The CRaZy calcium cycle.

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Summary.

Calcium is an essential cation for a cell. This cation participates in the regulation of numerous processes in either prokaryotes or eukaryotes, from bacteria to humans. *Saccharomyces cerevisiae* has served as a model organism to understand calcium homeostasis and calcium-dependent signaling in fungi. In this chapter it will be reviewed known and predicted transport mechanisms that mediate calcium homeostasis in the yeast. How and when calcium enters the cell, how and where it is stored, when is reutilized, and finally secreted to the environment to close the cycle. As a second messenger, maintenance of a controlled free intracellular calcium concentration is important for mediating transcriptional regulation. Many environmental stimuli modify the concentration of cytoplasmic free calcium generating the "calcium signal". This is sensed and transduced through the calmodulin/calcineurin pathway to a transcription factor, named calcineurin-responsive zinc finger, CRZ, also known as "crazy", to mediate transcriptional regulation of a large number of genes of diverse pathways including a negative feedback regulation of the calcium homeostasis system.

A model of calcium regulation in yeasts

In higher eukaryotes entry of calcium in the cell starts concatenated signaling events some of them are of enormous importance in animals such as initiation of the heartbeat or the synapses between neurons. In the budding yeast calcium mediates adaptation to a variety of stimuli such as the presence of mating pheromones (Iida et al., 1990), a damage to endoplasmic reticulum (Bonilla and Cunningham, 2003), and different ambient stresses like salinity, alkaline pH or high osmolarity [reviewed in (Cunningham, 2005)]. A general model for calcium homeostasis is depicted in Figure 1 and the elements participating in this process will be reviewed in this chapter.

Essentially, calcium enters the cell through different transport mechanisms generating an increase of cytosolic free calcium concentration. A feedback control system enables an stable concentration of calcium in cytoplasm of 50-200 nM (Aiello et al., 2002; Dunn et al., 1994; Miseta et al., 1999). The elevation of calcium levels in the cytoplasm sequentially activates the calcium binding proteins calmodulin (CaM) and the serine/threonine phosphatase calcineurin, which is composed of the Cna1/Cnb1 or Cmp2/Cnb1 heterodimer. An important final effector for regulation of transcription is the the transcription factor Crz1 a target of calcineurin. Dephosphorylation of Crz1 by calcineurin activity causes its immediate entry in the nucleus. Nuclear Crz1 regulates the transcription of a range of genes. Among these Crz1-dependent genes are those encoding for calcium pumps Pmr1 and Pmc1. These two calcium transporters play a key role in regulating cytoplasmic calcium by either pumping calcium to the ER and Golgi, PMR1, or to the vacuole, PMC1. In addition to the previous transport mechanism, calcium is stored in the vacuole through the activity of the $\text{Ca}^{2+}/\text{H}^{+}$ exchanger Vcx1. In the vacuole, calcium associates with polyphosphates constituting the "non-reusable" stock of calcium. However a low level of vacuolar calcium remains

free and it can be transported back to the cytoplasm via the specific channel Yvc1.

Release of calcium from the internal stores it is tightly regulated and occurs in response a number of stimuli. Calcium in the ER and Golgi may preferentially follow a secretion process depleting the cell of this cation. In a way, calcium exocytosis closes the cycle of calcium in the yeast.

How calcium enters the cell?

Since calcium is a basic element of cell signaling it is expected that specific transport mechanisms functionally located at the plasma membrane (PM) mediate the influx of this cation. Three different mechanisms of transport have been postulated for Ca^{2+} entry (Figure 1). To date, the only identified transport mechanism is a calcium channel based on two components shown to be responsible of calcium entry in the cell (Courchesne and Ozturk, 2003; Locke et al., 2000). Mid1p and Cch1p are proteins of the PM that are thought to assemble into a calcium channel (Paidhungat and Garrett, 1997), however evidence also suggests that they may function independently (Locke et al., 2000). Cch1, for calcium channel homologue, was primarily identified as a Ca^{2+} channel homologue due to its sequence similarity to the pore-forming subunit ($\alpha 1$) of a plasma membrane, voltage-gated, calcium channel from higher eukaryotes (Paidhungat and Garrett, 1997). Cch1p is a large protein of 2039 amino acids with 22-24 predicted transmembrane domains (TMHMM, <http://www.yeastgenome.org/locus/S000003449/protein>) organized into four hydrophobic repeats (Paidhungat and Garrett, 1997).

