partially overlays on the ER distribution (Fig. 3A). This suggests that on this gradient the nuclear envelope is not distinguishable from the ER membranes, and that the localization of Cdc25^{NES} protein in cellular membranes is not significantly different from the wild-type Cdc25. Unfortunately, the resolution of intracellular membrane compartments in the sucrose gradients in such conditions is too low to avoid partial overlapping of the different fractions. In order to better resolve the internal membranes, cellular fractionation was performed changing buffer compositions, replacing EDTA by 2 mM $MgCl_2$ in all solutions. In fact, in the presence of $MgCl_2$, the endoplasmic reticulum has been reported to have much higher density than Golgi membranes, likely because a Mg^{+}-dependent association of ribosomes with rough ER is preserved [46]. In these experimental conditions, most of the ER membranes were localized in the last gradient fractions corresponding to higher sucrose concentrations (Fig. 4A), dragging a minor quote of the other cellular membranes to the bottom, too. Ras2, Cyr1, Cdc25 and Ira proteins were also massively shifted to the bottom of the gradient, and a minor percentage could be observed in the middle, confirming that these proteins are mainly associated with ER membranes. Ras2 and Ira proteins were still found also in low density membranes, containing mitochondrial membranes (Figs. 3B and 4B), confirming previously reported data for Ras [23] and here for Ira2 (Fig. 2). The association of a minor fraction of Cyr1 with mitochondria could not be clearly revealed with this technique.

Both Ras2 and Cdc25 were recently reported to be affected by nutrient availability or by the hyper-activity of a PKA catalytic subunit, Tpk1. In order to investigate if Cyr1 and Ira2 protein localization