Mid1, from Mating pheromone-Induced Death, was identified in a screen for mutants deficient in survival after mating differentiation and in calcium uptake (Iida et al., 1994). Later, Mid1 was classified as a stretch-activated channel, with similarities to higher

eukaryotes SA-Cat channels (Kanzaki et al., 1999). Mid1 is a 548 amino acid protein with four hydrophobic regions (named H1 to H4) and two cysteine rich regions (C1 and C2) (see PFAM entry PF12929). In addition to a plasma membrane localization, Mid1 was found to be also present in ER as a 200-kDa oligomer by covalent cysteine binding (Yoshimura et al., 2004), probably through the cysteine rich regions. The role of hydrophobic regions in cellular distribution of Mid1 was established, being H1 to H3 required for PM and ER localization and H1 alone for PM localization in response to mating pheromone (Ozeki-Miyawaki et al., 2005).

Importantly, *mid1*Δ *cch1*Δ double mutants are indistinguishable of single mutants, this and physiological data early suggested that Mid1 and Cch1 might be actually components of a single yeast Ca²⁺ channel (Paidhungat and Garrett, 1997). Both proteins together could act as a voltage-gated Ca²⁺ channel (VGCC) becoming activated in response to depolarization (Catterall, 2000; Cui, J. et al., 2009a). The presence of mating pheromone or depletion of manganese from the medium (Paidhungat and Garrett, 1997), depletion of calcium from the ER (Bonilla et al., 2002), medium alkalisation (Viladevall et al., 2004), and cold, osmotic or saline stresses (Matsumoto et al., 2002; Peiter et al., 2005; Viladevall et al., 2004), are among others, signals that trigger calcium entry in the cell through the Mid1/Cch1 VGCC. Of importance to understand how calcium entry is regulated, is the fact that absence of either or both components of yeast VGCC does not completely perturb calcium homeostasis. Based on calcium resistance/tolerance experiments and mathematical models another two transport systems have been postulated (Cui and Kaandorp, 2006). These have not been identified so far in *S. cerevisiae*, these transporters or pumps have been termed as transporter X and transporter M. Activities of these Ca²⁺ influx transporters are modulated by extracellular Mg²⁺ and the possible identity of these transporters is speculated below.

Finally, it is interesting to note that as important as those mechanisms present in the yeast are those mechanisms which are absent. Different studies have shown the absence of calcium ATPases of the SERCA family at the plasma membrane (reviewed in (Cunningham, 2005)), as well as the mechanism that directly couples entry of calcium through the PM towards the ER. In animal cells, the store-operated calcium channels (SOCs) allows replenishment of the ER when becomes depleted of calcium by the action of resident calcium ATPases (Zhou, Y. et al., 2010a; Zhou, Y. et al., 2010b). This is a mechanism that ensures the adequate level of exchangeable pool of calcium in animal cells, but lacking these systems in *S. cerevisiae* entry of calcium through the PM is the major bottle neck for appropriate storage of intracellular calcium. Thus it is important to understand how calcium is kept into the yeast cell.

How calcium is stored in a fungal cell?

Under certain ambient conditions, a massive entry of calcium in the cell occurs and this represents a major stress for the yeast. Excessive free calcium is toxic because it may interact with numerous proteins or oligomolecules (ie. polyphosphate-derived compounds such as NTPs) in the cytoplasm. To prevent deleterious effects, the excess of calcium is rapidly eliminated by the activity of different Ca^{2+} pumps and exchangers and cytosolic Ca^{2+} is maintained at very low concentration, ranging 50–200 nM (see (Cui, J. et al., 2009b) and references therein). In fact, there are two major mechanisms in this process of calcium sequestration (Figure 1). Calcium can be either stored in the vacuoles or in secretory compartments of ER and Golgi. Most researchers identify the vacuole as the main organelle for storage and sink, and the compartmentalization of Ca^{2+} in the ER/Golgi revealed a pathway for depleting and recycling intracellular calcium (reviewed in (Cui, J. et al., 2009a)).

For vacuolar storage of Ca^{2+} two principal transporters have been identified; the P-type ATPase Pmc1 pump [(Cunningham, K. W. and Fink, G. R., 1994a; Cunningham, K. W. and Fink, G. R., 1994b)] and the calcium/hydrogen exchanger, Vcx1 [(Cunningham and Fink, 1996; Miseta et al., 1999)].

Pmc1 is a 1173 amino acid calcium ATPase. Three highly conserved domains among fungal and higher eukaryote Pmc1 homologues, the E1-E2 ATPase (PF00122), the haloacid dehalogenase-like hydrolase motif (PF00702) and the C-terminal ATPase domain (PF00689). Notably, these motifs are shared with other P-type ATPases, such as the Na^+/Li^+ pumps Ena (Ena1, 2 and 5), the plasma membrane proton pump (H^+ -ATPase), the proton-potassium pump (H^+/K^+ -ATPase), and the calcium ATPase PMR1. Since absence of Pmc1 activity is required for tolerance to elevated extracellular calcium levels, it is not surprising that expression of *PMC1* is up-regulated when intracellular calcium levels elevate (Marchi et al., 1999). Loss of Pmc1 function reduces the amount of non-exchangeable calcium in vacuole, however inactivation of calcineurin restores calcium sequestration in the vacuole of a null *pmc1* mutant (Cunningham, K. W. and Fink, G. R., 1994b). The explanation for this phenotype is that tolerance of null *pmc1* mutants to calcium is dependent on the activity of Vcx1 which, on the other hand, it is dependent on calcineurin activity (Cunningham and Fink, 1996). Vcx1, named as vacuolar H⁺/Ca²⁺ exchanger (Cunningham and Fink, 1996), is also known as HUM1, for "high copy number undoes manganese". Mutations in HUM1 were identified because conferred sensitivity to Mn^{2+} and this phenotype was exacerbated in hypofunctional calcineurin mutants (Pozos et al., 1996). Vcx1 shares similarities to other cation exchangers, among them are those involved in the antiport of Na^+ , K^+ with H^+ (Nhx1 and Vnx1) located the vacuole or prevacuolar compartment/vesicles (Nhx1) (Cagnac et al., 2010). Despite the similarity of Vnx1p to other members of the CAX (calcium exchanger) family of transporters, Vnx1p is unable to mediate Ca^{2+} transport but is a low affinity Na^+/H^+ and K^+/H^+ antiporter (Cagnac et al., 2007).

Biochemical data using purified vacuoles and vacuole membrane vesicles have evidenced that Ca^{2+} transport activity of Pmc1 and Vcx1, more dramatically for the latter, depends on the pH gradient. Calcium uptake is promoted when the interior of these compartments is acid and lost when alkaline. This optimal acidification of vacuoles is maintained by the vacuolar H^+ V-ATPase activity (Dunn et al., 1994) and mutations in subunits of this ATPase strongly reduced tolerance to calcium (see below). In 1994 it was discovered that the main reservoirs of intracellular calcium were vacuoles and ER/Golgi (Figure 1). Pmc1 in the vacuole and a second calcium P-type ATPase at the ER/Golgi, Pmr1 were essential for viability of *S. cerevisiae* (Cunningham, K. W. and Fink, G. R., 1994a). Pmr1 is a high affinity calcium pump. Initially, it was located to the vacuoles but subcellular fractionation studies located to ER and Golgi vesicles [(Cunningham, K. W. and Fink, G. R., 1994a; Sorin et al., 1997; Strayle et al., 1999)]. In fact, Pmr1 is a P-type $\text{Ca}^{2+}/\text{Mn}^{2+}$ ATPase (Antebi and Fink, 1992; Rudolph et al., 1989) and the transport of either cation can be specifically modified by affecting distinct amino acids, D778A and Q783A, both locating in transmembrane segment M6 (Mandal et al., 2000). Important for the Pmr1 functionality is the presence of a EF hand like motif at the N-terminal region of this pump (Wei et al., 1999). Mutations in this domain change the affinity of the protein for Ca^{2+} , Mn^{2+} , or both. Pmr1 activity is essential for growth of a double *pmc1 vcx1* mutant. The activity of both P-type Ca^{2+} ATPases Pmr1 and Pmc1, and the antiporter Vcx1 are key in maintaining the low cytosolic free calcium concentration which it is needed to avoid inappropriate calcineurin activation and other effects due to the presence of this cation (Cunningham, K. W. and Fink, G. R., 1994a). Deletion of *PMR1* cause the elevation of cytoplasmic levels of free calcium and a massive accumulation within vacuoles (Halachmi and Eilam, 1996). In fact, a deletion of *PMC1* and *PMR1* is a lethal genetic combination, leading to elevation of calcium levels, hyper-activation of calcineurin, and subsequent inactivation of Vcx1 antiporter. Transport activity of Vcx1 is inhibited by calcineurin,

possibly in a post-translational mechanism (Cunningham and Fink, 1996). Since Rcn2 is a negative regulator of calcineurin (see below), it participates in the regulation of Vcx1 (Kingsbury and Cunningham, 2000), which in turn involves the activity of Crz1, that modulates transcription of this calcineurin regulator (Mehta et al., 2009). Expression of Pmc1 is dependent on the transcription factor Crz1 (Matheos et al., 1997; Stathopoulos and Cyert, 1997), but also activity of Pmc1 is regulated. In a screen for negative regulators of Pmc1, the Nyv1 protein, a vacuolar v-SNARE, was found to inhibit the calcium transport activity of Pmc1 in the vacuole membrane, without affecting its expression levels (Takita et al., 2001).