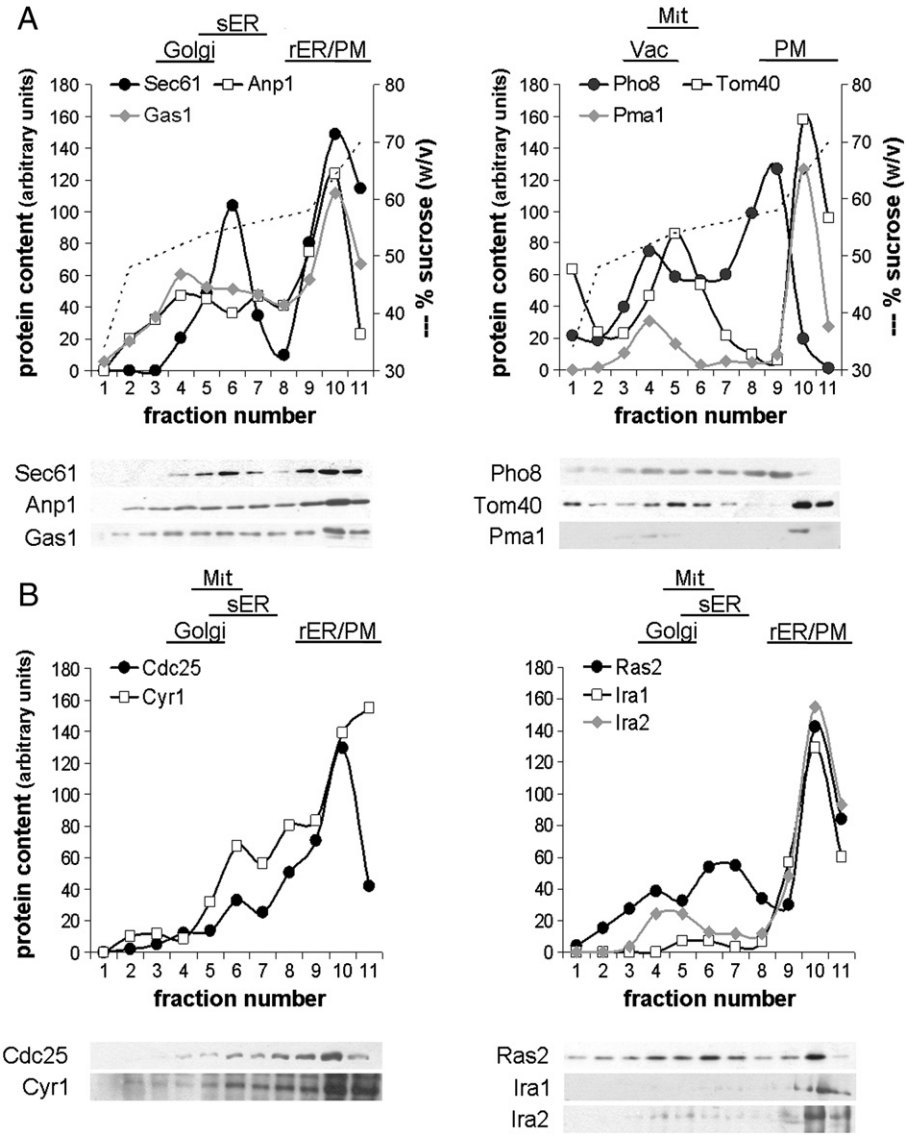

Fig. 4. Ras signaling complex is mainly localized on the ER membranes. 2 mM $MgCl_2$ replaced EDTA in all fractionation buffers. (A) Membrane marker distribution; B) signaling transduction complex component localization.

was also sensitive to such regulation, RT1230 and RT1240 strains were observed during exponential growth on glucose and after nutrient starvation in MES buffer (100 mM, pH 6.5) for 4 h. No changes were observed in either Cyr1 or Ira2 localization in ER membranes, where both the proteins were still observed in all tested conditions and in almost all of the cells observed. Nor did nutrient starvation affect localization of Cyr1 or Ira2 in mitochondria (data not shown).

To investigate if Cyr1 and Ira2 protein localization was sensitive to PKA hyper-activity, RT1210 and RT1220 strains were sporulated and the haploid strains expressing Cyr1-GFP or Ira2-GFP fusion proteins as unique version of the protein were transformed with an episomic plasmid (YEpTPK1) carrying *TPK1* gene. Co-localization with ER membranes was observed by co-transforming the strain with the YIplac204-T/C-Hmg1DsRED plasmid, and co-localization with mitochondria with *in vivo* DAPI staining. Tpk1 overexpression did not affect Cyr1 localization in ER or in the mitochondria (data not shown), but while co-localization with ER membranes was observed in all the cells expressing Ira2-GFP protein, either carrying YEpTPK1 plasmid or not, the overexpression of Tpk1 PKA subunit made Ira2-GFP co-localization with mitochondria much less evident, so that it was detectable in 40% of the cells, *versus*

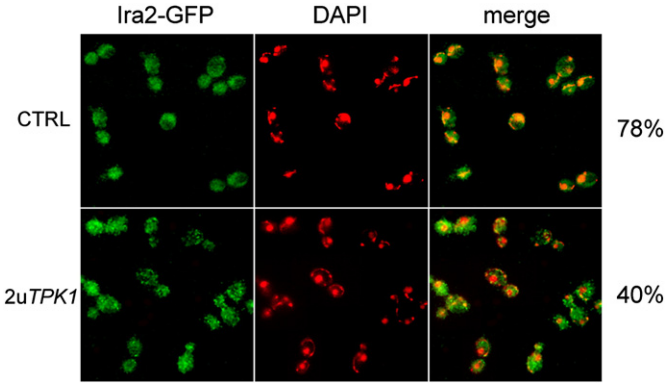

Fig. 5. Overexpression of PKA catalytic subunit Tpk1 counteracts Ira2 localization in mitochondria, driving it to ER membranes. Ira2 localization was observed in RT1211 (CTRL) and in RT1211 transformed with YEpTPK1 plasmid (2u*TPK1*), and the fraction of cells where co-localization of Ira2-GFP and DAPI-stained mitochondria was evident was calculated on at least 300 cells and indicated in the panel. Cells from different microscope fields were mounted in these panels to show representative patterns observed in the population.

the 78% in cells with physiological PKA activity showing evident Ira2-GFP localization in the mitochondria (Fig. 5).

Localization of Ras signaling complex in different compartments could be an effect of or a mechanism for feedback regulation by PKA, and could in its turn have effects on PKA pathway activation.