In contrast to Vcx1 and Pmc1, Pmr1p activity is not posttranscriptionally modulated by calcium binding proteins. Pmr1 apparently lacks the calmodulin binding domain at the C-terminus present in other plasma membrane Ca^{2+} ATPases (PMCAs). However, importance of transport of calcium and manganese to ER extends beyond being an storage for these cations and different studies evidence the role of Pmr1 in the proper functioning of ER, as for the normal secretion of proteins, protein maturation and/or degradation (Durr et al., 1998).

Efflux of calcium from internal stores and its recycling.

In addition to calcium entry in the cell, a second source for this cation is the return to cytoplasm from the internal stores. Only part of this intracellular calcium, about 10% of the total, can be released back to the cytoplasm and it is designated as the "exchangeable" pool of calcium (reviewed in (Cunningham, K. W. and Fink, G. R., 1994a)).

Release of calcium from vacuoles

In vacuoles calcium is present in two forms: a free and a non-usable pool. The latter is designated as "non-exchangeable" since calcium is associated to polyphosphates. The

concentration of free calcium in vacuoles is at the micromolar range (30 μM) meanwhile the total calcium was estimated at the millimolar range (2mM) (Dunn et al., 1994). However, this reduced free-calcium pool can be returned to the cytoplasm when required. In the yeast vacuole it was identified a ion channel responsible for efflux of vacuolar calcium to the cytoplasm. This activity was designated as the yeast vacuolar conductance and the *YVC1* gene (yeast vacuolar channel 1) was identified (Palmer et al., 2001). Yvc1 is a 675 amino acid protein containing six transmembrane domains and is solely detected in vacuolar membranes. This specific localization in *S. cerevisiae* contrasts with that shown by members of the same family of transporters in higher eukaryotes which locate at the plasma membrane (reviewed in (Cunningham, 2005)). Yvc1, is a calcium-activated cation channel of the transient receptor protein family (Denis and Cyert, 2002; Palmer et al., 2001). When yeast cells need the activity of this mechano-sensitive Ca^{2+} channel? In the case of a severe hypertonic shock, the vacuolar free Ca^{2+} can be released into the cytosol through the activity of the Yvc1. This calcium release serves to stimulate the calmodulin/calcineurin network and activates Crz1, thus enabling the transcriptional response to this stress.

Calcium release from ER and Golgi; the exocytic pathway.

The absence genes coding for inositol tri-phosphate (IP3) receptors and ryanodine receptors, RyR, in the yeast genome, and in general all fungal genomes (reviewed in (Cunningham, 2005)), clearly indicates that the role of ER and Golgi compartments in calcium homeostasis is different in fungal cells. Since calcium is stored in the ER and Golgi by the activity of PMR1 ATPases, and release seems not to be immediate because other calcium transporters for this activity are unknown a mayor role of exocytosis is proposed to liberate this compartment of any excess in calcium. In this way, it has been measured that when the calcium concentrations in Golgi and ER

exceed their resting levels of 300 μM (Pinton et al., 1998) and 10 μM (Aiello et al., 2002; Strayle et al., 1999), respectively, the calcium in ER and Golgi will be secreted along with the canonical secretory pathways. Most importantly, calcium and manganese, both transported by the Pmr1 ATPase, are necessary for the proper processing and trafficking of peptides and proteins through the secretory pathway. While Mn^{2+} has a role in protein glycosylation, Ca^{2+} is required for normal protein sorting (Durr et al., 1998). As for calcium, the excess of manganese is eliminated via Golgi and the secretory vesicles (Culotta et al., 2005). Hence, exocytosis is a major mechanism to largely reduce the intracellular, compartmentalized, pool of calcium, and other cations, but increasing the extracellular content of these ions, becoming available for a re-start of calcium transport and signaling.

Calcium signaling

S. cerevisiae grows in a wide range of extracellular concentrations of calcium. Yeast cells are able to adapt to large and rapid modifications in environmental calcium, ranging from a low concentration of 1 μM to more than 100mM (Anraku et al., 1991). Part of this adaptation process relies on the activation of a signaling cascade leading to the modification of the gene expression pattern (Figure 2).