In order to analyze if Cdc25 nuclear export affects PKA activity, W303-CDC25^{NES} strain phenotype traits related to PKA activity were further investigated: no difference was found in glycogen accumulation in stationary phase (data not shown) or in growth rate and percentage of budded cells on glucose, but W303-CDC25^{NES} strain revealed a smaller cell size both on glucose (Fig. 6A) and on galactose (data not shown), and a higher heat shock resistance during exponential growth on glucose (Fig. 6B), which suggests a reduction in PKA activity in this strain. This is consistent with the phenotype recently reported for a strain carrying a double mutation in Cdc25 coding sequence, which substitutes Ser806 and Ser807 with glutamic acid. This mutant protein is no longer localized in the nuclear compartment, and shows phenotype traits typical of PKA-activity down-regulation [25].

Furthermore, the physiological relevance of the localization of the bulk of Ras signaling complex components in the ER membranes could be related to an active role of ER membranes in Ras signaling, or the complex could simply be inactive on ER membranes and represent a storage for Ras signaling components, where to drop them when the signal has to be reduced. In order to address this question, the presence of active Ras on ER membranes was analyzed by taking advantage of a Ras-GTP specific probe based on the Ras-binding domain of Raf-1, fused to GFP. Observation of cells expressing EGFP-RBD-3 probe and co-transformed with the YIplac204/TKC-DsRED-HDEL plasmid allowed to observe that during exponential growth on glucose active Ras2 is present mainly in the nuclear compartment besides in the plasma membrane, as previously reported [26], while an evident localization of the probe in the ER was difficult to evidence (data not shown) suggesting that Ras2-GTP is only marginally present in ER membranes. However, an active signaling complex, or at least an endogenous exchange activity, can be detected on internal membranes: proteins isolated in gradient fractions 1 to 5, corresponding to internal membranes, were incubated for 15 min either with 1 mM GDP or GTP, and then Ras2-GTP was purified by pull-down assay and visualized by Western blot. In all the analyzed fractions, the amount of purified Ras2-GTP was higher in the presence of GTP than in the presence of GDP, suggesting that an exchange activity exists on these membranes. The guanine nucleotide exchange on Ras2 was a Cdc25-dependent process since the GTP loading on Ras2 in a *cdc25*Δ YepTPK1 strain was abolished (Fig. 7).

4. Discussion

The reported results indicate a largely intracellular localization of Ras signaling complex: in fact, the bulk of Cyr1, Ras, Cdc25 and Ira proteins, examined both by fluorescence tagging and/or cellular fractionation, co-localized with internal membranes, while only a consistent but minor amount of these proteins was found associated with the plasma membrane fractions in exponentially growing cells. Moreover, the membrane fractionation performed in the presence of $MgCl_2$ demonstrated that these proteins are mainly associated to ER membranes; only Ras2 and Ira proteins appear to be also significantly present in lighter membranes, likely mitochondria. This is in agreement with fluorescence tagging experiments, which in turn reveal that also a minor fraction of Cyr1 protein is present in this last compartment. A mitochondrial localization was previously reported only for Ras2 [23], and it is consistent with the preliminary identification of Ira1 in the mitochondrial proteome by two distinct high-throughput approaches [47,48], while this is the first report stating the presence of Cyr1 in mitochondria. None of these proteins show any consensus for mitochondrial localization signals, so they probably associate to the mitochondria following a non-classical pathway. Consistently, also Ras targeting to the plasma membrane does not require the classical secretion pathway, while it requires endosomal and vacuolar membrane fusion class C *VPS* proteins and mitochondrial functionality [23].