Upon elevation of extracellular levels of calcium a massive influx of this cation occurs in the yeast. For the free calcium now existing in the cytoplasm, one of the targets is the small and essential protein calmodulin, Cmd1 (reviewed in (Cyert, 2001); see Figure 2A). Calmodulin has 4 EF-hand moieties (Davis et al., 1986). Each EF-hand is able to bind a Ca^{2+} atom, but in the case of *S. cerevisiae* Cmd1 the fourth EF-hand is divergent and most likely is not able to bind calcium (Starovasnik et al., 1993). Calmodulin has many roles in the yeast cell which can be classified into calcium independent and dependent functions (Davis et al., 1986). Among the calcium-dependent roles is to

activate the protein phosphatase 2B calcineurin (see Figure 2A) . In *S. cerevisiae* calcineurin is composed of a catalytic subunit, the A subunit encoded by either CMP2 or CNA1 isoforms (Cyert et al., 1991), and the regulatory, B, subunit encoded by CNB1 (Cyert and Thorner, 1992) . Activity of Cnb1, and subsequently of the calcineurin, is modulated by the α -arrestin Aly1 (O'Donnell et al., 2013) and through myristoylation (Connolly and Kingsbury, 2012) in response to a reduction in calcium signal.

The calcineurin phosphatase may have numerous targets. However, these are directly recognized through a short motif namely the calcineurin docking domain (Rodriguez et al., 2009; Roy et al., 2007). An important target of calcineurin is the Crz1 transcription factor (Stathopoulos and Cyert, 1997) . Crz1 is a three zinc-finger, two classical Cys2His2 fingers and a non-canonical CysCysHisCys finger, transcription factor that is inactivated and activated in a cyclic , pulsatile dynamic, phosphorylation/dephosphorylation process in the cell (Dalal et al., 2014) . Crz1 mediates in yeast tolerance to high concentrations of different cations, alkalinity and other types of stresses (reviewed in (Cyert, 2003)).

Calcineurin modulates the activity of Crz1, at least, regulating its nucleocytoplasmic trafficking (Stathopoulos-Gerontides et al., 1999). Crz1 is mainly cytoplasmic when in a phosphorylated state. A nuclear export signal (NES) becomes activated and recognized by the exportin Msn5p (Boustany and Cyert, 2002) . After calcineurin dependent dephosphorylation, in response to an elevation of the cytoplasmic concentration of calcium, the nuclear import signal is dephosphorylated as is the NES. A nuclear transporter Nmd5 (karyopherin Kap119) recognizes this dephosphorylated form of Crz1 and translocates it in nucleus following a general nuclear transport process through the pores located at the nuclear envelope (Polizotto and Cyert, 2001) . A recent work has shown that Crz1 may enter nucleus in a stochastic mode. This is a response to a brief calcium dependent stimuli that , in a non coordinate mode among cells , promote temporal accumulation of Crz1 and other transcription factors into the nucleus (Dalal et

al., 2014). This mechanism is proposed as the basis to provide a fast response to ambient stress signals. However, following our case of an elevation of cytosolic calcium levels, this network of calcium binding proteins and dephosphorylation process activates the nuclear entry of Crz1 in all cells in a culture.

In the nucleus Crz1 will bind to precise DNA sequences known as CDREs, calcineurin-dependent regulatory elements, present in the promoter of genes under its regulation (Mendizabal et al., 2001; Stathopoulos and Cyert, 1997). Among these genes are *PMR1* and *PMC1* (for review, see [(Cyert, 2001)]) and Crz1 have a positive role on their expression levels (Figure 2A). Thus, Crz1 is responsible for a rise in the levels of *Pmr1* and *Pmc1* pumps that would locate at the ER/Golgi and vacuoles, respectively. This effect was early noted by (Beeler et al., 1994; Dunn et al., 1994) when found that a rise in the cytosolic calcium concentration increased the non-exchangeable pool of Ca^{2+} . Mutants lacking *PMC1* grow poorly in calcium stress conditions, although growth can be restored by overexpression of *PMR1* or *VCX1*. This reflects the importance of calcium sequestration in tolerance to elevated concentrations of extracellular calcium (Cunningham, 2005; Cunningham, K. W. and Fink, G. R., 1994b). Therefore, the activity and an adequate level of expression of these pumps have a direct effect on depleting cytosol of calcium causing the attenuation of calcineurin-dependent signaling. Crz1 activity also influences calcineurin function. Expression of calcineurin regulators *RCN1* (regulator of calcineurin) and *RCN2* are positively modulated by Crz1 (Figure 2B). *Rcn2* is a negative regulator but *Rcn1* could act as a positive and negative modulator of calcineurin (Kingsbury and Cunningham, 2000; Mehta et al., 2009). Modulation of the phosphorylation levels of Rcn proteins by homologues the glycogen synthase 3 kinase, Gsk3, is the mechanism to activate or inactivate these calcineurin regulators (Hilioti et al., 2004).

A reduction of calcineurin activity have two major consequences in calcium homeostasis. Firstly, the negative effect on *Vcx1* activity is reduced and activity of this

antiporter is restored, with an immediate consequence in lowering calcium levels in the cytoplasm (Figure 2B). Secondly, a low calcineurin activity will consequently reduce the dephosphorylation process of Crz1, and the kinase activities acting on this TF will restore the pool of inactive Crz1 in the cell. HRR25 encodes for a casein kinase I with multiple roles in the cell (Hoekstra et al., 1991; Mehlgarten and Schaffrath, 2003), among them Hrr25 opposes to the activity of calcineurin on the posttranslational modification of Crz1 (Kafadar et al., 2003). Other kinases (see below) may act on restoring phosphorylated levels of Crz1, rendering a cytoplasmic and, thus, inactive form of this TF.

In summary, calcium homeostasis relies on a meticulous regulatory system that senses, transduces and transcriptionally responds to provide with tools (transporters) to "clean" the excess of calcium, and with regulators to modulate and attenuate signal transduction. Survival of cells to calcium stress not only depends on these specific elements but other players are accessory in some ambient conditions and mutant backgrounds of need to sustain yeast's health.

Other protein activities involved in calcium homeostasis.

In 1986, Ohya and collaborators published their work on characterizing recessive mutations affecting growth in the presence of high calcium concentration in the media. Eighteen genes, designated as *c/s* (calcium sensitive mutants) were identified and many of them showed to be necessary for maintaining the structure of function of the vacuole (Ohya et al., 1986). Among these are genes coding for subunits of the vacuolar proton ATPase. *c/s* mutations were isolated in different subunits of the heterocomplex VMA, the vacuolar proton ATPase, constituting the type IV *c/s* mutants. Among these are Vma3, Vma1, Vma11, Vma13 as parts of the ATPase, and Vma12 the assembly

factor ((Ohya et al., 1986; Tanida et al., 1996) and references therein). The role of Vma complex is to provide the correct amount of protons at the vacuole to allow exchange with Ca^{2+} via Vcx1 activity. In this screen it was found mutations in other two genes of interest, *csg1* and *csg2*. CSG2 encodes a endoplasmic reticulum membrane protein; required for mannosylation of inositolphosphorylceramide and for growth at high calcium concentrations; protein abundance increases in response to DNA replication stress (Tanida et al., 1996). Csg2 protein is a transmembrane protein, located in the ER. Biochemical and functional studies indicated that Cls2/Csg2 is necessary for mobilization of non-exchangeable pool of calcium distinct from that of the vacuole and plays an important role in calcium tolerance in the yeast, and would cooperate in stimulating the activity of calcineurin (Tanida et al., 1996). It is interesting to note that Csg2 could fulfill the role of ER-calcium efflux transporters from higher eukaryotes (IP3 receptors and RyR), however this might be an specific mechanism of *S. cerevisiae* since orthologues for Csg2 are not found in other fungi. CSG1 (also known as SUR1, suppressor of *rvs161* and *rvs167* mutations, (Beeler et al., 1997)) encodes for a mannosylinositol phosphorylceramide synthase catalytic subunit, and forms a complex with regulatory subunit Csg2 at the ER (Desfarges et al., 1993). Notably, the study of membrane composition, specially the synthesis of sphingolipids and related molecules has discovered a novel role in calcium homeostasis via influx of calcium from the ER to the cytoplasm (Birchwood et al., 2001; Desfarges et al., 1993; Dickson and Lester, 2002). Also related with the composition of membranes and specifically in aminophospholipids organisation are the type IV of P-type ATPases, constituted by aminophospholipid translocases APTs or flippases. Of importance in maintaining calcium homeostasis are Drs2 (Ripmaster et al., 1993) and Neo1 (Prezant et al., 1996). These flippases are located in late Golgi and are important for proper constitution of plasma membrane and exocytosis. Also the P-type ATPase, type V, is *SPF1*(YEL031), located to the ER and required for calcium homeostasis. Absence of both Spf1 and

Pmr1 function greatly elevate cytoplasmic calcium levels (Cronin et al., 2002).

Reinforcing the importance of ER, Golgi and vacuoles in the overall calcium managing in the yeast cell is the participation of proteins located in these compartments, for example, Gdt1, is transmembrane protein involved in calcium and pH homeostasis in yeast and higher eukaryotes. It localizes to the cis- and medial-Golgi apparatus, but the a GFP-fusion protein localizes to the vacuole. The exact role of this protein is unknown, but possibly related to glycosylation since deficiency in its human homologue TMEM165, a human gene which causes congenital disorders of glycosylation (Demaegd et al., 2013). At the vacuole also locate Ccc1 (Cross-Complements Ca^{2+} phenotype of *csg1*), this is a vacuolar $\text{Fe}^{2+}/\text{Mn}^{2+}$ transporter that also may participate in the respiration process (Fu et al., 1994; Lapinskas et al., 1996), and ECM27 and YDL206W genes coding for two members of the CAX (cation exchangers) family of vacuolar transporters, closely related to Vcx1 and Vnx1 ,

Finally, it is worth to mention the only mutation found causing the need of large amounts of extracellular calcium for survival, the *ca1-1* mutation (Ohya et al., 1984).