Though Ras2 localization on internal membranes is not surprising, since it was already reported [20,27] and, trivially, enzymes required for Ras2 post-translational modifications were found on ER membranes

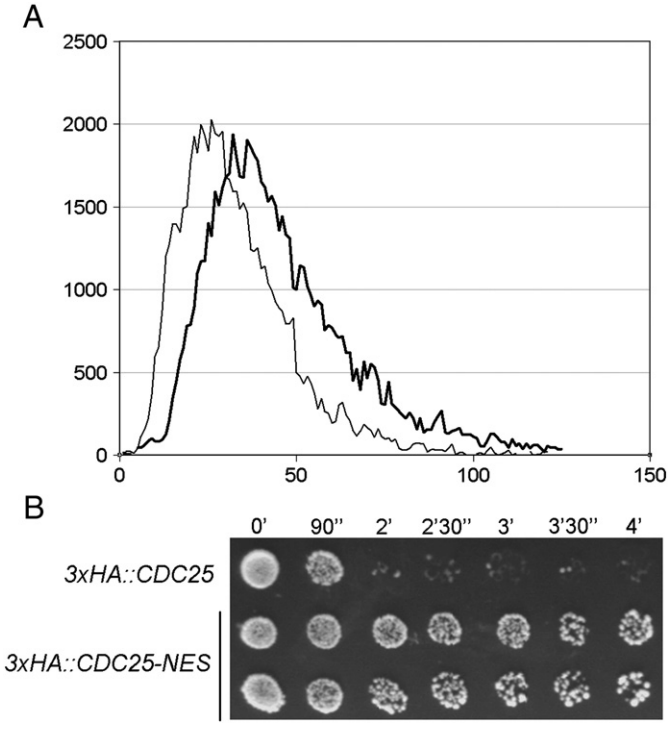

Fig. 6. Cdc25^{NES} strain shows features associated to a down-regulation in PKA activity. OL568-1C (W303 with *3xHA::CDC25*) (heavy line) and W303-CDC25^{NES} strain (thin line) cells exponentially growing in YPD medium were analyzed for cell volume with a Coulter Counter associated Channelyzer (panel A) and 10^4 cells were spotted on fresh YPD medium after being exposed to 51 °C for the indicated time (panel B).

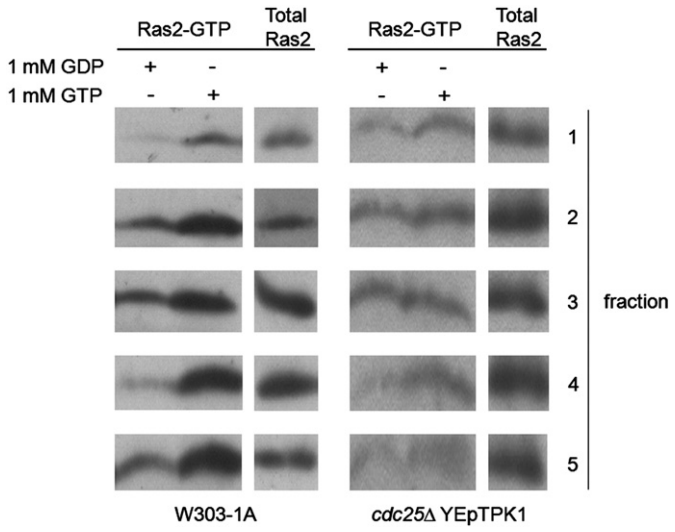

Fig. 7. GDP/GTP exchange can be detected in internal membranes. Membranes were extracted from YPD medium exponentially growing cells of W303-1A or *cdc25*Δ YEp*TPK*1 strains and fractionated on a sucrose gradient. Proteins from the fractions 1 to 5 were incubated with 1 mM GDP or GTP for 15 min and then with GST–RBD prebound to glutathione–Sepharose. Purified Ras2-GTP and total Ras2 protein were detected by Western blot using anti Ras2 antibodies (Santa Cruz Biotechnology).

[21], the findings that its effector Cyr1, and its regulative factors, Cdc25 and Ira proteins, are also localized on internal membranes suggest that Ras2 localization could have a functional meaning and it would not merely be linked to a post-translational modification process. Consistently, mammalian Ras isoforms, H-Ras, N-Ras and K-Ras, are found associated not only to plasma membrane but also to intracellular membranes: many studies have shown that in other organisms Ras proteins can control different pathways by acting in distinct cellular compartments such as plasma membrane, endosomes, Golgi or mitochondria [49–51]. In yeast, Ras signaling is important for several cellular pathways (PKA activity, filamentous or invasive growth), but up-to-now no clear evidence exists that different pools of Ras or Ras regulating proteins could be differently involved in these different signals, beyond data suggesting that Ras2 uses the ER as a signaling platform from which it negatively regulates the first step in the production of GPI-anchors for cell surface proteins [52].

Shuttling of Ras2 and Cdc25 from plasma membrane to internal membranes was reported to be connected to PKA activity and nutritional conditions, as well as control of nuclear localization of Cdc25 [25,27], while Cyr1 protein was reported to be more soluble in mutants with PKA hyper-activity [8]. Observation of fluorescence tagged Ira2 and Cyr1 proteins did not evidence any difference in cells growing on carbon sources different from glucose or after nutrient starvation (data not shown), suggesting that this nutritional regulation is a specific feature of Ras2 and Cdc25 localization. Though, PKA activity is involved in driving Ira2 away from the mitochondria (Fig. 5), as well as Cdc25 out of the nucleus and both Ras2 and Cdc25 away from the plasma membrane [25]. Driving all of these proteins to the ER compartments in case of PKA hyper-activity is expected to have a functional meaning.

Bhattacharya et al. [53] have suggested that *S. cerevisiae* Ras2 signaling function is confined to plasma membrane level, while normal cell growth does not require Ras2 attachment to plasma membrane. In fact, the mutant $Ras2^{C318S}$, that is not palmitoylated and subsequently not able to bind to the plasma membrane, supports a normal mitotic growth but fails to induce cAMP increase in response to glucose. It was suggested that Ras would sense the energetic condition within the cell [54], or the level of some glycolytic intermediates; none of these signals requires the localization of this complex on the plasma membrane. Anyway, the presence on the plasma membrane of another adenylate cyclase activating complex should also be considered: it is assessed that both the activating systems, Gpr1/Gpa2 and Ras complex, are required to cooperate for rapid response to glucose re-addition to glucose-starved or derepressed cells, while during exponential growth on glucose this is not necessary. In fact, it was reported that PKA hyper-activation causes Ras2 relocalization from plasma membrane to cytoplasm [27], while GPCR complex is unable to activate adenylate cyclase in the absence of active Ras, although very small amounts of active Ras are sufficient for GPCR system to activate glucose-induced transient cAMP spike [55]. A working model could be that during exponential growth PKA activity is finely tuned according to the nutritional condition by Ras complex working on internal membranes, while in the absence of glucose a higher fraction of Ras proteins and Cdc25 RasGEF are re-located to the membrane to cooperate with GPCR system and to achieve the fast and transient cAMP signal necessary to rapidly adapt to an incoming glucose availability. Accordingly, active Ras and an exchange activity can be detected on ER membranes during exponential growth on glucose (Fig. 7).

Mitochondrial membranes might represent another platform for Ras signaling. Phosphorylation-induced relocalization of mammalian K-Ras to endomembranes has been demonstrated to be associated with induction of apoptosis [50]. In yeast, the shutdown of cAMP–PKA signaling activity in wild-type cells involves targeting of Ras2p to the vacuole for proteolysis. Cells lacking Whi2p function exhibit an aberrant accumulation of activated Ras2 at the mitochondria in response to nutritional depletion, inducing actin-mediated apoptosis as a result of inappropriate Ras–cAMP–PKA activity [26]. Further work will be needed to clarify if mitochondrial Ras activity is specifically required for apoptosis induction.

Moreover, nuclear localization of Cdc25, Ira1 and active Ras2 suggests that nuclear compartment too could have a role in Ras signaling. It is well-known that PKA subunits are localized in the nucleus [56,57], and the interaction among these proteins in the nuclear compartment should be considered. For example, Cdc25 export from the nucleus is controlled by PKA activity, but on the other hand the exclusion of Cdc25 from the nuclear compartment is sufficient to affect PKA activation ([25] and this work).

Acknowledgements

We thank M. Jacquet, M. Vai, J. Winderickx, S. Munro, T. Endo, P. Coccetti, P. Branduardi, B. Glick, and JF. Cannon for the generous gift of strains, plasmids or antibodies, and M. Ceriani for technical assistance in confocal microscopy. This work was partially supported by UNICELLSYS to EM.

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