This mutation locates in CDC43 gene, that encodes for a β -subunit of geranylgeranyltransferase type I, which catalyzes geranylgeranylation to the cysteine residue in proteins containing a C-terminal CaaX sequence ending in Leu or Phe. In addition to this novel role in calcium homeostasis, the substrates under its regulation are important for morphogenesis (Adams et al., 1990).

As for the latter, the precise role in calcium homeostasis of many of these proteins remains obscure and needs clarification. Some of them open new venues for understanding how calcium is regulated and associated factors and mechanisms that modulate calcium storage and managing. However proteins are not the only new regulatory elements found, but other cations and macromolecules are known to participate in calcium homeostasis.

The role of magnesium in regulating the calcium response

Cui and collaborators (Cui, J. et al., 2009a) discovered that the calcium sensitivity displayed by a *pmc1* mutant was dependent not only on the concentration of extracellular calcium but on the composition of medium. Further analyses revealed that this suppressing factor was the presence of variable concentrations of magnesium in media. In this way, increasing concentrations of Mg^{2+} elevated the IC_{50} to calcium toxicity of a *pmc1* mutant but also modified sensitivity to calcium caused by the combination of double or triple *pmr1*, *vcx1* and/or calcineurin (*cnb1*) mutations. Furthermore, this study indicated the existence of a Mg^{2+} -inhibited calcium transport system located at the PM. Calcium toxicity displayed by a *yvc1 cch1 pmc1 vcx1* quadruple mutant was suppressed by addition of magnesium to the medium. In fact, a mathematical modeling of calcium transport across the PM predicted the presence of at least two transport systems, in addition to that integrated by Mid1 and Cch1, in the plasma membrane. Only in this way it could be accommodated the experimental data of calcium sensitivities in a variety of single and double mutants involving pumps and channels, and measurements intracellular levels of calcium. Magnesium will have a major role in regulating, at least one of these calcium transport systems. These authors conclude that transporter M is regulated by Mg^{2+} and transporter X respond to a hypertonic calcium shock (see also Figure 1).

One of the most abundant divalent cations in cells is magnesium. Mg^{2+} participates acting as a counterion in stabilizing many macromolecules such as RNA and DNA or single nucleotides (i.e. ATP). It also mediates in important catalytic processes and in stabilizing large molecules or membranes (see (Wiesenberger et al., 2007) and references there in). Cellular concentrations of Mg^{2+} are in the millimolar range (from 15-20mM), about three orders of magnitude lower than those of Ca^{2+} (100 to 200 nM). In mammals, entry of Mg^{2+} in the cell is an electrogenic process requiring a negative

charge at the inner side of the PM and the activity of two transporters (TRPM6 and TRPM7) (Schlingmann and Gudermann, 2005; Schmitz et al., 2003), or by members of a heterogeneous protein family, designated as CorA homologues, and found in lower and high eukaryotes (plants and animals), allowing growth or development even in the presence of very low concentrations of magnesium. In yeast, Mrs2 and Alr1 are orthologues of CorA.

Mg²⁺ enters the cell through the activity of two members of the CorA family of transporters, Alr1 and Alr2 (Graschopf et al., 2001; Macdiarmid and Gardner, 1998). These are the metal ion transporter superfamily, MIT. Alr1 and Alr2 may form oligomeric transporter at the plasma membrane constituting a high affinity Mg²⁺ uptake system (Wachek et al., 2006). Mg²⁺ can be stored in two subcellular compartments, the mitochondria, vacuole and ER/Golgi. For the first store the activity of two transporters located in the inner membrane of mitochondria are needed, Mrs2 and Lpe10. Mg²⁺ is stored in the trans-golgi, and possibly in the vacuole by the activity of a Mg²⁺/H⁺ antiporters (Pisat et al., 2009) (Borrelly et al., 2001). Storage of magnesium in the vacuole requires the activity of the vacuolar proton-ATPase, and for Mg²⁺ efflux it is required the Mrs2 transporter, also belonging to the MIT superfamily (Pisat et al., 2009).

Important for understanding calcium homeostasis was the results of a RNA profile analysis of changes in expression dependent on Mg²⁺ depletion. These showed the intimate relationship between magnesium and calcium homeostasis (Wiesenberger et al., 2007). A reduction in external Mg²⁺ upregulate the ENA1, encoding the P-type ATPase sodium pump, and PHO89, encoding a sodium/phosphate cotransporter, which are also upregulated under calcium and alkaline pH stress. In that work they demonstrated that Mg²⁺ starvation caused an increase in cytoplasmic calcium. A rise in cytosolic free calcium activate the calmodulin/calcineurin network, which led to the

activation of Crz1 TF. ENA1 and PHO89 are among the genes regulated by Crz1 (Hu et al., 2007; Mendizabal et al., 2001).

How Mg^{2+} also influences calcium homeostasis? Immediately from what has been exposed before, a negative role in calcium efflux through the PM is expected, causing a reduction in the intracellular pool of Ca^{2+} . To this effect, an elevation of intracellular Mg^{2+} will reduce the release of calcium from the internal stores. But a third effect is also predictable, based on the capacity of EF-hands to bind Mg^{2+} in addition to Ca^{2+} (see review (Grabarek, 2011)). Calmodulin and other EF-hand containing proteins, such as the regulatory subunit of calcineurin, may bind Mg^{2+} rendering an alternative conformation to that originated by calcium, in fact magnesium helps to release calcium from these proteins allowing the pass from the holo-enzyme to the apo-enzyme state (Grabarek, 2011). Thus, Mg^{2+} will directly affect the functionality of the calmodulin/calcineurin network, attenuating calcium signaling. Altogether, an excess of Mg^{2+} will cause a reduction on calcium signaling and probably increase tolerance to this cation, meanwhile low extracellular levels of Mg^{2+} increase calcium signaling (Wiesenberger et al., 2007).

Role of inorganic phosphate in calcium homeostasis.

The property of inorganic phosphate or polyphosphates in chelating cations is fundamental to understand calcium and magnesium homeostasis. Actually calcium and phosphate homeostasis have major importance in vertebrates since it is crucial in bone formation, among other cellular and tissue specific processes (Shaker and Deftos, 2000).

In the yeast, the presence of polyphosphates in vacuoles is the basis for the immobilization of calcium. In this way, the capacity to maintain the vacuolar Ca^{2+}

concentration up to 2 mM is a result of Ca^{2+} binding to vacuolar polyphosphate (Dunn et al., 1994). However, Ca^{2+} can be completely released from isolated vacuoles or from whole cells using the ionophores A23187 or ionomycin, suggesting that the non-exchangeable pool of Ca^{2+} is soluble (Cunningham, K. W. and Fink, G. R., 1994a). The conserved pathway governing phosphate homeostasis in yeast is composed of the PHO genes (reviewed in (Tomar and Sinha, 2014)). Interestingly there is well established interconnection between calcium- and phosphate-dependent regulation, for example at the level of the Crz1 transcription factor. As cited before, Crz1 up-regulates *PHO89* in response to calcium stress or alkalinization, and, in the absence of calcium stress, among the cyclin-dependent kinase Pho85 targets is the transcription factor Crz1p (Sopko et al., 2006).

Concluding remarks and future prospects

Numerous elements have been described in this chapter participating in transport, signaling and storage of a principal messenger in cells, the calcium ion. Along years, *S. cerevisiae* has served as a model to understand how cells deal with a messenger that is essential but lethal at the same time. Most of the principal elements are well known, and many functional analyses have been performed to understand the molecular and biochemical mechanisms involved, e.g. the calmodulin/calcineurin pathway and its fungal effector Crz1. *S. cerevisiae* has served as a model to study calcium homeostasis in other fungi, and in some cases particular variations are found, specially in how Crz homologues are signalized and the transcriptional function of this TF. But a general mechanism underlies in almost all cellular systems under study, from bacteria to human cells, and for that the budding yeast has largely provided with basic and elemental findings to understand this complex homeostatic system. Future research lines will

provide with a more detailed view of the exocytic process of calcium and the interrelationships among different ions and ambient stress signals to modulate and generate a coordinated response.

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Figure legends.

Figure 1. Main transport elements of calcium in *S. cerevisiae*. The chart depicts the relationships between calcium transporters and their preferential locations in the cell. In the text are found a detailed description of these components.

Figure 2. Calcium signaling cascade in *S. cerevisiae*. A) the calmodulin/calcineurin cascade towards activation of the transcription factor Crz1. The protein phosphatase calcineurin dephosphorylates this transcription factor leading to its transcriptional activation. Among Crz1 targets are the genes *PMR1* and *PMC1*, coding for the P-type ATPases, and B) calcineurin regulators *RCN2* and *RCN1*. Modulation of calcineurin activity by Rcn2 and Rcn1 alters signaling of Crz1 and the negative effect of this phosphatase on Vcx1 activity.

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Figure 1

Figure 2